Stabilization of the CLOCK:BMAL1 Heterodimer Decreases Circadian Rhythm Amplitude

by

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To the people who never gave up on me…

ABSTRACT

Rhythmic changes in behaviour and/or physiology are governed by the daily light-dark cycle in most organisms to increase their fitness to the environment. These rhythmic changes are generated from the biological clock and last about 24 hours. In mammals, a hierarchically ordered circadian clock that is highly regulated by complex transcriptional-translational feedback loops (TTFLs) is observed. At the molecular level, CLOCK and BMAL1 form a heterodimer to bind E-box sequences within the promoter region of the clock-controlled genes (including *Cryptochrome* (*Cry*) and *Period* (*Per*)) to initiate the transcription. Then CRYs and PERs which are accumulated within the cytosol, translocate into the nucleus along with Casein Kinase Iε to represses BMAL1:CLOCK-driven transcription.

Small molecules regulating the activities of these core-clock proteins could offer temporal control over the circadian clock. Considering the pathologies- such as cancer, metabolic diseases, and accelerated aging- that can arise from the lack of a robust circadian clock, identification of such molecules carries great importance. Therefore, I characterized the CLK95, which is previously identified by our group, as a novel CLOCK and BMAL1 binding small molecule. I showed that CLK95 stabilizes the interaction between CLOCK and BMAL1 which, in turn, enhances their nuclear localization both *in vitro* and *in vivo*. Further experiments revealed that the CLK95 dampens the amplitude and increases the period of the circadian rhythm by stabilizing the positive loop of the primary TTFL. Considering elevated CLOCK and BMAL1 levels inhibit cell growth, CLK95 may show therapeutic effects against cancer cells due to its enhancing effect on CLOCK and BMAL1.

ÖZETÇE

Organizmaların çevreye adapte olabilmek için gösterdiği davranış ve/veya fizyolojilerindeki ritmik değişiklikler, gece gündüz döngüsüne göre yönetilir. Bu ritmik değişiklikler, biyolojik saat tarafından üretilir ve yaklaşık 24 saat sürer. Memelilerde, kompleks transkripsiyonel-trasnlasyonel geri bildirim döngüleri tarafından düzenlenen hiyerarşik yapıda bir sirkadiyen saat gözlenir. Moleküler düzeyde, CLOCK ve BMAL1, transkripsiyonu başlatmak için saat-kontrollü genlerin (Kriptokrom (Cry) ve Periyod (Per)'u da içeren) promotör bölgesindeki E-box dizilerine bağlamak için heterodimer oluşturur. Daha sonra sitozol içinde biriken CRY ve PER'ler, BMAL1:CLOCK tarafından başlatılan transkripsiyonu bastırmak için Kazein Kinaz Iε ile birlikte çekirdeğe gider.

Bu gibi temel saat proteinlerinin aktivitelerini düzenleyen küçük moleküller, sirkadiyen saat üzerinde geçici kontrol sağlayabilir. Sağlıklı bir sirkadiyen saatin olmamasından kaynaklanabilecek kanser, metabolik hastalıklar ve hızlanmış yaşlanma gibi patolojiler göz önüne alındığında, bu tür moleküllerin tanımlanması büyük önem taşımaktadır. Bu nedenle, daha önce grubumuz tarafından tanımlanan CLK95'i CLOCK ve BMAL1'i bağlayıcı yeni bir küçük molekül olarak karakterize ettim. CLK95'in CLOCK ve BMAL1 arasındaki etkileşimi stabilize ettiğini ve bunun da bu proteinlerin nükleer lokalizasyonlarını hem *in vitro* hem de *in vivo* olarak arttırdığını gösterdim. Yapılan diğer deneyler, CLK95'in sirkadyen ritimin genliğinde azaltıcı, periyodunda arttırıcı etksini birincil transkripsiyonel-translasyonel geri bildirim döngüsünün pozitif kolunu stabilize ederek gösterdiğini ortaya çıkardı. Yüksek CLOCK ve BMAL1 seviyelerinin hücre büyümesini engellediği düşünüldüğünde, CLK95, CLOCK ve BMAL1 üzerindeki arttırıcı etkisinden dolayı kanser hücrelerine karşı terapötik etkiler gösterebilir.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AMPK	AMP-Activated Protein Kinase
bHLH	Basic Helix-Loop-Helix
BMAL1	Aryl Hydrocarbon Receptor Nuclear Translocator-Like Protein 1
CCGs	Clock-Controlled Genes
CDK5	Cyclin Dependent Kinase 5
CHX	Cycloheximide
CKIε	Casein Kinase 1ε
CLOCK	Circadian Locomotor Output Cycles Caput
Co-IP	Co-Immunoprecipitation
CRY	Cryptochrome
CT	Circadian Time
DBP	D-Box Binding Protein
FASPS	Familial Advanced Sleep Phase Syndrome
HAT	Histone Acetyltransferase
HEK 293T	Human Embryonic Kidney Cells
ipRGC	Intrinsically Photoreceptive Retinal Ganglion Cells
JA	Jasmonic Acid
LUC	Luciferase
MEF	Mouse Embryonic Fibroblast
NEAA	Non-Essential Amino Acids
NES	Nuclear Export Signals
NFIL3	Nuclear Factor Interleukin 3
NLS	Nuclear Localization Signal
NMDA	N-methyl-D-Aspartic Acid
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PAS	Per-Arnt-Single-minded
PER	Period

PHR	Photolyase Homology Region
PTM	Posttranslational Modification
RHT	Retinohypothalamic Tract
SCF	SKP1-CUL1-F-box
SCN	Suprachiasmatic Nucleus
TAD	Trans-Activating Domains
TNFα	Tumour Necrosis Factor A
TTFL	Transcriptional-Translational Feedback Loop
U2OS	Human osteosarcoma cells
ZT	Zeitgeber Time
β-TrCP	β-Transducin Repeat-Containing Protein

Chapter 1:
INTRODUCTION

The ability of various species to coordinate biological activity into daily cycles, so called circadian rhythm, provides a significant benefit in terms of fitness. Circadian rhythm increases the chance of survival by regulating the physiological and behavioural decisions of organisms according to environmental cues, such as light and dark cycle [1]. Circadian rhythm is conserved from cyanobacteria to higher eukaryotic organisms including mammals and plants [2-8].

Light is one of the most important external stimuli that can reset the circadian clock. When light received by retina of a mammal, suprachiasmatic nucleus (SCN) which is the central circadian pacemaker receives light information and resets the peripheral clocks through hormonal and neuronal signalling [9-11]. At molecular levels, both SCN and peripheral cells show similar mechanism. There are interlocking transcription-translational feedback loops (TTFLs) that generates molecular oscillations. The primary TTFL has two loops: a positive and a negative loops. In the positive loop, circadian locomotor output cycles caput (CLOCK) and aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) heterodimerize and bind to E-box elements (CACGTG) of clock-controlled genes (CCGs) to activate their expression. The expressions of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes which encode PER, and CRY are also controlled by these transcription factors. In the negative loop, PER and CRY proteins form a heterodimer to translocate into nucleus with casein kinase 1ε (CK1ε) to repress the CLOCK:BMAL1 driven transcription. Once CRYs and PERs get degraded by ubiquitin dependent 26S proteasomal degradation, CLOCK:BMAL1-driven transcription re-starts a new circadian rhythm. Additionally, the transcription of *Bmal1* gene is controlled by a second TTFL which consists retinoic acid receptor-related orphan receptors RORs and REV-ERBs. While RORs initiate the *Bmal1* transcription, REV-ERBs represses it [9, 12, 13]. However, these alone are not enough to give a robust rhythm. Posttranslational modifications which affect the stability, localization and activity of core-clock proteins are also required for such a phenotype [14].

A recent study shows 43% of protein coding genes of mice are under circadian control in at least one tissue [15]. This finding indicates the importance of circadian clock

for mammalians. Indeed, an innate malfunctioning of mammalian circadian system can have devastating outcomes such as development of cancer, sleep disorders, metabolic diseases, cardiovascular diseases, and neurological disorders (schizophrenia, bipolar disorders, unipolar major depression) [16-19]. Therefore, several studies have identified several small molecules that affect circadian rhythm and, in turn, treat these diseases. Many of the small molecule modifiers of circadian clock, are identified through high-throughput screening by targeting axillary circadian clock proteins. There few small molecules are being identified against core clock proteins. For example, KL001 which is a CRY stabilizer, regulates circadian rhythm at cellular level [20].

Recently, our group identified a small molecule called CLK8 that binds to CLOCK, using *in silico* approach. Further experimental studies revealed that CLK8 reduces the interaction between CLOCK and BMAL1, and, in turn enhances circadian rhythm amplitude [21]. In the same screen another molecule called CLK95 shown to alter circadian rhythm. Detailed *in silico* analyses suggest that CLK95 binds to CLOCK:BMAL1 complex (performed by Onur Özcan (PhD candidate from Kavakli Lab, Koç University). In this thesis, I performed *in vitro* and *in vivo* studies to fully characterize the effect of the CLK95. CLK95 binds to CLOCK:BMAL1 complex, it also stabilizes the interaction between these two binding partners which results in nuclear accumulation of CLOCK and BMAL1 proteins. Our results indicate that the decreased amplitude and increased period is the result of increasing the amount of CLOCK and BMAL1 in the nucleus. This is because CLK95 changes the stoichiometric amount of the BMAL1 and CLOCK in the nucleus. Uses of CLK95 on CLOCK:BMAL1 could help us better understand the mechanisms behind circadian rhythm.

Chapter 2 of the thesis provides detailed information about circadian rhythms focusing on mammalian clocks. Additional information such as identified small molecule modifiers of circadian clock is also available in this chapter.

Chapter 3 of the thesis provides materials and methods employed in this research.

Chapter 4 of the thesis provides the results of the study.

Chapter 5 of the thesis provides a discussion about the observed results with concluding remarks.

Chapter 2:
LITERATURE REVIEW

2.1. Types of Biological Rhythms

From unicellular to multicellular, almost all organisms possess endogenous biological rhythms to cope with the environmental changes. These environmental changes could show daily, tidal, lunar, or seasonal cycles resulting from earth's rotation around its own axis and the sun, and its position to the sun and moon [22-24]. According to length of their periodicity, biological rhythms are classified into three categories. First category of biological rhythms is called infradian rhythms which oscillates with a period greater than 24 hours such as migration and hibernation of animals [25, 26]. Second category is called circadian rhythms which means "about a day" and shows approximately 24 hours oscillations. Sleep-wake cycles, metabolic and several hormonal regulations are under circadian control [9, 27]. The last category is called ultradian rhythms that exhibits less than 24 hours cycles for example urination, breathing and feeding shows ultradian rhythms [22, 25]. The research field that focuses on studying the biological timing of living organisms is called Chronobiology. Among the biological rhythms that were discussed previously, circadian rhythm gets the most attraction by the chronobiology researchers in these last decades.

2.2. Circadian Rhythms and Clocks

Almost all organisms have an innate timing system called circadian clock that allows them to anticipate daily environmental changes and impacts their fitness by influencing the physiological and behavioural decisions [1-8]. Biotic and abiotic environmental changes that shows periodicity are believed to enforce a selective pressure to organisms which eventually led circadian clock to evolve. It's highly conversed among species. Several examples of circadian rhythm can be found in nature [28].

In cyanobacteria, nitrogen fixation and photosynthesis show high circadian regulation. It has been shown that compared to wild-type strains of cyanobacteria, mutants with defective clock have lower fitness under competition [29-31].

Plants, one of the higher organisms, also show circadian behaviour. Considering that plants use photosynthesis for energy production, role of circadian rhythm cannot be underestimated. Because they can't change their location, they are more prone to get affected by the unfavourable environmental conditions. *Arabidopsis thaliana* is the most studied plant in circadian clock research. Light, hormones and ions can alter the period of its circadian clock [32, 33]. Studies have shown that at least 30% of mRNA expression of *A. thaliana* show circadian oscillations [34]. Plant immunity is also tightly associated with circadian clock. It has known that during day stomata of plants are bigger compared to night. While plants use these epidermal openings for gas exchange, they can also serve as passages for pathogens [35-38]. *A. thaliana* shows higher expression levels of pattern-triggered-immunity genes at dawn which is the perfect time for a pathogen to invade due to high humidity levels and opening of stomata [35, 39, 40]. In addition to that, while level of jasmonic acid (JA) which is responsible to defend plants against necrotrophs and herbivores peaks at the midday, activity of caterpillars gets highest level just before dusk. So, these two events correlate with each other. Studies have shown that, like mutant cyanobacteria strains that have defective clock, mutant plants with defective clock also exhibit lower fitness. Caterpillars favouring clock-deficient plants against wild-type plants is one of the best examples of this situation [41, 42].

Animals also have a hierarchically organized circadian clock which allows them to adapt to their niche. Food intake, regulation of metabolism, predator prey interactions are only a few examples of physiological and behavioural decision that are under control of circadian rhythm [43]. In fact, a study has shown that 43% of all protein coding genes in at least one tissue show circadian oscillations in mice which can state the importance of circadian rhythmicity in animals [15]. Loss of circadian rhythmicity can drastically change the survival chance of an animal. A research conducted on chipmunks has shown that clock deficient chipmunks are more prone to a prey for predators [44].

For a biological rhythm to be classified as a circadian, three common characteristics must be displayed. First, it should have a self-sustainable endogenous rhythm with a period of 24 hours. When put in constant conditions such as constant light or constant darkness, rhythmicity must be sustained. This phenomenon is known as free running. Free running periods can show variety from organism to organism (23.5 hours in mice, 25 hours in human) [45]. Secondly, it should exhibit temperature compensation which means the period length of the rhythms shouldn't differ under changing

temperatures [46]. A third characteristic is that it should be entrainable. External stimuli such as light can synchronize circadian rhythm [9]. Even though, circadian rhythms are free running, they can be aligned to environmental cues. The term Zeitgeber which literally means time-giver is used for such external stimuli that can entrain circadian clock.

Circadian time (CT) and Zeitgeber time (ZT) are standardized units of time. While CT is based on the intrinsic free-running period of rhythm, ZT is based on the period of a zeitgeber. CT0 and CT12 implies the beginning of subjective day and night, respectively. In 12:12 light:dark cycle, ZT0 is used for beginning of the day while ZT12 is used for beginning of night [47]. These terms are explained because circadian rhythms are generally plotted against these two units of time. A plot of circadian rhythm demonstrates features such as amplitude, phase, and period. Amplitude could be referred as distance between peak and through values. A large amplitude indicates a robust circadian rhythm. Phase on the other hand could be described as the timing of a refence point in the oscillation to a fixed event. Finally, period can be defined as the time required for a defined phase to repeat itself in an oscillation [22, 48]. All these features are fairly represented in Figure 2.1. As mentioned previously, when circadian rhythms are put under constant conditions such as constant darkness, they oscillate with a free running period. Depending on the time a Zeitgeber is introduced to such an oscillation, shifts in circadian rhythms can be seen. These shifts are categorized as phase delay or phase advance. While the period length increases in the phase delay, in phase advance period length of an oscillation gets shortened [48].

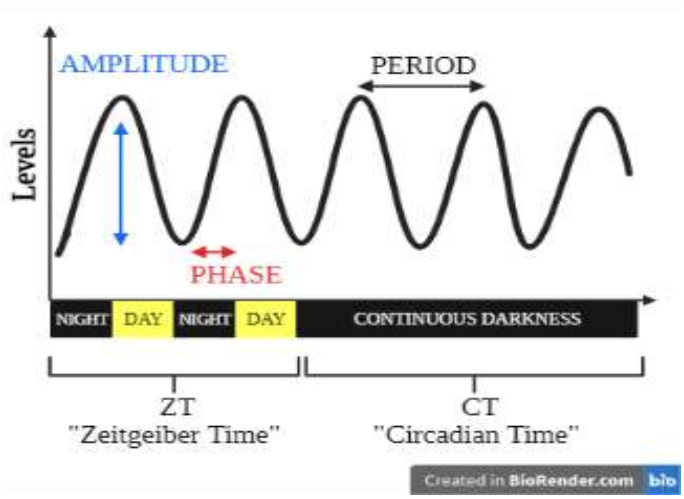

Figure 2. 1 Circadian rhythm parameter. Adapted from [48]

2.2.1. Milestones in the research field of Circadian Rhythms

The first ever noted experiment of this field is conducted by Jean-Jacques d'Ortous de Mairan in 1729. He observed, even under constant darkness, the leaves of the *Mimosa pudica* still opened at daytime and closed at night-time which implies the existence of an internal clock [22, 49]. However, he was not the first scientists that described the endogenous circadian clock. He concluded that the daily movements of the plant persisted in the absence of light. But his interpretation was that the plants were able to sense the Sun without ever seeing it. Later, Augustin Pyramus de Candolle, a Swiss botanist, brought the concept of an internal clock [50]. In 1832, he performed an experiment on *Mimosa pudica*. When he placed the plant in constant light condition, he observed that the opening and closing of the leaves had a period of approximately 22 hours which is 2 hours less than the 24 hours periodicity of Earth's light-dark cycle. He hypothesized that there must be an internal clock that is responsible for this rhythmicity.

After these two studies, researchers start to focus on circadian biology. Prominent scientists in the field such as Jurgen Aschoff and Colin Pittendrigh conducted several experiments and coined the terms like Zeitgeber and Aschoff's rules to chronobiology. But until 1971, no one had a clue about the molecular mechanism behind the endogenous circadian clock. The first discovered molecular component of the circadian clock was *Period* (*Per*) gene. It was discovered by Ronald J. Konopka and Seymour Benzer in 1971 [51]. They made screenings on mutant fruit files (*D. melanogaster*) to find clock genes. Experiments on the three of the selected mutants which exhibited either shorter (19 hours), longer (28 hours) or arrhythmic phenotype were revealed that all these mutations were mapped to same region on the X chromosome which later called period. Subsequently, Joseph S. Takahashi and his group identified the *circadian locomotor output cycles kaput* (*Clock*) gene in 1994 by screening mutant mice with defective clock [52]. Two years later, *CRYPTOCHROME2* (*CRY2*) were identified in humans by Aziz Sancar and his group [53, 54]. CRYs play an important role on mammalian circadian clock as shown in 1999 by Gijsbertus van der Horst and his group [55] [56]. Their result implied that while CRY1 deficiency shortens the period, CRY2 deficiency increases the period of circadian rhythm in mice. In addition to these findings, John B. Hogenesch and his group identified the last core-component of the mammalian circadian clock which is Brain and muscle Arntl-1 like (BMAL1) in 1997 [57, 58].

2.3. Mammalian Circadian Rhythms and Clocks

Mammalian circadian clock consists of a hierarchically ordered structure system. Suprachiasmatic nucleus (SCN) which resides in anterior hypothalamus of the brain and composes approximately 20,000 neurons, functions as the central circadian pacemaker [59]. It synchronises the peripheral clocks by receiving the light information from the environment. What happens is when intrinsically photoreceptive retinal ganglion cells (ipRGC) senses blue light, SCN gets stimulated through direct retinal innervation via retinohypothalamic tract (RHT) [60]. Studies have shown that in addition to ipRGCs, rod and cone cells also acts as photic input source to the SCN. Synchronization of SCN occurs when the expression of immediate early genes such as *Period1* (*Per1*) is induced through activating N-methyl-D-aspartic acid (NMDA) receptor and pituitary adenylate cyclase-activating polypeptide (PACAP) signalling pathway. Next, photically entrained SCN transmits signals to peripheral clock via both neural (sympathetic and parasympathetic) and hormonal pathways to synchronize them [17, 61].

There have been two major theories that tried the explain the relationship between SCN and the peripheral clocks. According to first theory, SCN and peripheral clocks have a master-slave relationship. This model gives all the power to SCN. It claims that the peripheral clocks cannot be synchronized by the external or internal stimuli if it's not coming from the SCN. Second theory which is referred as the orchestra model, claims that while SCN plays the role of conductor to guide for an efficient physiological output rhythm, and peripheral clocks can adapt their rhythm to its environment. This model portrays a more balanced relationship between SCN and peripheral clocks. Because there are several studies supporting the independence of peripheral clocks, second model is more accepted [62, 63].

2.3.1. Molecular Mechanism behind Mammalian Circadian Clock

Both central and peripheral clocks have the same operational unit of the mammalian circadian system which is called as cell-autonomous molecular oscillator [64, 65]. Interlocking transcription-translational feedback loops (TTFLs) drive that molecular oscillations [66]. The primary TTFL has a negative and a positive arm. The positive arm consists of CLOCK and BMAL1 proteins which belong to basic helix-loop-helix; Per-Arnt-Single-minded (bHLH-PAS) family. When produced, BMAL1 and CLOCK forms

a heterodimer that shuttles to nucleus to induce the expression of clock-controlled genes (CCGs) by binding to E-box motifs of them. *Cry* (*Cry1* and *Cry2*) and *Per* genes (*Per1*, *Per2*, and *Per3*) are also among to these clock-controlled genes [67-69]. They interact with each other to form a heterodimer. Then this complex interacts with casein kinase 1 isoforms (CK1 ε/δ) and translocates into nucleus to repress the transactivation by CLOCK:BMAL1 heterodimer. After repression, PER:CRY complex gets ubiquitinated by E3 ubiquitin ligases which eventually leads to its proteasomal degradation [70, 71].

Secondary TTFL is mainly about the regulation of *Bmal1* gene expression. *Bmal1* gene expression is regulated by retinoic acid receptor-related orphan receptors REV-ERBα/β and RORα/β/γ. The expression of *NR1D1/2* (encodes REV-ERBs) is driven by CLOCK:BMAL1 complex. When produced, they compete with RORs to bind to RORE elements of *Bmal1*. While binding of RORs to RORE elements of *Bmal1* results with activation of the gene, binding of REV-ERBs inhibits the gene expression [9, 13].

The last TTFL is composed of D-box binding protein (DBP) and nuclear factor interleukin 3 (NFIL3). While the transcription of *Dbp* is directly under control of rhythmic binding of CLOCK:BMAL1 complex to its E-box elements, transcription of *Nfil3* is under controlled by RORs and REV-ERBs. Like in BMAL1 gene expression control, they compete to bind to RORE elements of NFIL3 to regulate the transcription. When produced, DBP binds to D-box elements of the genes such as *Rora/b*, *Nr1d1/2* and *Per1/2/3* and activates their expression. On the other hand, binding of the NFIL3 to D-box elements of the genes leads to repression of the genes. Altogether these three interlocking TTFLs, can create transcriptional cycles with diverse expression phases depending on the cis elements of the target genes [13, 66].

In addition to transcriptional and translational controls, the circadian rhythm is also regulated by the posttranslational modification (PTM), translocation, and protein turnover rates of the core-clock components [14]. When core-clock components checked, various PTMs could be seen. Phosphorylation which is one of the most studied PTMs is especially important for PER2 stability. It's been shown that PERs are phosphorylated at different regions by casein kinase 1δ and 1ε. Subsequent to phosphorylation, PERs get ubiquitinated by β-transducin repeat-containing protein (β-TrCP) for proteasomal degradation. In addition to its effect on PER2 stability, it's also known that CK1-mediated phosphorylation is a necessity for PER1 nuclear entry [14, 72-74]. Beyond that,

modifications such as O-GlcNAcylation and acetylation could be seen in PERs. While acetylation increases the stability, O-GlcNAcylation shows dual effect on PER levels which are accelerated repression activity and decreased stability [14, 75, 76].

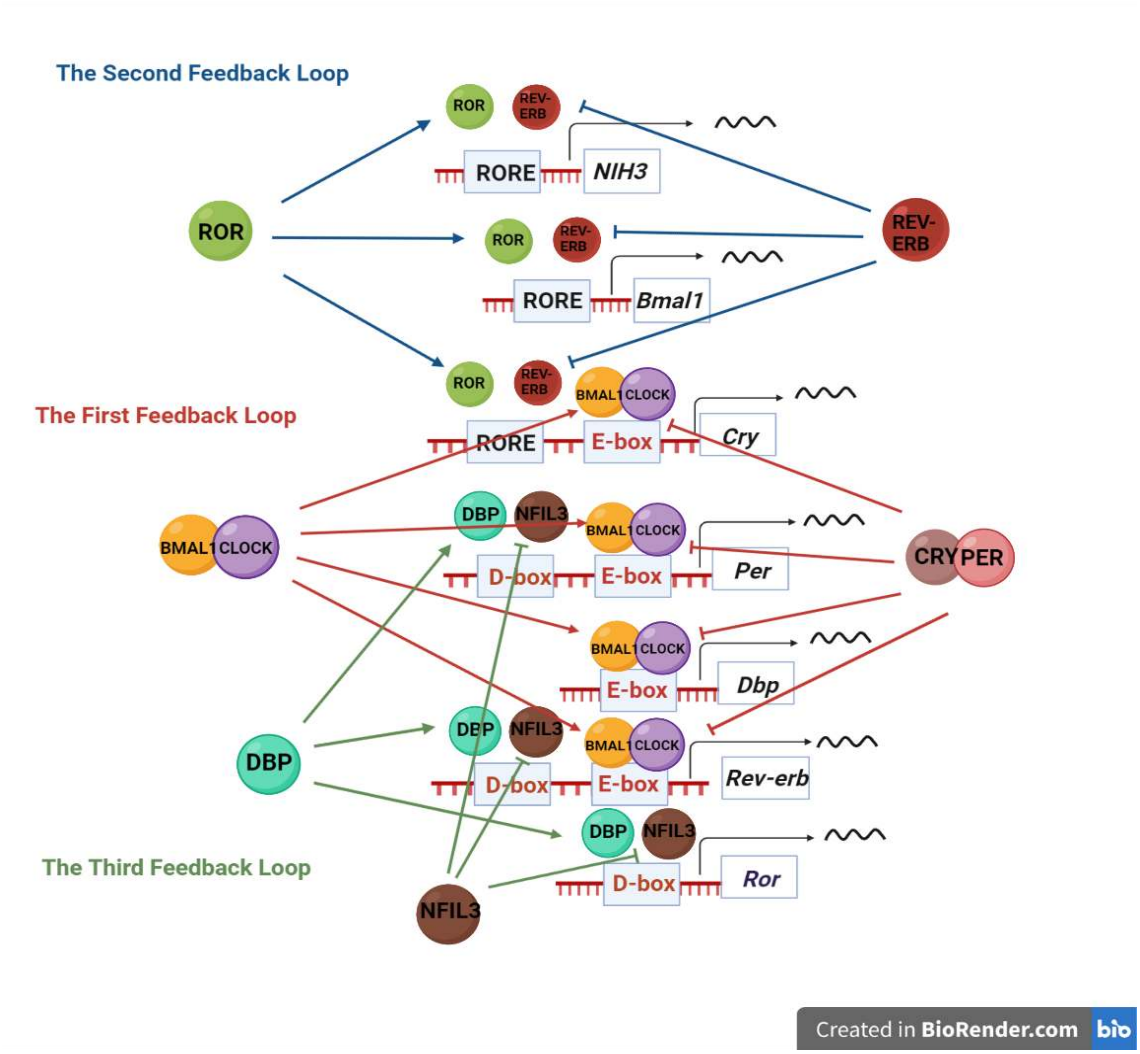

Figure 2. 2 Intervening TTLs of mammalian circadian clock. Adapted from [71]

Compared to PERs, CRYs exhibit higher repression activity on CLOCK:BMAL1 complex. For CRYs to show such a strong phenotype, PTMs are crucial. As in PERs, phosphorylation and ubiquitination are also very important for stability of CRYs. It's been show that phosphorylation by AMP-activated protein kinase (AMPK), primes CRY proteins for poly-ubiquitination [77]. If the poly-ubiquitination of CRYs were performed by SKP1-CUL1-F-box together with FBXL3 (SCF_{FBXL3}) in the nucleus, then the proteins get targeted by 26S proteasomes for degradation [78, 79]. However, if the ubiquitination

was done by FBXL21 in nucleus, an another E3 ubiquitin ligase, CRYs get stabilized. It's been shown that FBXL21 stabilizes CRY proteins by antagonizing the FBXL3 through ubiquitinating CRYs at different residues that eventually interferes binding of FBXL3. Besides that, if the CRYs get ubiquitinated in cytoplasm by FBXL21, degradation gets promoted [14, 80].

It's known that CLOCK doesn't oscillates at protein level. However, studies have shown that PTMs on this protein exhibits circadian cycling. They play important roles at regulating stability, localization, and degradation of the CLOCK:BMAL1 complex [14, 81]. Phosphorylation which is one of the many modifications of these complex, is vital for CLOCK:BMAL1 driven transactivation. Phospho-dead mutant forms of CLOCK and BMAL1 displays phenotypes such as increased stability but decreased transcriptional activity. Protein kinases such as GSK-3β and cyclin dependent kinase 5 (CDK5) are believed to phosphorylate CLOCK protein [82-84]. For BMAL1, only the phosphorylation by GSK-3 is known [85]. Except from phosphorylation, ubiquitination, SUMOylation, and O-GlcNAcylation were seen in both CLOCK and BMAL1. While ubiquitination and SUMOylation enhances the degradation and transactivation [86-89], O-GlcNAcylation increases the stability of the complex [90]. It has been also known that BMAL1 gets acetylated by CLOCK which harbours histone acetyltransferase (HAT) activity [91]. Acetylation of BMAL1 leads to CRY1 recruitment to complex which eventually results with repression of the transactivation. SIRT1 which is a deacetyl-transferase, deacetylates BMAL1 to remove the repression by the CRYs [92].

Preserving the 1:1 stoichiometric ratio between positive and the negative loops of the primary feedback loop is critical for robustness of the circadian rhythm [93]. Studies have shown that whenever stoichiometry between these two loops changed, alterations on circadian oscillations were observed. For instance, CLOCK and BMAL1 overexpression in MEF cells resulted with a dampened rhythm [94]. The same study suggested that observed dampened oscillation could be the result of the insufficient feedback inhibition of the positive arm that is relatively high. To support this theory, they also overexpressed the PER1 or PER2 in MEF cells. What they saw was the amplitude of the circadian oscillation enhanced compared to their controls which means the repression by the negative arm is vital for robust circadian rhythm.

Having the proper ratios of core-clock components is not enough. To fulfil their functions, these proteins should enter to nucleus so the correct phase of circadian rhythms could be maintained. CLOCK can only enter the nucleus when it forms a heterodimer with BMAL1. Unlike BMAL1, CLOCK neither have nuclear localization signal (NLS) or nuclear export signal (NES). It's reported that NLS and NES take place in N-terminal and PAS domains of BMAL1 [95-97]. In case of PERs, both NLS and NES sequences are present in all three isoforms. However nuclear accumulation of PERs is CRY dependent. As PERs enter nucleus, CRY1 which also have NLS sequence binds to them. This interaction protects PERs from both ubiquitin dependent proteasomal degradation and nuclear export [95, 98, 99].

2.3.2. Structure of Core-Clock Proteins

Except from CRY proteins, other core-clock components contain significantly similar domains that enable them to interact with each other and DNA. For instance, PAS domains can be found in CLOCK, BMAL, and PER proteins. Both CLOCK and BMAL1 proteins belong to bHLH-PAS-containing family of transcription factors and build from four similar domains. These domains are called PASA, PASB, bHLH and trans-activating domains (TAD). For BMAL1 to heterodimerize with CLOCK, interaction of corresponding domains of both CLOCK and BMAL1 is required. What it means is, PASA, PASB and bHLH domains of CLOCK interacts with PASA, PASB and bHLH domains of BMAL1, respectively. Also, bHLH domains plays role in DNA binding of CLOCK:BMAL1 complex. Though these domains are quite similar, they also have distinct characteristics which enable differential binding of PER and CRY proteins to CLOCK and BMAL1. While CLOCK has an overall negative electrostatic potential, BMAL1 has an overall positive electrostatic potential. In addition to that, spatial arrangements of PASA and bHLH domains of CLOCK and BMAL1 also show difference [100, 101].

CRY proteins are composed of a photolyase homology region (PHR) and a C-terminal tail which shows high variability. CRYs heterodimerize with PERs through interaction between C-terminal of PER proteins with coiled-coil (CC) helix of CRY proteins [102, 103]. Heterodimerization of CRY and PER proteins is further stabilized through binding of zinc ion to C1210 and C1213 residues of PER2 along with C432 and

H491 residues of CRY2 [104]. C-terminal tail, secondary pocket, and FAD-binding pocket of CRYs are important for its interactions with BMAL1, CLOCK, and FBXL3/21 respectively [78, 104-106]. The more recent studies have revealed Serine (Ser) loop at the gate of secondary packet selectively regulates CRY1's binding to CLOCK [107, 108].

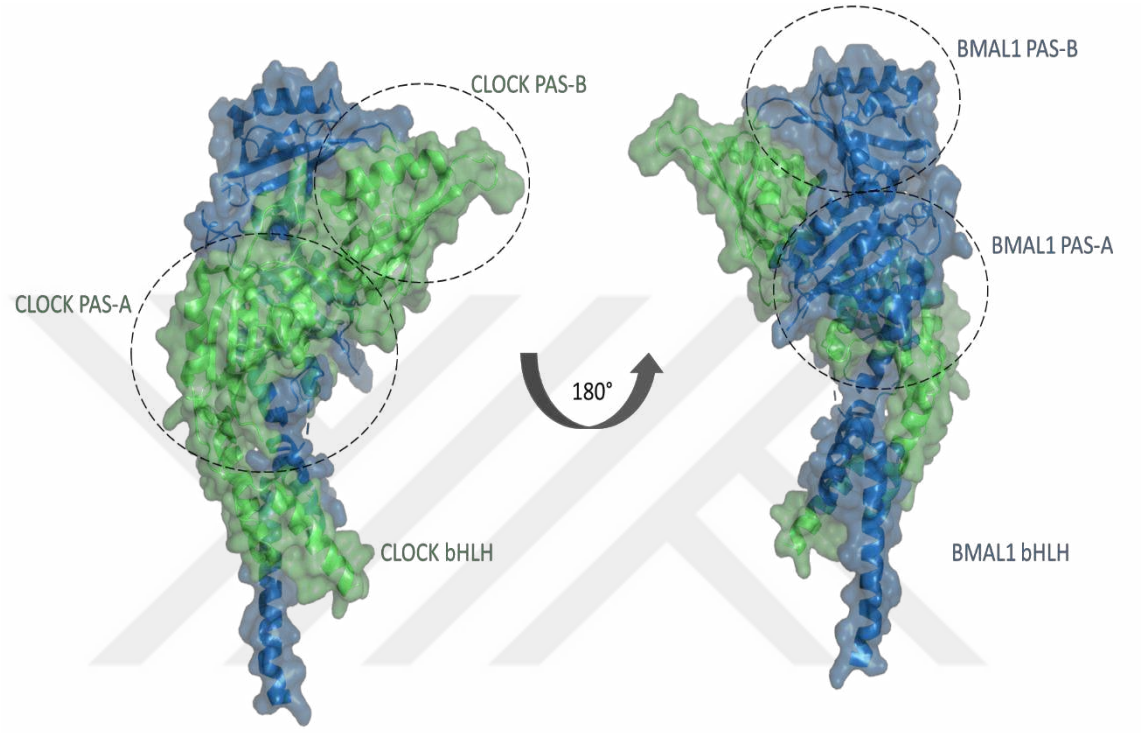

Figure 2. 3 Crystal structure of mouse CLOCK and BMAL1. Adapted from [101].

2.4. Relationship between Circadian Rhythm and Diseases

As mentioned before, circadian clock plays a huge role in well-being of organisms. Disruption of it can lead to serious pathologies such as metabolic and cardiovascular diseases, cancer, sleep disorders and Alzheimer's disease. For instance, recent studies have reported that the night shift workers carry a greater potential to develop diabetes, obesity, metabolic syndromes, cardiovascular diseases, and breast cancer [16-19, 109, 110].

Clock$^{\Delta 19}$ is a mutant version of *Clock* gene that encodes CLOCK proteins with 51 amino acids less at the C-terminus. While mutant CLOCK preserved its ability to heterodimerize with BMAL1, it's lost its transactivation ability [52]. Experiments conducted on mice with mutant *Clock*$^{\Delta 19}$ transgene revealed that these animals later

developed obesity, and metabolic syndrome with insulin resistance. Also, this mutation caused a prominent decrease in pancreatic islets which resulted with low levels of insulin secretion and weakened glucose tolerance. Another study also showed that mutant *Clock*$^{\Delta 19}$ transgene carrying mice exhibited enhanced cholesterol levels [111-113].

BMAL1 is as known as a binding partner of CLOCK. However, deficiency of BMAL1 doesn't exhibit same phenotypes as seen in the case of the CLOCK mutant mice. Studies indicated that *Bmal1*-null mice exhibited phenotypes such as premature aging, pancreatic β-cell dysfunction, disruption of mitochondrial respiration in liver tissues, lower blood glucose levels and attenuated blood triglyceride [112-117].

CRYs and PERs also effect metabolism of mice. Studies have shown that *Per2*-null mice displayed obesity due to regulatory effect of PER2 on lipid metabolism [118, 119]. The regulation of lipid metabolism was belied to be the result of repressor activity of PER2 on PPARγ which is nuclear receptor in adipogenesis [119]. On the other hand, *Cry1/2*-double knockout mice but display reduced body weight and enhanced cortisone levels [113, 120]. All these findings on mice underline the importance of that clock components for healthy metabolism. Finally, mutations in *CRY1* are associated with familial delayed sleep phase and attention deficit/hyperactivity disorders [121, 122].

Associations between cancer and circadian clock are highly researched. Studies have revealed that core-clock proteins could associate with cancer. Elevated levels of tumour growth were observed on *Per2* mutant mice [123]. In addition to that, another study revealed that *Per1* overexpression could synthesize the human cancer cells to DNA damage-induced apoptosis. This result is believed to be triggered by interactions of PER1 with DNA damage checkpoint proteins ATM and CHK2 [124]. *Bmal1* depletion resulted with enhancement of tumour growth and cell proliferation with reduced expression levels of *Wee1* and *p53* genes [125]. Also, Sakamoto et al. showed that *CLOCK* and *BMAL1* overexpression inhibits cancer cell growth by preventing cell proceeding from G1 to S phase [126]. Opposite to other core-clock proteins, mutations on CRYs proteins protects p53-null mutant mice from early onset of cancer. Later it was shown that CRY mutation leads to tumour necrosis factor α (TNFα)-initiated apoptosis [127, 128].

Sleep disorders born from circadian clock dysregulations can be caused by mutations on endogenous clock or change in environmental factors. They are categorized as delayed sleep disorders, advanced sleep disorders, irregular sleep-wake rhythm, and

non-24 hours-sleep-wake disorders [129]. It has been shown that variable-number tandem repeat polymorphism in *PER3* gene causes familial cases of delayed sleep disorders [130]. Also, S662G mutation in PER2 or T44A mutation on CK1δ results with familial advanced sleep phase syndrome (FASPS) [131, 132]. Change in environmental conditions such as traveling to a place with different time zone can also introduce chronic sleep and mood irritations.

2.5. Small Molecules as Circadian Modifiers

Considering the importance of circadian clock to human life and increasing risks of circadian impairment due to modern lifestyle, number of studies that focused on manipulating the circadian machinery to ease the problems that could arose with the circadian misalignment is increased. Use of small molecules that targeting clock proteins is one of the ways to modulate circadian rhythmicity. Compared to gene knockout and RNAi-based techniques, small molecules offer a temporal control of circadian rhythmicity. They have many advantages such as their allosteric effect on target proteins are reversible, could be controlled by time, and the dose of circadian effect can be determined with the change of small molecule concentrations [20].

There are two approaches that were harboured by researchers to identify small molecule modifiers of circadian clock. The first method is high-throughput screening of small molecule libraries on luciferase expressing cell lines (*Bmal1*-d*Luc* or PER2::LUC). After cells synchronized with forskolin or dexamethasone, treatment with small molecules was performed and bioluminescence signalling was recorded for 5-7 days. Molecules altering phase, period and/or amplitude were selected as candidates. Many molecules have been identified using this method. However, except from KL001 which is a CRY stabilizer, none of the molecules identified with high-throughput screening, targets core-clock proteins [20, 133]. Along with availability of crystal structures of circadian proteins, structure-based approaches started to use on identification of small molecule modifiers of circadian clock. In this approach, molecules were first screened according to their affinity to target protein, in-silico. Then the molecules were tested on luciferase expressing cell lines and categorized according to their effect on circadian rhythm. CLK8, which is a CLOCK targeting molecule, is identified using this method [21].

Second approach for identification of small molecule modifiers is evaluating the direct interaction of small molecule with target protein. For instance, when effect of IC261 which is a known CK1 inhibitor was tested on circadian rhythm, increase on period length was observed [73]. There are also other molecules that were later identified as circadian modifiers due to their known effect on GSK-3β, and REV-ERBα (Table 2.1). A recent study used virtual screening on the CRY1 FAD binding packet and discovered a small molecule (M47) that interacts with the CRY1 Arg-293 and Trp-399 amino acid residues [134]. The FAD-binding pocket is required for the FBXL3 (E3 ligase) interaction [135], which controls the ubiquitination of CRYs [135, 136]. M47 lowers the half-life of CRY1 by increasing its ubiquitination, which lengthens the circadian period (Table 1), according to other experimental investigations [134].

Table 2. 1 A table of selected small molecule modifiers of circadian clock. Adapted from [20, 21, 137]

Name	Target Protein	Categorized as	Reference
CLK8	CLOCK	Amplitude enhancer	[21]
KL001	CRYs	Period lengthener	[133]
KS15	CRYs	Amplitude reducer	[138]
M47	CRY1	Period lengthener	[134]
KL101	CRY1	Period lengthener	[137]
TH301	CRY2	Period lengthener	[137]
GSK4112	REV-ERBα	Amplitude reducer	[139]
SR9011/SR9009	REV-ERBs	Amplitude reducer	[140]
PF-4800567	CKIε	Period lengthener	[141]
LH846	CKIδ	Period lengthener	[142]
IC261	CKIδ/ε	Period lengthener	[73]
CK01	CKIδ/ε	Period lengthener	[143]
CKI-7	CKIδ/ε	Period lengthener	[144]
D4476	CKIδ/ε	Period lengthener	[145]
PF-670462	CKIδ/ε	Period lengthener	[146]
PD169316	CKIδ/ε, P38	Period lengthener	[147]
SB202190	CKIδ/ε, P38	Period lengthener	[147]
Roscovitine	CKIδ/ε, CDK	Period lengthener	[147]
Longdaysin	CKIδ/α, CDK7, ERK2	Period lengthener	[148]
Cmpd-1	CKIε, CKIδ?	Period lengthener	[149]
Cmpd-2	CKIε, CKIδ?	Period lengthener	[149]
Cmpd-3	CKIε, CKIδ?	Period lengthener	[149]
SU5416	CKIδ/ε, VEGFR PTK	Period lengthener	[147]
TG003	CKIδ/ε, CLK	Period lengthener	[147]
DRB	CKIδ/ε, CK2	Period lengthener	[147]
PPT	CKIδ/ε, ERα agonist	Period lengthener	[147]
SP600125	CKIδ/ε, JNK	Period lengthener	[147]
CGS-15943	CKIδ/ε, AR agonist	Period lengthener	[147]

Chapter 3:
MATERIALS AND METHODS

3.1. Cell Culture

Human embryonic kidney cells (HEK 293T), human osteosarcoma cell line stably express destabilized luciferase under the control of the *Bmal1* promotor (U2OS *Bmal1*-d*Luc*), NIH 3T3 cell line stably express destabilized luciferase under the control of the *Bmal1* promotor (NIH 3T3 *Bmal1*-d*Luc*), wild-type mouse embryonic fibroblast cells (WT MEF), *Clock*-knockout mouse embryonic fibroblast cells (MEF *Clock* KO) and/or *Bmal1*-knockout mouse embryonic fibroblast cells (MEF *Bmal1* KO) were used in different experimental setups. Cells were maintained at 37°C under 5% CO_2 in a 95% humidified incubator using D10 medium (Dulbecco's Modified Eagle Medium with high glucose supplemented with 10% heat-inactivated Fetal Bovine Serum (Thermo Scientific) and 100µg/mL Penicillin/Streptomycin cocktail). Subculturing was regularly performed when cells reached confluency, or the medium was depleted of nutrients. First, the old medium was aspirated, and the cells were washed with 5mL 1X PBS. Then the cells were trypsinized with 1mL Trypsin-EDTA (Multicell, Cat. No 325-043-EL) solution. After incubation with Trypsin-EDTA for 2 min to detach the cells, D10 medium was added to cells for trypsin inactivation. Subsequently, cells were split into new 10cm cell culture dishes with a 1:6 ratio.

3.2. Cell Cytotoxicity Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability in NIH 3T3 *Bmal1*-d*Luc* and U2OS *Bmal1*-d*Luc* cells. To perform this calorimetric assay, cells were seeded as triplicates into a clear 96-well plate ($4x10^3$ cells/well) and incubated for 48 hours at 37°C in a humidified incubator under 5% CO_2. After incubation cells were treated with appropriate amounts of CLK95 (control cells were treated with 0.25% DMSO) and incubated for another 48 hours at 37°C under 5% CO_2. Then, cell media was replaced with MTT reagent (final concentration: 1mg/mL)/DMEM mix. After 4 hours incubation, the media was exchanged with

DMSO:EtOH (1:1) mixture to dissolve formazan salts to measure absorbance (A) of the wells at 600 and 690nm by Synergy H1 Microplate Reader (Bio-Tek Instruments). The cell viability was calculated according to Equation 3.1. by using recorded absorbance values.

$$\%\ Cell\ viability = \frac{(A600nm - A690nm)sample}{(A600nm - A690nm)DMSO} \times 100$$

Equation 3.1. Cell viability calculation formula.

3.3. Protein Degradation Assay

Protein degradation assay was executed in transiently transfected HEK 293T cells which were seeded as triplicates into an opaque 96-well plate ($4x10^4$ cells/well). To carry out this experiment, reverse transfection technique was employed. Depending on the experiment, cells were transfected with PEI (final concentration: 6ng/µL) using either 4ng pcDNA4-*Luc* plasmid or 50ng pcDNA4-*Bmal1*:*Luc* with or without 250ng pCMV-Sport6-*CLOCK* plasmids. For each condition, maximum plasmid amount was limited to 300ng. If the total plasmid amount was below 300ng, empty pCMV-Sport6 plasmid was used to achieve the desired plasmid amount. After reverse transfection, cells were treated with appropriate doses of CLK95 and incubated at 37°C under 5% CO_2. Next day, medium of the cells was supplemented with HEPES-NaOH (final concentration: 10mM, pH 7.2) and Luciferin (final concentration: 0.4mM) and further incubated for an hour at 37°C under 5% CO_2. Then, cells were treated with CHX (final concentration: 20µg/ml) to inhibit protein synthesis. Finally, the plate is sealed with an optically clear film and placed to Synergy H1 Microplate reader to record luminescence readings every 15 minutes at 32°C for 24 hours. To calculate the protein half-life, one-phase exponential decay function was used.

3.4. Real-time Monitoring of Circadian Oscillation

NIH 3T3 *Bmal1*-d*Luc* and U2OS *Bmal1*-d*Luc* cells were used for real-time monitoring of circadian oscillation. While LumiCycle 32 (Actimetrics, USA) was used for recording the luminescence signals of NIH 3T3 *Bmal1*-d*Luc* cells, Synergy H1 (BioTek, USA) was used for recording the luminescence signals of U2OS *Bmal1*-d*Luc* cells. NIH 3T3 *Bmal1*-d*Luc* cells were plated in 35mm cell culture dish at density of 45×10^4 cell/plate and incubated overnight at 37°C under 5% CO_2. The next day, cell medium was replaced with 0.1µM Dexamethasone (Sigma D-8893) containing DMEM to synchronize the cells. After 2 hours incubation, cell media was exchanged with appropriate amount of CLK95 containing recording medium (10g low glucose DMEM powder (Sigma D2902), 3.5mg/mL D(+) glucose powder (Sigma G7021), 0.35mg/mL sodium bicarbonate (Sigma S5761), 10mM HEPES (Gibco 15630106), 5%FBS (Gibco, 16000044), 1% NEAA (Multicell 321-011-EL), 25U/mL streptomycin-penicillin (Gibco 15140-122) which was supplemented with 0.1mM Luciferin. Then plates were sealed with silicone grease to prevent evaporation and immediately placed in LumiCycle. This instrument continuously recorded the luminescence for each plate for 5-7 days with 10 minutes intervals at 37°C. LumiCycle Analysis software (Actimetrics) was used to analyse the changes in circadian rhythm. The data of the first day was excluded from analysis due to transient deviations caused after medium change.

For the experiments that were performed using Synergy-H1, U2OS *Bmal1*-d*Luc* cells were plated as triplicates into an opaque 96-well plate (5×10^4 cell/well). After synchronizing and treating the cells with CLK95, opaque 96-well plate was sealed with an optically clear film and placed under Synergy-H1 instrument. Luminescence signals were recorded for 5-7 days with 24 minutes intervals at 32°C.

3.5. Lentivirus Production and Transduction

Wild type (WT), *Clock* knockout (KO), and *Bmal1* KO mouse embryonic fibroblast (MEF) cells were transduced with *Bmal1*-d*Luc* lentiviral particles to enable us to monitor the effect of CLK95 on circadian oscillation. To this end, virus particles were produced as follow: HEK 293T cells were transfected with 10µg pLV6-*Bmal1*-d*luc*, 9µg pCMV-ΔR8.2dvpr packaging and 1µg pCMV-VSVG envelope vectors at ~70-80%

confluency. The plasmid cocktail was mixed with 20µL PEI in 400µL DMEM and incubated at room temperature for 10 minutes and cells were transfected. After 16 hours of incubation at 37°C under 5% CO_2, 3mL fresh D10 medium were added to plates. 48 hours after transfection, cell medium was replaced with 8mL fresh D10 medium. Lentiviral particles were harvested at 72 and 96 hours post transfection. Collected particles were filtered through 0.45µM syringe filter and aliquoted (750µL) for storage at -80°C for further use.

WT, *Clock* KO, and *Bmal1* KO MEF cells were transduced with *Bmal1*-d*luc* lentiviral particles as follow: Cells were seeded in 35mm cell culture plates at the density of 1×10^5 cell/plate. Next day, cells were transduced with lentiviral particles by replacing the cell medium with virus-containing master mix which was prepared by mixing the virus-containing aliquots (750µL) with 250uL DMEM and 8µg protamine sulphate. 16-hours after incubation, cell media was replaced with 1.5mL fresh D10. 72-hours post-transduction cells were synchronized with Dexamethasone (0.1µM) and prepared for luminescence recording of the circadian oscillation as indicated at section 3.4

3.6. Pull-Down Assay

To perform pull-down assay HEK 293T cells were transiently transfected with either 10µg pCMV-Sport6-*CLOCK* or 10µg pCMV-Sport6-*Bmal1* plasmids. First, cells were seeded in a 10cm cell culture dish at a density which will give ~70-80% confluency next day. Then cells were transfected with 10µg of the appropriate plasmid using 30µL PEI in 1mL DMEM. Cells were harvested 24 hours post-transfection and divided into four 1.5mL centrifuge tubes. The conditions that were employed to probe the binding of the molecule to proteins contained a)only CLOCK overexpressed, b)only BMAL1 overexpressed and c)CLOCK-BMAL1 overexpressed mixtures. Pellet for each condition was lysed with 500µL Lysis Buffer (50mM Tris-HCl pH 7.4, 2mM EDTA, 1mM $MgCl_2$, 0.2% NP-40, 1mM Na_3VO_4, 1mM NaF, 1X PIC) and incubated on ice for 20 minutes. Then samples were centrifuged at 7000xg for 10minutes at 4°C. The supernatants of the CLOCK and BMAL1 lysates were mixed at 1:1 ratio. Following this step, collected lysate mixture was mixed with 2X binding buffer (100mM Tris-HCl pH 7.4, 300mM NaCl, 0.2% NP-40, 2mM Na_3VO_4, 2mM NaF, 2X PIC) at 1:1 ratio. While 5% of the mixture

was kept as input, the remaining part was divided equally into three separate microcentrifuge tubes and incubated by continuous mixing with either DMSO, or 20µM biotinylated-CLK95 (bCLK9) with or without 200µM non-biotinylated CLK95 (competitor) at 4°C for 90 minutes. Meanwhile, the NeutrAvidin Agarose resin (Thermo Scientific 29201) was equilibrated (30µL settled resin/sample). Equilibration was performed according to manufacturer's protocol. Lysates were supplemented with equilibrated NeutrAvidin resin and incubated for 24 hours at 4°C with constant shaking. The next day, samples were centrifuged at 2500xg for 2 minutes and then supernatants were discarded. The resin of each sample was washed 3 times with 1X binding buffer for 5 minutes with constant mixing at 4°C. Subsequently, resin of each sample was boiled with 1xLaemmli buffer for 7 minutes at 95°C for SDS-PAGE analysis. After samples subjected to the SDS-PAGE, proteins were transferred to PVDF membrane (Millipore) for Western blot study. Upon completion of transferring proteins, the membrane was incubated with 5% milk powder in 0.15% TBS-T for an hour. Then the membrane was incubated with anti-CLOCK (Bethyl, A302-618A) and anti-BMAL1 (Santa Cruz Biotechnology, SC-365645) antibodies diluted in 5% milk powder in 0.15% TBS-T. Finally, membrane was washed three times with TBS-T and incubated with secondary antibodies conjugated wit with HRP. Membranes were subjected to homemade ECL, and images were taken with chemiluminescence (Bio-Rad).

3.7. Site Directed Mutagenesis

To investigate the binding sites of the CLK95 on CLOCK and BMAL1, promising residues which were computationally determined to interact with small molecule were mutated via site-directed mutagenesis. Computational study revealed that R126 of the BMAL1 and F80-K220 residues of the CLOCK were important for the binding of the CLK95. These amino acid residues were introduced to *Bmal1* and *CLOCK* genes by PCR based mutagenesis as previously described [21]. FLAG-tagged pcDNA4a-*Bmal1* and pCMV-Sport6-*Bmal1* were used as template DNAs. Each PCR reaction consisted of 50ng of template DNA, 0.2µM primer pair, 0.2mM dNTPs and 3 units of Pfu DNA polymerase. Then the PCR reaction was performed under this conditions: 5minutes at 98°C followed by 12 cycles of 1 minute at 98°C, 1 minute at 55°C and 15 minutes at 72°C. The PCR reaction completed with a final extension step at 72°C for 15 minutes.

PCR products were analysed with 0.8% of agarose. Bands at the right size were further treated with 1µL FastDigest Dpn I (Thermo Scientific, FD1704) for 1 hour at 37°C to digest methylated-parental DNA. Subsequently, DH5$_\alpha$ (a strain of *E. coli*) cells were transformed with the 7µL PCR product. Standard heat-shock transformation protocol was used. Transformed cells were plated on Ampicillin (100µg/mL) containing Luria-Bertani (LB) agar plates and incubated overnight at 37°C. Later, colonies formed on the LB ager plate were selected to culture in 5mL LB broth containing Ampicillin (100µg/mL). After overnight incubation at 37°C, cells were harvested and plasmid isolation was performed with Macherey Nagel's NucleoSpin Plasmid (NoLid), Mini kit. Isolated plasmids were then sent for Sanger sequencing (Macrogen) to confirm desired single-site mutation.

3.8. Co-immunoprecipitation (Co-IP)

To assess the effect CLK95 on BMAL1-CLOCK interaction Co-IP was employed as previously described [21]. Briefly, HEK 293T cell were transfected with FLAG-tagged pcDNA4a-*Bmal1* or pCMV-Sport6-*CLOCK* plasmids. The FLAG-tagged pcDNA4a-*Bmal1*-R126A and pCMV-Sport6-*CLOCK*-F80A-K220A mutants were used in this assay. The R126 residue in the BMAL1 and F80-K220 residues in the CLOCK were identified to be important for the interaction of CLK95 by computational analysis. Therefore, these mutations were used as negative control during the Co-IP studies. After the transfection cells were harvested and were lysed with 500µL Lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% Triton X-100, 1X PIC). After centrifugation at 15000xg for 20 minutes, supernatants were transferred into new tubes. The protein concentration of each lysate was determined with the Pierce™ 660nm Protein assay (Thermo Scientific, 22660). For each fraction 150µg protein was used with a 2:1 ratio of CLOCK and BMAL1 proteins, respectively. CLOCK and BMAL1 lysates were and divided into two fractions which were treated with DMSO and CLK95. BMAL1-FLAG and BMAL1-R126A-FLAG were precipitated with Anti-Flag M2 Affinity Gel (Sigma-Aldrich, A2220). Then, the bound proteins were eluted with 30µL 4X Laemmli buffer at 95°C for 7 minutes after washing the resin with 1X TBS buffer (3 times). After quick centrifuge, 15µL of each sample was subjected to 8% SDS-PAGE following by Western blot as described above using anti-CLOCK and anti-FLAG (Sigma, A9469-1MG) antibodies.

3.9. Transactivation Assay

To confirm Bmal1-R126A mutation has no effect on its binding to CLOCK, transactivation assay was employed. HEK 293T cells were transiently transfected via reverse transfection with 125ng pCMV-Sport6-*CLOCK*, 50ng pGL3-*Per1*::*Luc* and 50ng pcDNA4a-*Bmal1* (wild type) or 50ng pcDNA4a-*Bmal1* (R126A) and 3ng of pGL4-Renilla (for normalization). Cells were seeded as triplicates into an opaque 96-well plate ($4x10^4$ cells/well) and maximum 300ng plasmid was used for each condition (completed with 72ng pCMV-Sport6 plasmid). 19 hours after incubation at 37°C under 5% CO_2, cells were lysed with Firefly Luciferase buffer and measured by Fluoroskan Ascent. (Final concentration: 5mM DTT, 0.2mM Coenzyme, 0.15mM ATP, 1.4mg/mL Luciferin, 0.03M Tris-HCl, 0.01M Tris-base, 25Mm NaCl, 1mM $MgCl_2$, 0.08%Triton X-100). After recording the firefly luciferase activity, renilla luciferase activity was measured by incubating the mixture with 1X renilla luciferase buffer which was diluted from main stock of 3X renilla luciferase buffer (0.06mM PTC124 (in DMSO), 0.01mM h-CTZ(in ethanol), 45Mm Na_2EDTA, 30mM $Na_4P_2O_7$, 1.425M NaCl) for 10minutes at room temperature. Relative luciferase activity (RLU) which was normalized to Renilla was calculated according to Equation 3.2.

$$RLU = \frac{Average\left(\frac{Firefly}{Renilla}\right)sample}{Average\left(\frac{Firefly}{Renilla}\right)DMSO}$$

Equation 3.2. RLU calculation formula.

3.10. The effect of CLK95 on core clock protein

The effect of CLK95 on core-clock proteins was assessed both on synchronized and unsynchronized U2OS *Bmal1*-d*Luc* cells. Cells were seeded into 6 well-plates with a density of 45×10^4 cell/well and incubated at 37°C under 5%CO_2 for 16 hours. For unsynchronized cells, they were treated with appropriate amounts CLK95 (final 0.25% DMSO) and further incubated for 24 hours. For synchronized cells, cell media was exchanged with 0.1µM Dexamethasone containing DMEM. After synchronization, cells were subjected to the CLK95 (final 0.25% DMSO). After 24 hours of incubation, cells

were harvested with 6 hours intervals for 7 time points (24H-60H). Then harvested cells were lysed with 90µL RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton x-100, 0.1% SDS, 1X PIC) and left for an incubation on ice for 30 minutes. Following, samples were centrifugated at 15000xg for 20 minutes at 4°C. After boiling samples with Laemmli buffer for 7 minutes at 95°C, 10µg of protein was loaded for each sample to 10% SDS-polyacrylamide gel. For Western blot analysis following antibodies used to determine the level of core clock proteins: anti-CLOCK(Bethyl, A302-618A), anti-BMAL1(Santa Cruz Biotech., SC-365645), anti-PER2(Proteintech, 67513-1-Ig), anti-CRY1(Bethyl, A302-614A), and anti-β-Actin (Cell Signaling, 8H10D10) antibodies.

3.11. Total RNA Isolation and RT-qPCR

The effect of small molecule on core-clock genes were explored through RT-qPCR. TRIzol reagent (Invitrogen, Cat. No. 15-596-018) was used for RNA isolation from unsynchronized and synchronized U2OS *Bmal1*-d*Luc* cells. After resuspending cells in 1mL TRIzol reagent, 200µL chloroform was added to each sample. Then the mixture was incubated at room temperature for 5 minutes. Samples were centrifugated at 14000xg for 20 minutes at 4°C after completing the incubation phase. Newly formed aqueous phase was transferred to a new microcentrifuge tube and mixed with 1:1 ratio with isopropanol and 12µg glycogen for each sample. After that, mixtures were incubated at -80°C for 1.5 hours. Next, samples were centrifugated at 14000xg for 20 minutes (4°C) and the pellets that were formed at the bottom of the tubes were washed with 1mL of 75% ethanol for 3 times. At the end of each wash step, centrifugation at 14000xg for 5 minutes (4°C) was done. Supernatants were discarded and the pellets were left for air-dry at room temperature following last wash. Then, RNA pellets were dissolved in 30µL nuclease-free water. Quantity and the quality of RNAs were controlled with Nanodrop 2000 (Thermo Scientific) and agarose gel electrophoresis, respectively. Agarose gel electrophoresis was performed at 100 Volt (V) for 15 minutes using 0.8% agarose gel which was prepared with DEPC (diethylpyrocarbonate) treated water.

Following RNA isolation, total RNAs were converted into cDNA using Thermo Scientific's RevertAid First Strand cDNA Synthesis Kit as described in product's manual. Each reaction consists of 0.5µg RNA template, 5µM Oligo$(dT)_{18}$ primer, 1X Reaction

Buffer, 1U/µL RiboLock RNase Inhibitor, 1mM dNTP mix, and 10U/µL RevertAid M-MuLV RT. Then, For Real-Time PCR (qPCR), cDNAs were further diluted with nuclease-free water and the qPCR experiments were conducted with 1:40 diluted cDNAs.

For qPCR assay, reaction mixture containing 10µL 2X SensiFAST SYBR® No-ROX Mix (Bioline), 1µL forward and reverse primer mix (final concentration 0.5µM from each), 6µL nuclease-free water, and 3uL 1:40 diluted cDNA was prepared for each sample. The complete list of the primers is given in Appendix B. *RPLP0* (ribosomal protein, large, P0) was used as the reference gene. After qPCR reaction mixtures were prepared, following cycling protocol was performed using CFX Connect Real-Time PCR detection system (Bio-Rad). 7 minutes of initial denaturation at 95°C followed by 40 amplification repeats which subsequently contained, 8 seconds at 95°C, 10 seconds at 60°C and 20 seconds at 72°C. At the end of each amplification step, fluorescence signals were recoded. After completing amplification steps, reaction mixtures were incubated 8 seconds at 95°C, 5 seconds at 70°C, and 5 seconds at 90°C, respectively. Last fluorescence recording was done in this step after incubating at 70°C for 5 seconds. The results were analysed with the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method.

3.12. Cycloheximide (CHX) Chase Experiment

To investigate if the small molecule has any effect on stability of CLOCK and BMAL1, Cycloheximide-chase experiment was conducted. At first day, HEK 293T cells were seeded into 6-well plates with a density of 37.5×10^4 cell/well. Next, these cells were transiently transfected with 65ng FLAG-tagged pCMV-Sport6-*CLOCK*, 875ng FLAG-tagged pcDNA4a-*Bmal1*, and 60ng pCMV-Sport6 by 3µL PEI. 24 hours after transfection, cells were treated with either DMSO (0.25%) or 5µM small molecule. 48hours after transfection, cells were treated with 100µg/mL CHX to stop protein synthesis. Following CHX treatment cells were harvested with 3 hours intervals for 12 hours. Cells harvesting and protein isolation were executed as previously described in Protein isolation and Western blotting section. Selective immunodetection was performed via anti-FLAG (Sigma-Aldrich, A9469-1MG) and anti-β-Actin (Cell Signaling, 8H10D10) antibodies.

3.13. Subcellular Fractionation

Subcellular fractionation was performed to explore if CLK95 effects the subcellular localization of core-clock proteins. U2OS *Bmal1*-d*Luc* cells were plated with a density of 40×10^4 cell/well in 6 well plate, a day prior to small molecule treatment. Following, cells were treated with either DMSO (0.25%) or 5µM CLK95. 24 hours after small molecule treatment, cells were harvested to get fractionated. Cells were harvested with 1mL ice cold- 1X PBS, and pelleted at 5000xg for 7 minutes at 4°C. After discarding supernatants, cells were resuspended with 200µL Cytosolic Lysis Buffer (CLB) (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA pH 8.0, 0.05% NP-40, 1X PIC) and incubated on ice for 10 minutes. Then, samples were centrifuged at 660xg for 5 minutes (4°C). While supernatants were transferred to new centrifuge tubes as cytosolic fractions, pellets were washed with 200µL CLB buffer for 3 times. At the end of each wash, samples were centrifugated at 660xg for 5 minutes at 4°C. Next, cell pellets were resuspended in 100µL Nuclear Lysis Buffer (NLB) (20mM HEPES pH 7.9, 0.4M NaCl, 1mM EDTA, 10% Glycerol, 1X PIC) and subjected to sonication (60% power, 10 seconds, 3 cycles) (BANDELIN SONOPULS HD 2070 homogeniser) for 2 times. This step was followed by centrifugation at 15000xg for 5 minutes with the cytosolic fractions. Each cytosolic and nuclear fractions were transferred into new tubes and subjected to the Western blotting analysis. For Western blot analysis following antibodies used: anti-CLOCK(Bethyl, A302-618A), anti-BMAL1(Santa Cruz Biotech., SC-365645), anti-PER2(Proteintech, 67513-1-Ig), anti-CRY1(Bethyl, A302-614A) anti-Histone H3 (Abcam, ab1791), and anti-αTUBULIN(Sigma-Aldrich, T9026) antibodies. While Histone H3 was used as an internal control for normalization of nuclear fractions, αTubulin was used for normalization of cytosolic fractions.

3.14. Maintenance of Animals

8-12 weeks old male C57BL/6J mice were used for *in vivo* studies. Mice weighing 24-28 grams were subjected to a single dose of CLK95 (50mg/kg) or vehicle (DMSO:Cremophor EL:0.9% NaCl;2.5:15:82.5, v/v/v) intraperitoneally. Animals which were obtained from Koç University Animal Research Facility were fed ad libitum. Four hours after treatment, animals were sacrificed, and the internal organs were harvested.

3.15. Protein Isolation and Western Blot Analysis from Mice Liver

Mice (C57BL/6J) treated with either vehicle or CLK95 (50mg/kg) were sacrificed 4 hours post-treatment to study the effect of molecule *in vivo*. Liver tissue was collected and snap-frozen in liquid nitrogen to further investigate the protein and RNA expression levels. Snap-frozen samples either immediately used or stored in -80°C for long term usage. To isolate proteins, 10mg liver tissue was dissected and washed with 500µL 1X ice cold PBS buffer. Dissected tissue then, homogenized for 2 minutes on ice using 500µL RIPA buffer (recipe is given in section 3.10.). After incubating the suspension for 20 minutes on ice, samples were centrifugated at 10000xg for 30 minutes at 4°C. Supernatants were harvested and transferred to new microcentrifuge tubes to perform Pierce 660nm Protein Assay to measure protein concentrations. Samples that were assayed, supplemented with Laemmli buffer to perform SDS-PAGE and Western-Blot techniques. 10µg from each sample was used. For immunodetection, anti-CLOCK, anti-BMAL, anti-PER2, anti-CRY1, and anti-ACTIN antibodies were used.

3.16. RNA Isolation and RT-qPCR Analysis from Mice Liver

RNA isolation from mice liver was operated according to RNeasy® Mini Kit from Qiagen (Cat no. 74106). After RNAs were eluted in 50µL nuclease-free water, quantity, and the quality of RNAs were assessed with Nanodrop 2000 (Thermo Scientific) and agarose gel electrophoresis, respectively. Following RNA isolation, cDNA synthesis and qPCR assay performed as previously described in section 3.11.

3.17. Subcellular Fractionation from Mice Liver

Subcellular fractionation from mice liver was performed by using 10mg tissue sample. Buffers that were used in this process are same as in given in section 3.13. First, the tissue was washed in 500µL ice-cold 1X PBS buffer and then resuspended in 300µL cytosolic lysis buffer by pipetting and incubated on ice for 10 min. Samples were centrifuged at 660xg for 5 minutes (4°C) to get rid of cell debris. While supernatants were collected and transferred to new microcentrifuge tubes for processing later to give cytosolic fractions, pellets containing intact nucleus were washed 3 times with 300µL cytosolic lysis buffer. At

the end of each wash, suspensions were centrifugated for 5 minutes at 660xg (4°C). Pellets were resuspended in 200µL nuclear lysis buffer and sonicated two times with 60% power for 10 seconds with 3 cycles. Supernatants were collected and labelled as cytosolic and nuclear fractions depending on the buffer they were dissolved. Samples were analysed with Western blot as described earlier using anti-CLOCK(Bethyl, A302-618A), anti-BMAL1(Santa Cruz Biotech., SC-365645), anti-CRY1(Bethyl, A302-614A), anti-Histone H3 (Abcam, ab1791), and anti-αTUBULIN(Sigma-Aldrich, T9026) antibodies were used.

3.18. Statistical Analysis

All data were represented as mean ± SEM (Standard Error of Mean) and statistical significancy was evaluated with Student's t-test by using GraphPad Prism (GraphPad Software, California, USA). For each experimental setup, number of biological replicates was at least n=3. [*p-value<0.05, **pvalue<0.01, ***p-value<0.001.]

Chapter 4:
RESULTS

4.1. Molecular Dynamics Simulation and Virtual Screening

Our previous virtual screening enabled us to identify several clock binding molecules that alter different properties of circadian rhythm at cellular level [21]. Among them CLK95 affects the amplitude and the period of the circadian rhythm. *In silico* analysis of the CLK95 indicated that it binds CLOCK:BMAL1 complex (PDB ID:4F3L). The CLK95 binds to the cavity between α1, and α2 helix of the bHLH domain, and PAS-A domain of CLOCK:BMAL1 complex with an energy of -9.19 kcal/mol (**Fig. 4.1A)** (Onur Özcan (PhD candidate from Kavaklı Lab, Koc University) performed the in-silico analysis). Close inspection revealed that CLK95 interacts with Arg126, Phe141, and Glu124 of BMAL1 with Pi-cation, Pi-lone pair, and Pi-anion bonds, respectively. In addition to that, the Ser179 and Lys220 residues of CLOCK interact with CLK95 by forming hydrogen bonds (**Fig. 4.1B)**.

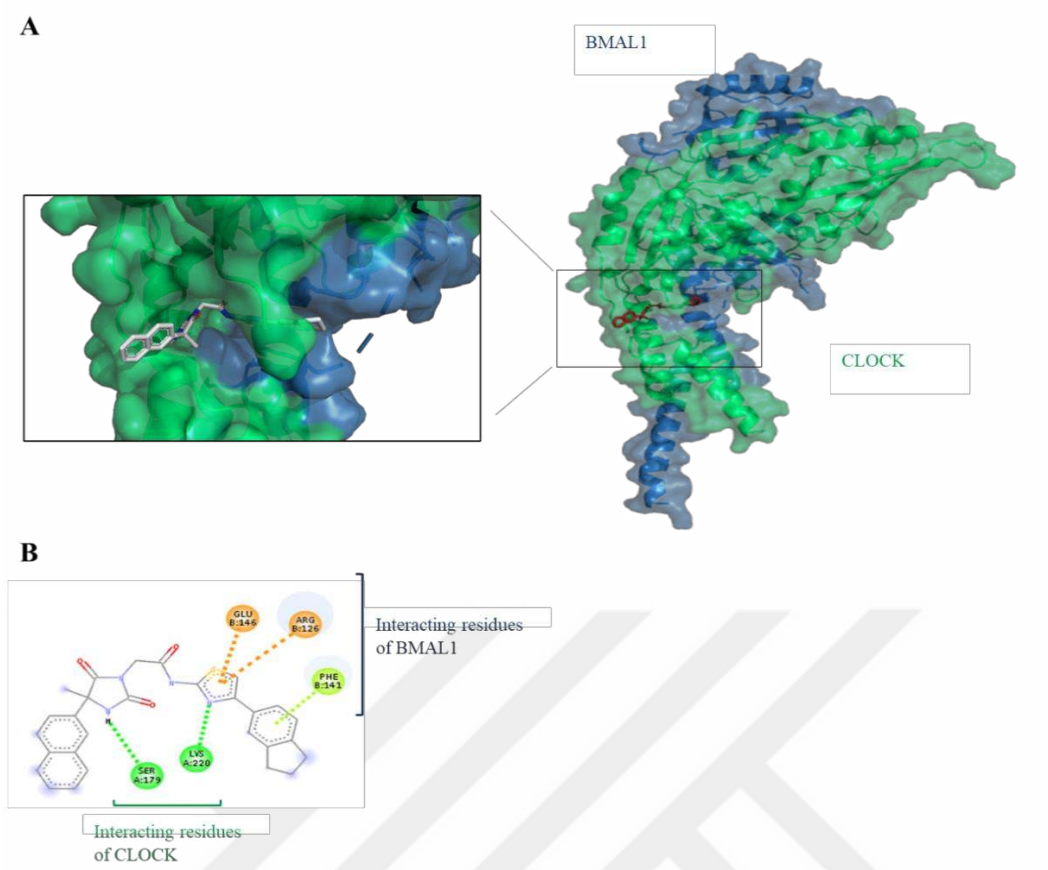

Figure 4. 1 CLK95's docking result to CLOCK:BMAL1 complex. (A) Best binding model of CLK95 to CLOCK:BMAL1 complex with surface representation of CLOCK:BMAL1 binding sites of CLK95. CLK95 binds to CLOCK:BMAL1 complex with -9.19 kcal/mol energy. CLOCK crystal structure is represented in green colour, while BMAL1 is represented in blue colour. (B) Chemical Structure of CLK95 with predicted interacting residues of CLOCK:BMAL1 complex

4.2. Cell Viability Test

Cellular cytotoxicity of CLK95 was assessed in NIH 3T3 *Bmal1*-d*Luc* and U2OS *Bmal1*-d*Luc* cells using MTT assay. Doses that showed less than 80% cell viability were eliminated and 5µM was selected as maximum dose to use in living cells (**Fig. 4.2A, B**).

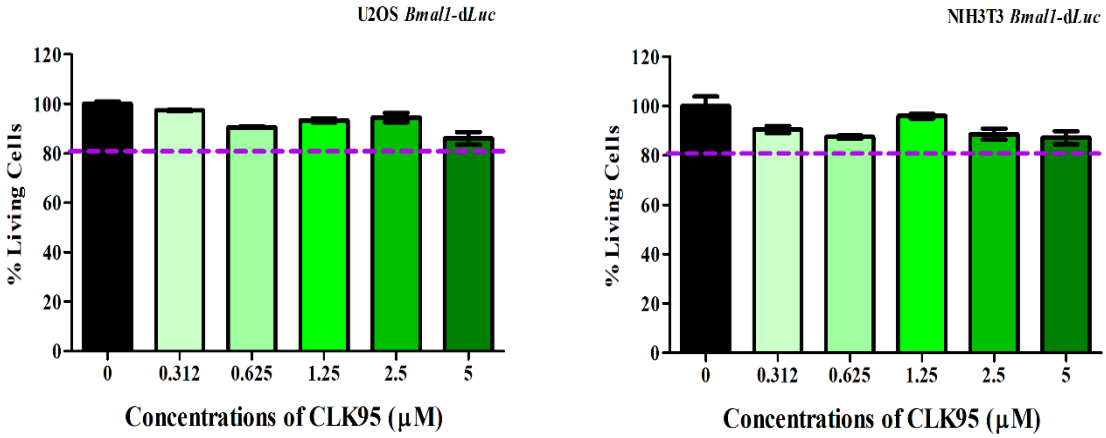

Figure 4. 2 Non-toxic doses of CLK95 were determined using MTT assay. (A) Assessment of cell viability in U2OS *Bmal1*-d*Luc* cells. (B) Assessment of cell viability in NIH 3T3 *Bmal1*-d*Luc* cells. Cells were treated with indicated doses of CLK95 (0.25%DMSO), 48 hours after cell seeding. Cells were incubated for 48 hours with CLK95 doses, and cell viability was determined using Synergy H1 Microplate Reader (Bio-Tek Instruments) as previously discussed. All measurements were normalized against control (0.25% DMSO). Doses that showed ≥ 80% cell viability were selected as non-toxic doses. (Data represent the mean ± SEM; n=3).

4.3. The effect of CLK95 on Circadian Rhythm

Several methods that I used in this thesis relies on luciferase-based assay. To make sure CLK95 doesn't interfere with LUCIFERASE (LUC) activity by either stabilizing or disturbing its luminometric properties, the effect of the CLK95 on LUC activity was determined. As seen in **Fig. 4.3**, CLK95 didn't affect any features of the LUC. The effect of CLK95 on circadian rhythm was determined using NIH3T3 *Bmal1*-d*Luc* and U2OS *Bmal1*-d*Luc* reporter cells. These cell lines specifically developed for the measurement of the cellular circadian rhythm and stable express *Luc* genes under the control of *Bmal1* promotor.

After synchronization of the cells with Dexamethasone, cells were treated with CLK95 in a dose dependent manner. Then the samples were placed into Lumicycle or Synergy H1 microplate reader to monitor circadian rhythm. Real-time monitoring of circadian rhythm revealed that while CLK95 decreases the amplitude, it increases the period of NIH3T3 *Bmal1*-d*Luc* (**Fig. 4.4A**) and U2OS *Bmal1*-d*Luc* cells (**Fig. 4.4B**) in a dose dependent manner. Observing the dose dependent effect in two different genetic

background suggested CLK95's specificity to CLOCK and BMAL1. Compared to effect in NIH3T3 *Bmal1*-d*Luc* cells, CLK95 demonstrated the maximal phenotypic change (loss of rhythmicity) at 1.25µM in synchronized U2OS *Bmal1*-d*Luc* cells. Doses between 1.25 to 5µM showed the similar circadian phenotype because of the saturation of the molecule's effect(**Fig. A.1. (Appendix A)**).

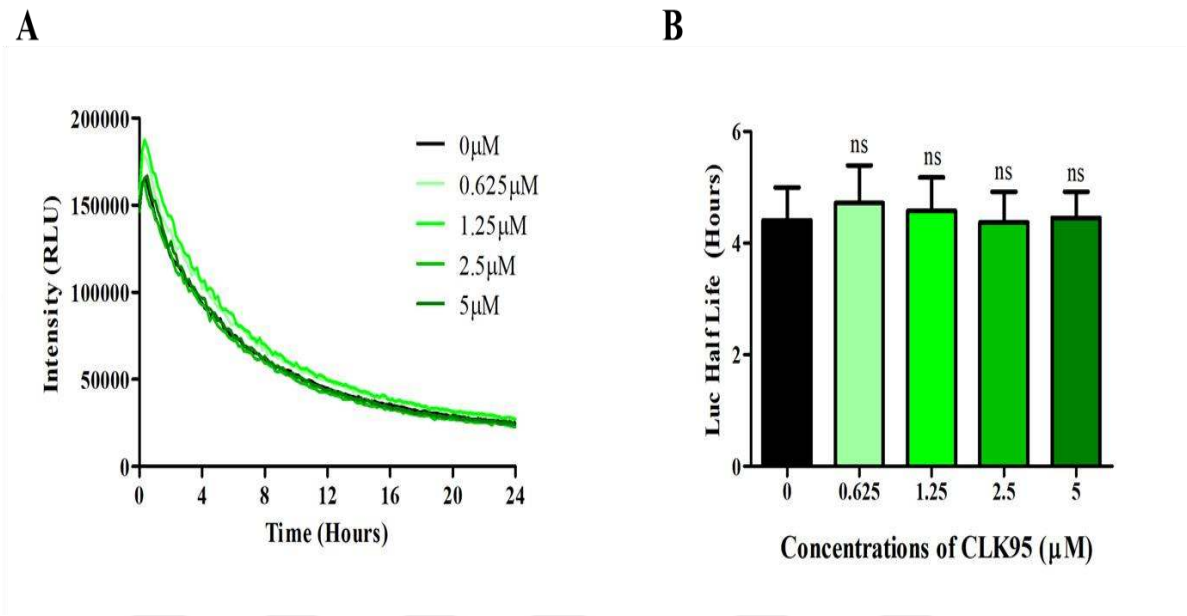

Figure 4. 3 CLK95 does not affect the half-life of LUCIFERASE. (A) Effect of CLK95 on LUCIFERASE half-life was assessed in HEK 293T cells that were transfected with pcDNA-*Luc*. Luciferase signal recorded after CLK95 (0.25%DMSO) treatment until it reached to a plateau (B) Graphical representation of LUC half-life quantification. (Data represent the mean ± SEM; n=3).

To further show that the observed circadian phenotype was caused by CLK95's binding to CLOCK and BMAL1, circadian rhythms of wild type, *Clock* knockout and *Bmal1* knockout mouse embryonic fibroblast (MEF) cells (**Fig. 4.5A**) were continuously monitored after CLK95 treatment for 7 days. Wild type (WT) MEF cells had measurable circadian rhythm while the *Clock* and *Bmal1* knock out cells had arrhythmic phenotype (**Fig. 4.5B,C and D**). Similar results were observed in WT MEF cells (**Fig. 4.5B**). However, CLK95 had no effect on circadian phenotype of *Clock* KO and *Bmal1* KO cells (**Fig. 4.5C, D**). Collectively all these results suggest the CLK95 affect circadian rhythm through CLOCK and BMAL1.

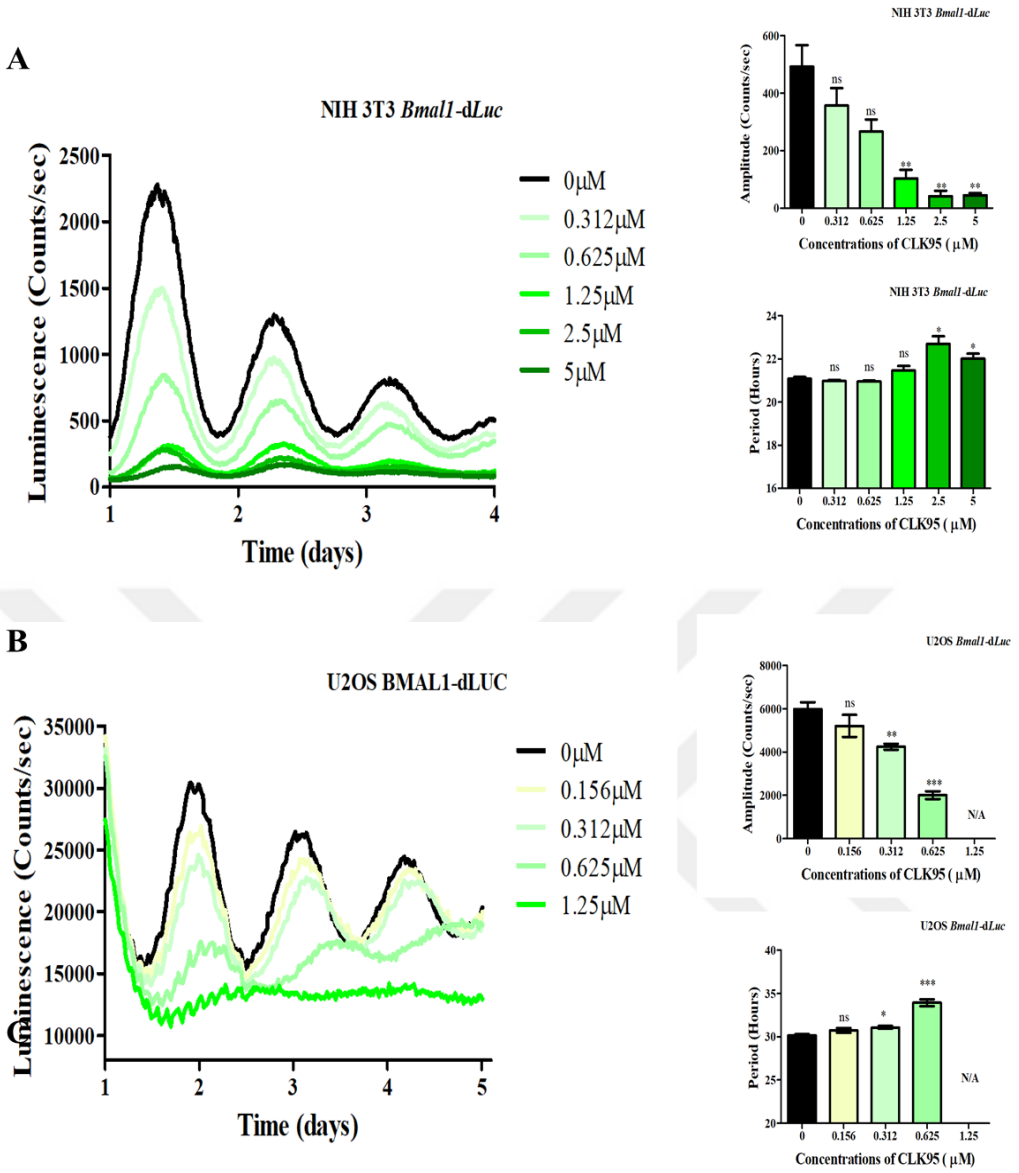

Figure 4. 4 CLK95 specifically decreases the amplitude and increases the period. (A) Continuous monitoring of circadian oscillations in NIH3T3 *Bmal1*-d*Luc* cells using Lumicycle, Actimetrics. (B) Continuous monitoring of circadian oscillations in U2OS *Bmal1*-d*Luc* cells using Synergy H1. After seeding, cells were synchronized and treated with indicated doses of CLK95 (0.25%DMSO). Then, cells were placed in Lumicycle or Synergy H1 to record luminescence signals for 5-7 days with 10 minutes or 24 minutes intervals, respectively. The data of the first day were omitted. Data represent the mean ± SEM; n=3. *p-value <0.05; **p-value <0.01; *** pvalue<0.001.

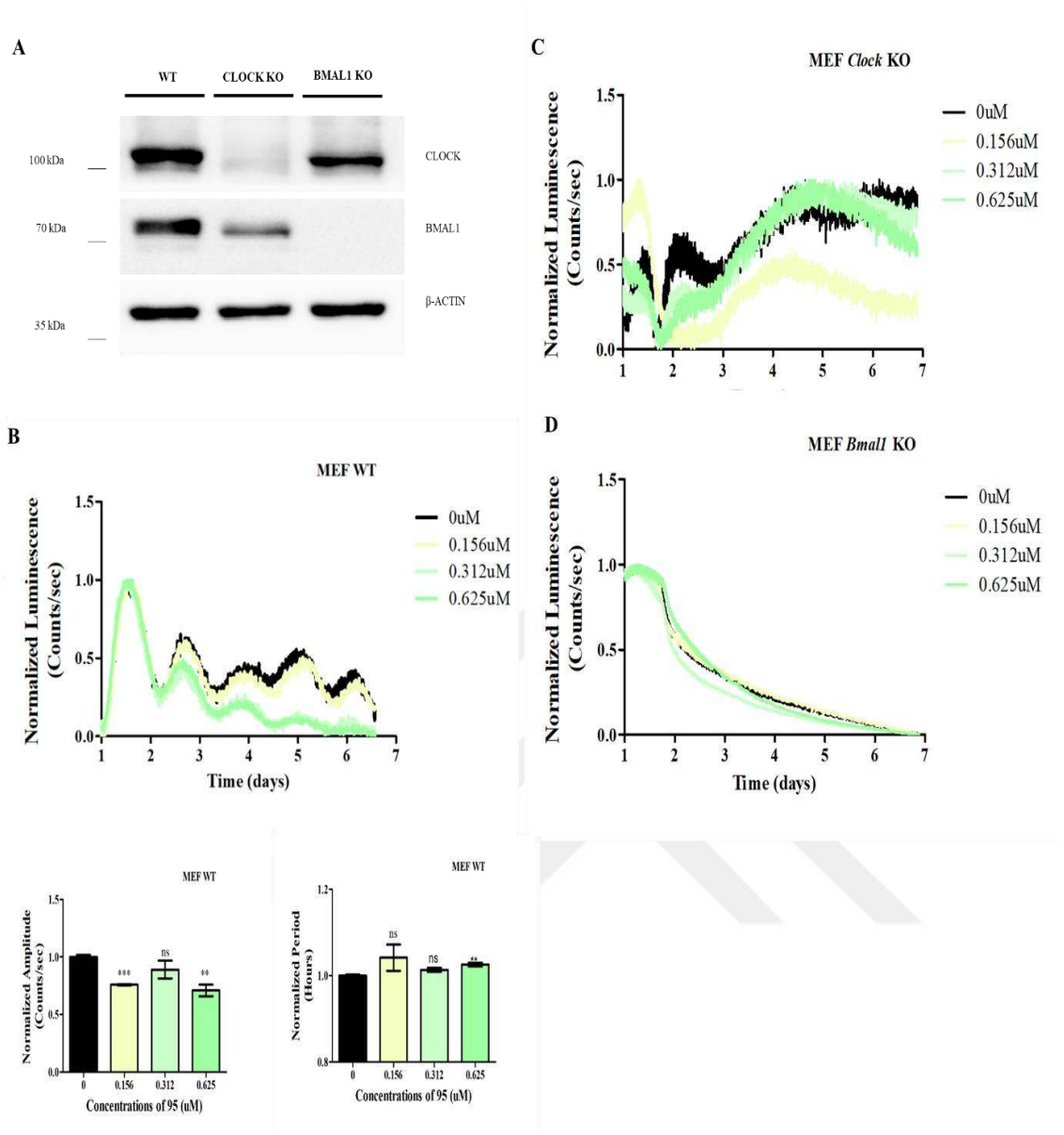

Figure 4. 5 CLK95 effects the circadian phenotype through CLOCK and BMAL1. (A) CLOCK and BMAL1 levels of WT, *Clock* KO, and *Bmal1* KO MEF cells. Continuous monitoring of circadian rhythm after CLK95 treatment in (B) WT, (C) *Clock* knockout, and (D) *Bmal1* knockout MEF cells. After cells were transduced with *Bmal1*-d*Luc* lentiviral particles to stably express *Luc*, they were treated with CLK95 and put in Lumicycle to record Luminescence signalling for 7 days with 10 minutes intervals. Data of first day is omitted. Data represent the mean ± SEM; n=3. *p-value <0.05; **p-value <0.01; *** pvalue<0.001.

4.4. Specific Binding of CLK95 to CLOCK:BMAL1 Complex

After CLK95 identified as a potential molecule that can regulate circadian rhythm, series of experiments were performed to characterize its effect. *In silico* assays implied that CLK95 has high binding affinity to CLOCK:BMAL1 complex. To test if CLK95 physically interacts with this complex, pull-down experiments with biotinylated-CLK95 (bCLK95) were performed. First, bCLK95 was synthesized by Enamine (Ukraine) (**Fig. 4.6A**). HEK 293T cells were transfected with plasmids that consist of *Bmal1* and *CLOCK*. After preparation of the cell lysates from these cells, the binding ability of the bCLK95 were assessed on CLOCK, BMAL1 and BMAL1/CLOCK dimer. Although the resin non-specifically binds to both BMAL1 and CLOCK, in the presences of the bCLK95 the amounts of both CLOCK and BMAL1 were significantly higher than that of DMSO control (**Fig. 4.6B, C**). To further verify the binding of CLK95 to BMAL1 and CLOCK, bCLK95 was precipitated in the presences of free CLK95. Expectedly, the amount of the CLOCK and BMAL1 was precipitated comparable amount to DMSO control (**Fig. 4.6B, C**). These results suggest that CLK95 binds to both BMAL1 and CLOCK. This observation is further confirmed by pull down of BMAL1/CLOCK dimer with bCLK95 (**Fig. 4.6D**). All these results suggest that CLK95 physically interacts with both CLOCK and BMAL1. To eliminate overexpression artifact by pull down assay, cell extract was prepared from U2OS cells to test whether CLK95 binds to endogenous CLOCK and BMAL1. Our results indicated that bCLK95 precipitated with both BMAL1 and CLOCK while other core clock proteins including CRY1, PER2 and NPAS2, did not precipitated with it (**Fig. 4.6E**).

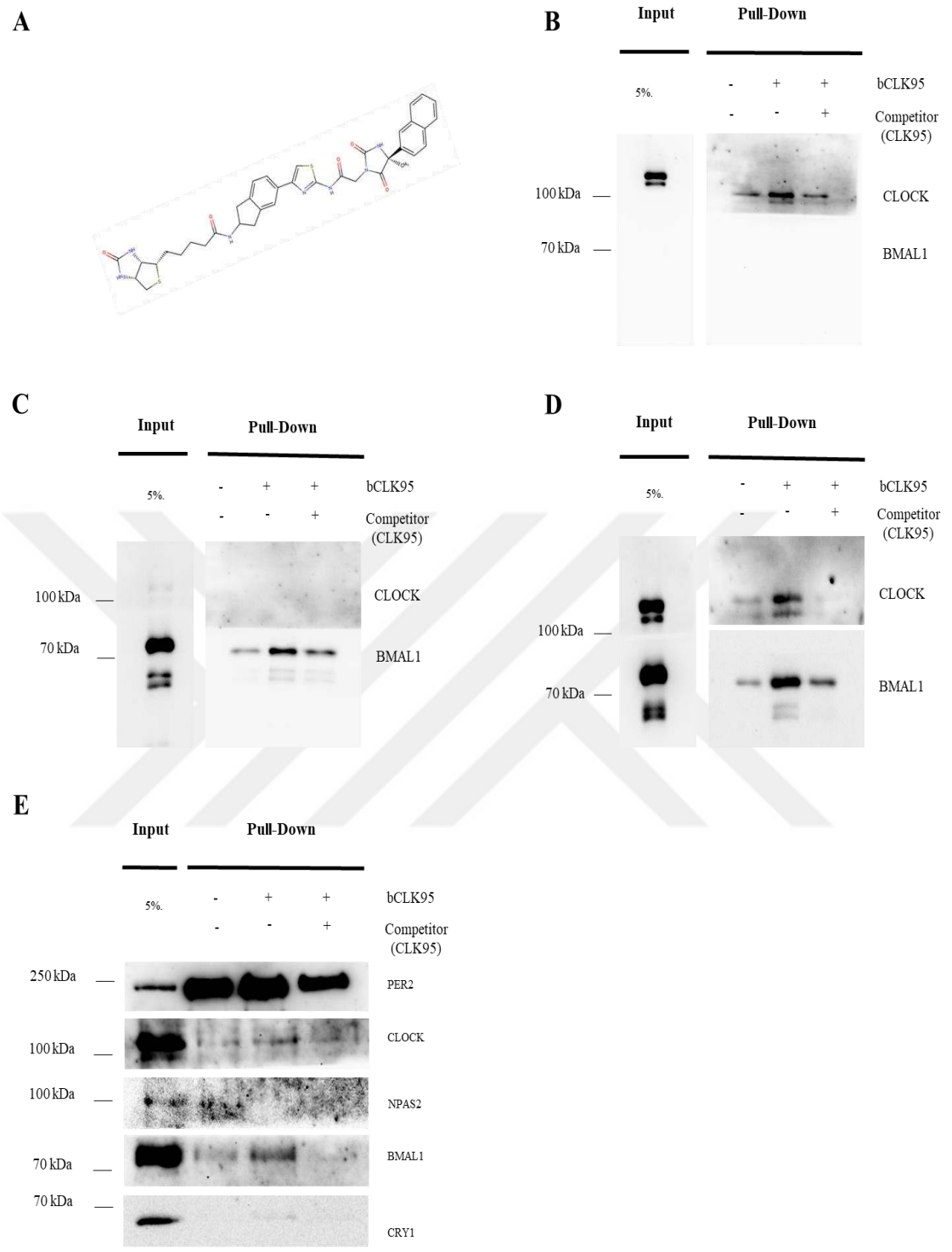

Figure 4. 6 CLK95 specifically binds to CLOCK:BMAL1 complex. (A) Chemical structure of biotinylated-CLK95 (bCLK95). Pull-down assays conducted by employing overexpression systems that used (B) only CLOCK overexpressing, (C) only BMAL1 overexpressing, and (D) CLOCK:BMAL1 co-expressing HEK 293T lysates. (E) Whole cell lysate of U2OS cells were used to explore binding at endogenous level. While 20µM bCLK95 was used as bait, 200 µM non-biotinylated CLK95 used as competitor. Data are representative for three independent studies for Fig. 4.5B-D. Data are representative for two independent studies for Fig. 5E.

4.5. The Effect of CLK95 on Subcellular Localization of CLOCK and BMAL1

Our previous study has indicated that CLK8 interferes with nuclear translocation of CLOCK:BMAL1 [21]. To see whether CLK95 also affects subcellular distribution of CLOCK:BMAL1, subcellular fractionation was performed on unsynchronized U2OS cells. After fractionations, we first checked the quality of the fractionated samples via probing cytosolic and nuclear fractions with anti-TUBULIN and anti-HISTONE H3 antibodies, respectively. After obtaining clean fractionations (**Fig. 4.7.),** the levels of core clock proteins were determined in cytosol and nucleus. The levels of core-clock proteins in the nucleus were calculated by normalizing values to HISTONE H3 while their level in the cytosol were determined by normalizing the values with α-TUBULIN. Results indicate that while the levels of both CLOCK and BMAL1 were significantly increased in nucleus, there was no considerable difference in cytoplasm (**Fig. 4.7.**). In addition to that, the levels of the CRY1 and PER2 were comparable in both cytosol and nucleus (**Fig. 4.7.**). These findings suggested that CLK95 can lead to prominent changes in core-clock protein stoichiometry by increasing the nuclear localization of CLOCK and BMAL1. Unlike BMAL1, CLOCK does not have neither Nuclear Localization Signal (NLS) nor Nuclear Export Signal (NES) [95-97]. So, it is possible that CLK95 enhances the assembly between CLOCK and BMAL1 that results in efficient nuclear translocation of complex without interfering the CRY and PER transloaction.

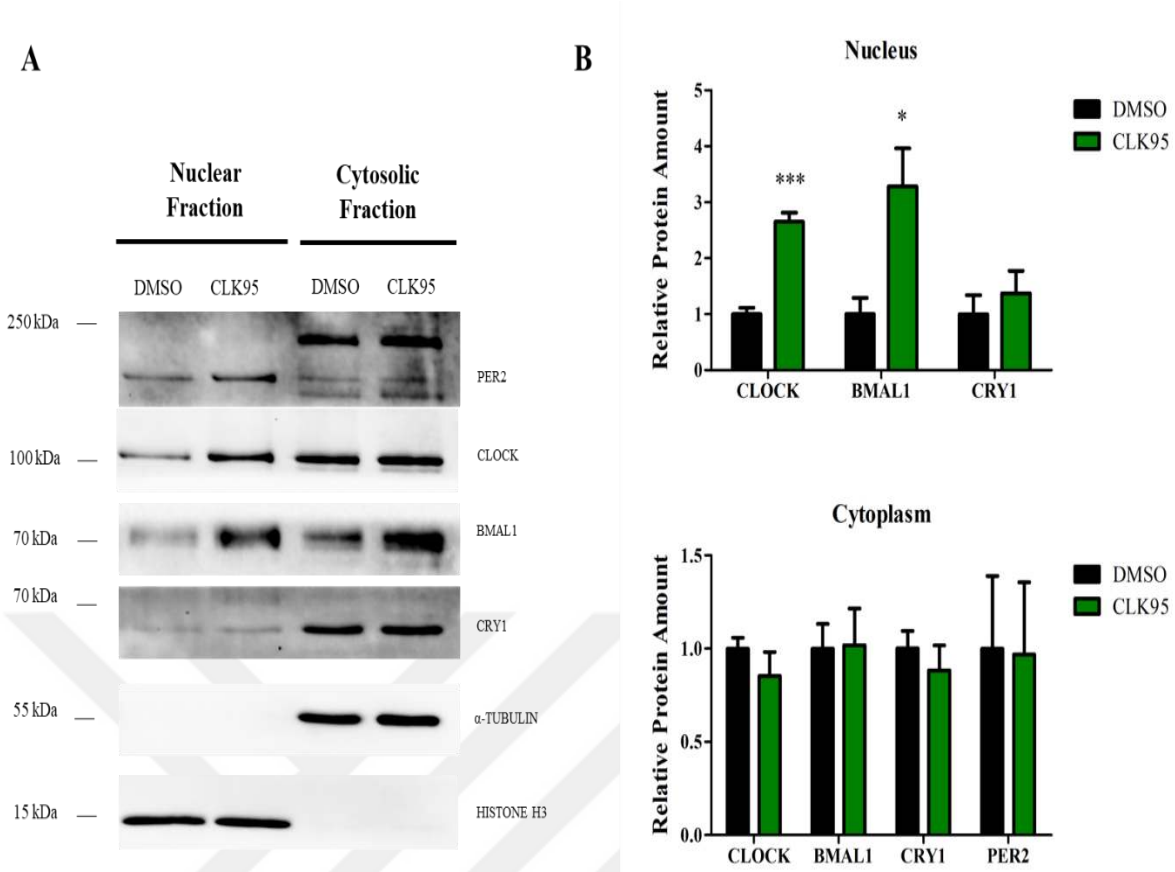

Figure 4. 7 CLK95 increases the nuclear localization of CLOCK and BMAL1. (A) Subcellular fractionation was performed on unsynchronized U2OS *Bmal1*-d*Luc* cells that were treated with 5μM CLK95 (0.25% DMSO) and. CLOCK, BMAL1, CRY1, and PER2 proteins were immunoblotted. Protein levels were normalized to HISTONE H3 and α-TUBULIN levels. (B) Quantification of core-clock protein levels in nucleus and cytoplasm after CLK95 treatment. Data are mean ± SEM; n=4, independent experiments. *p-value <0.05; **p-value <0.01; *** p-value<0.001.

4.6. Enhancement of CLOCK:BMAL1 Interaction in the Presence of CLK95

To test whether CLK95 enhances the assembly between the CLOCK and BMAL1, we performed Co-IP assay between the CLOCK and BMAL1 in the presences of the CLK95. The plasmids consist of FLAG-tagged *Bmal1,* and *CLOCK* were transfected to HEK 293T cells. After preparation of the cell lysates, FLAG-BMAL1 were precipitated with anti-FLAG resin. Then, the amount of CLOCK was quantified with western blotting. Analysis of the result revealed that FLAG-BMAL1 levels were equal while the level of the CLOCK was higher in the presences of the CLK95 (**Figure 4.8A, B**). To verify this observation the same experiment was repeated with a BMAL1 mutant (BMAL1-R126A).

This mutant was selected because of the critical contribution of the Arg-126 of the BMAL1 for the binding of CLK95. I hypothesized that BMAL1-R126A mutant should not be able to bind the CLK95, thus the enhancement in the precipitated CLOCK levels shouldn't be observed After replacing Arg to Ala at the position 126 of the BMAL1, the functional assay was performed to make sure this residue had no effect on the transactivation. HEK 293T cells were transfected to co-express either *Bmal1/CLOCK* or *R126A Bmal1/CLOCK* along with *Per1:* d*Luc*. Comparing wild-type BMAL1's *Per1:* d*Luc* activity to R126A BMAL1's *Per1:*d*Luc* activity showed that the activity levels were indeed equivalent (**Figure 4.8C**). Co-IP experiments were performed with the BMAL1-R126A with the CLK95. Results indicated that CLK95 had no effect on CLOCK and BMAL1-R126A dimerization, which suggest that CLK95 enhances interaction between CLOCK and BMAL1 (**Figure 4.8A, B**).

Collectively all data suggest that the CLK95 enhances the interaction between CLOCK and BMAL1 and, in turn, promotes their nuclear translocation. Therefore, I investigated the effect of the elevated nuclear CLOCK:BMAL1 levels on clock output genes.

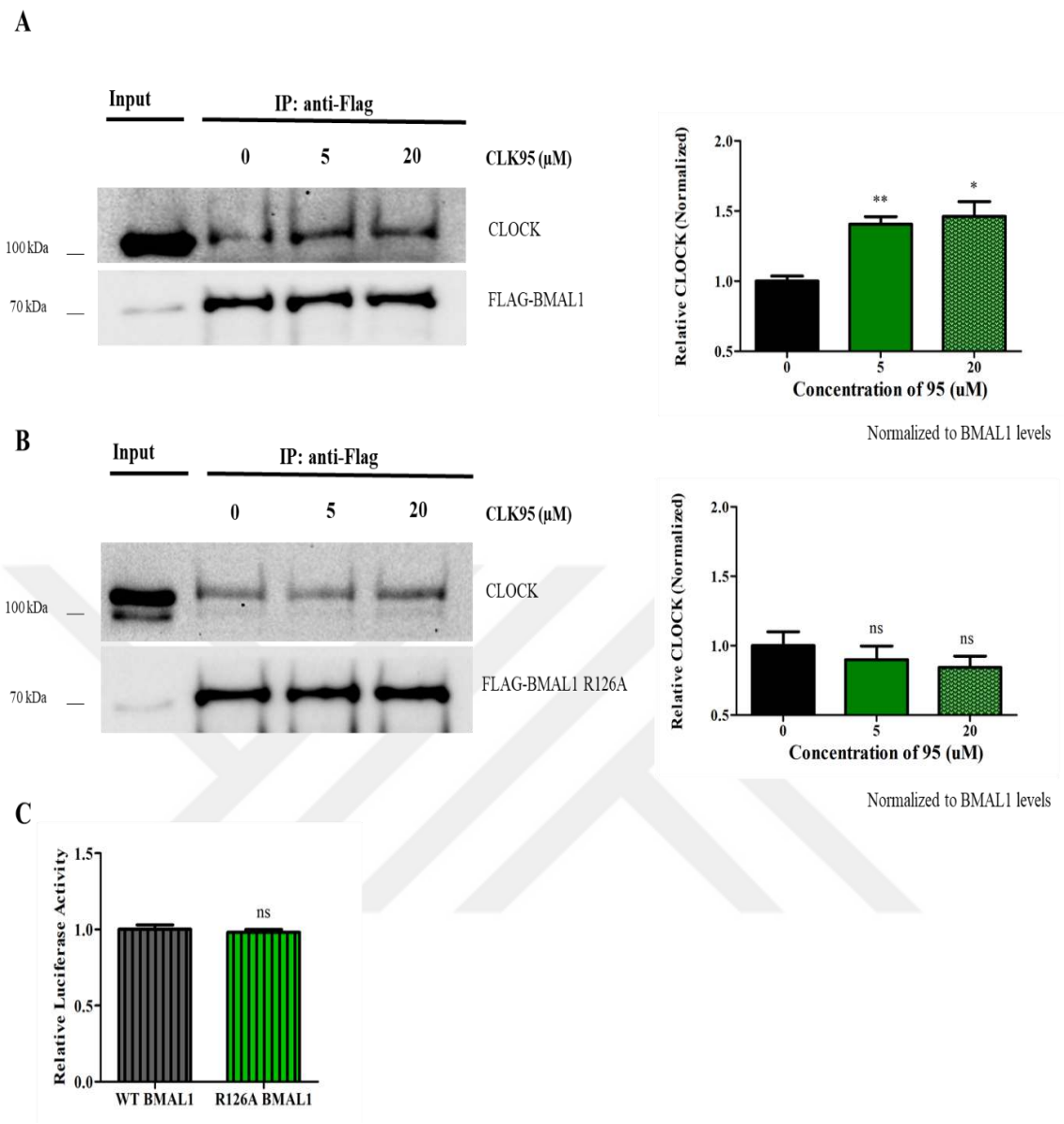

Figure 4. 8 CLK95 increases the interaction between CLOCK and BMAL1, and Arg126 of BMAL1 is essential for CLK95's binding to CLOCK:BMAL1 complex. (A) Interaction levels of CLOCK:BMAL1 were assessed under CLK95 presence via Co-IP assay. FLAG-BMAL1 predicated by anti-FLAG resin and the amounts of CLOCK it came with were determined with western blotting. (B) Graphical representation of the quantification of CLOCK:BMAL1 interaction after CLK95 treatment. Level of CLOCK proteins that were precipitated along with FLAG- BMAL1 were normalized to BMAL1 levels. (C) Co-IP assay were used to evaluate the interaction levels of CLOCK:R126A BMAL1 under CLK95 treatment. (D) Graphical representation of the quantification of CLOCK:R126A BMAL1 interaction after CLK95 treatment. (E) Functional evaluations of R126 BMAL1 in comparison to WT BMAL1. Evaluations were performed by using transactivation assay on HEK 293T cells. Data are mean ± SEM; n=3. *p-value <0.05; **p-value <0.01; *** pvalue<0.001.

4.7. The Effect of the CLK95 on Core-Clock Components

The unsynchronized U2OS *Bmal1*-d*Luc* cells were treated with CLK95 in a dose dependent manner. Results showed that while CLOCK, and BMAL1 proteins levels were increased, CRY1 and PER2 levels did not change (**Fig. 4.9A, B**). When CLK95's effect on core-clock proteins were also tested in synchronized U2OS *Bmal1*-d*Luc* cells, similar results were observed (**Fig. 4.10A, B**).

After investigating the effect of molecule on core-clock components at protein levels, it's effect on transcriptional levels was analysed in unsynchronized U2OS *Bmal1*-d*Luc* cells. expression levels of *BMAL1* and its controlling genes *RORα* and *NR1D1* did not change, while expression levels of *CLOCK*, *CRY1* and *PER2* were decreased under CLK95 treatment (**Fig. 4.11**). These results could be interpreted according to transcription cycling concept. According to this concept, removal of the transcription factor from DNA after RNA polymerase recruitment is crucial for gene expression [14]. This concept is highly validated for transcription factors, i.e., estrogen receptor [150]. Considering the high levels of CLOCK and BMAL1, CRY-PER gets insufficient to repress this complex. So, the gene expression controlled by CLOCK:BMAL1 gets inhibited.

All these results suggest a hypothesis in which the positive arm of the TTFL is getting strengthened by increased amounts of CLOCK:BMAL1, whereas the negative arm is unchanged, resulting in decreased circadian amplitude and increased circadian period.

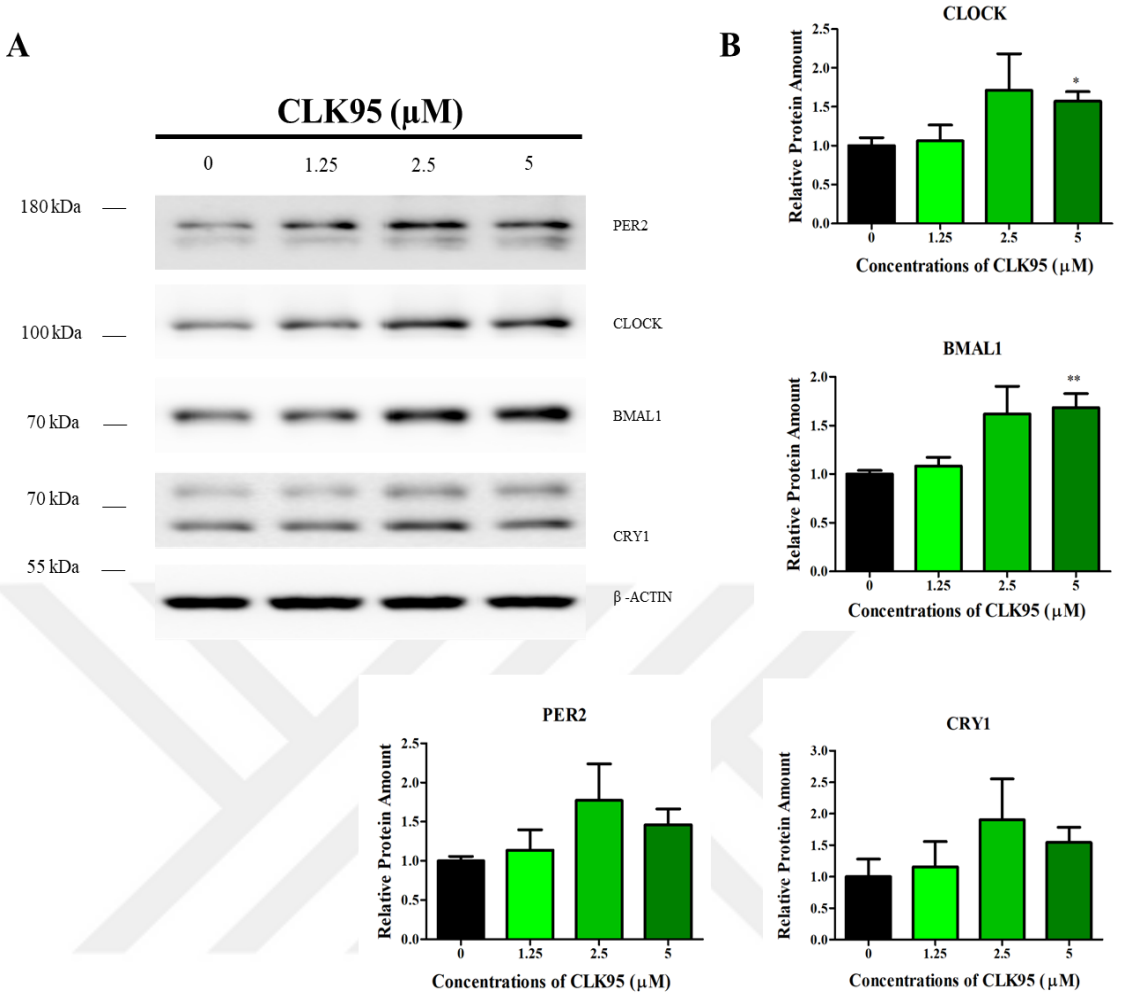

Figure 4. 9 CLOCK and BMAL1 protein levels increased after CLK95 treatment. (A) Unsynchronized U2OS *Bmal1*:d*Luc* cells were treated with indicated doses of CLK95. 24 hours after molecule treatment, cells were harvested, and western blot was performed. Protein levels were normalized to β-ACTIN levels. (B) Quantification of protein levels in unsynchronized U2OS *Bmal1*:d*Luc* cells. Data represents mean ± SEM; n=3, independent experiments. *p-value <0.05; **p-value <0.01.

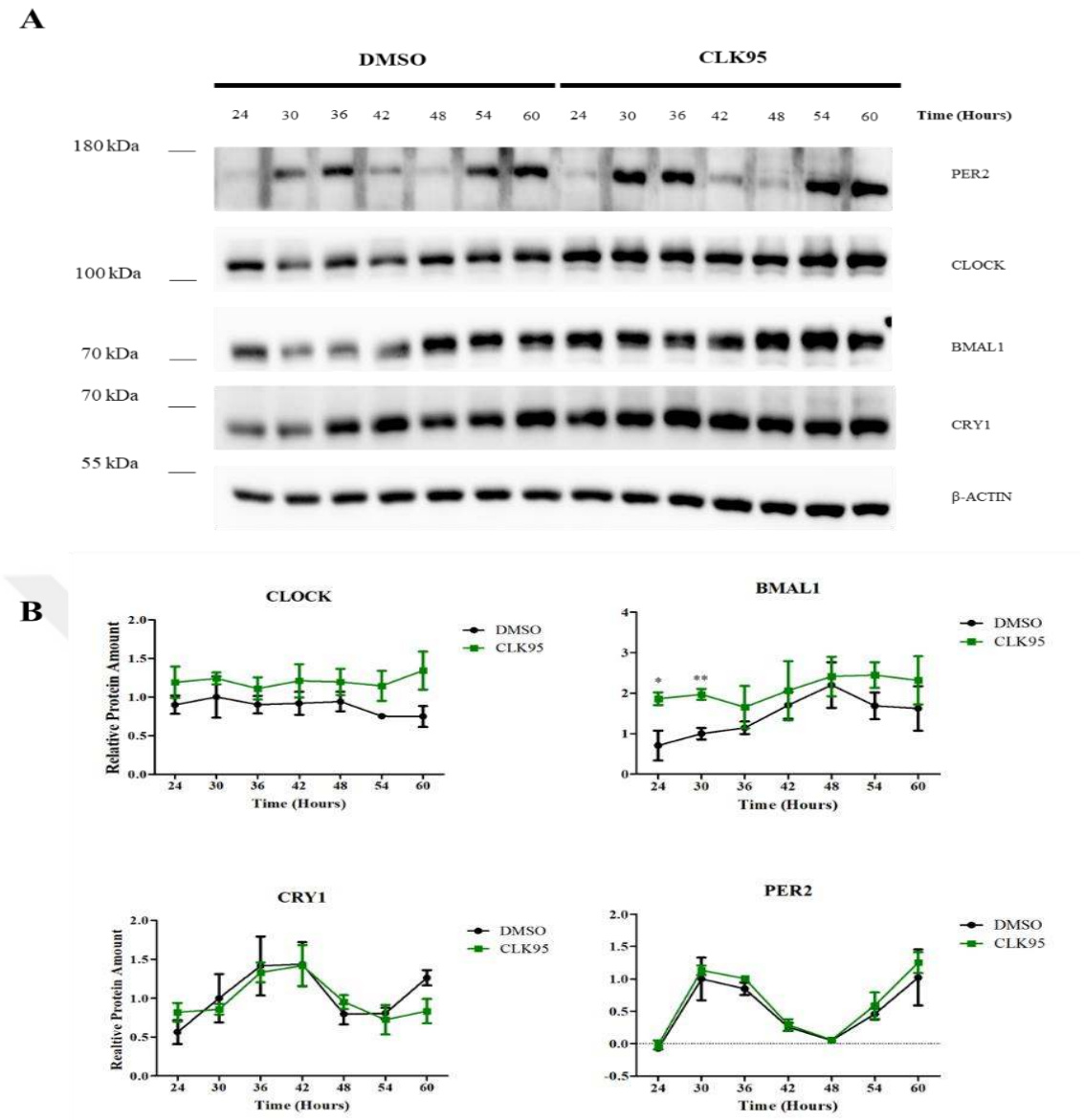

Figure 4. 10 Alterations at protein levels after CLK95 treatment in synchronized U2OS Bmal1:dLuc cells. (A) U2OS *Bmal1*:d*Luc* cells that were synchronized with 0.1µM dexamethasone, were treated with 5µM CLK95 (0.25% DMSO). 24 hours after molecule treatment, cells were harvested at indicated times and western blot was performed. Protein levels were normalized to β-ACTIN levels. (B) Quantification of protein levels of synchronized U2OS *Bmal1*:d*Luc* cells. Data represents mean ± SEM; n=3, independent experiments. *p-value <0.05; **p-value <0.01.

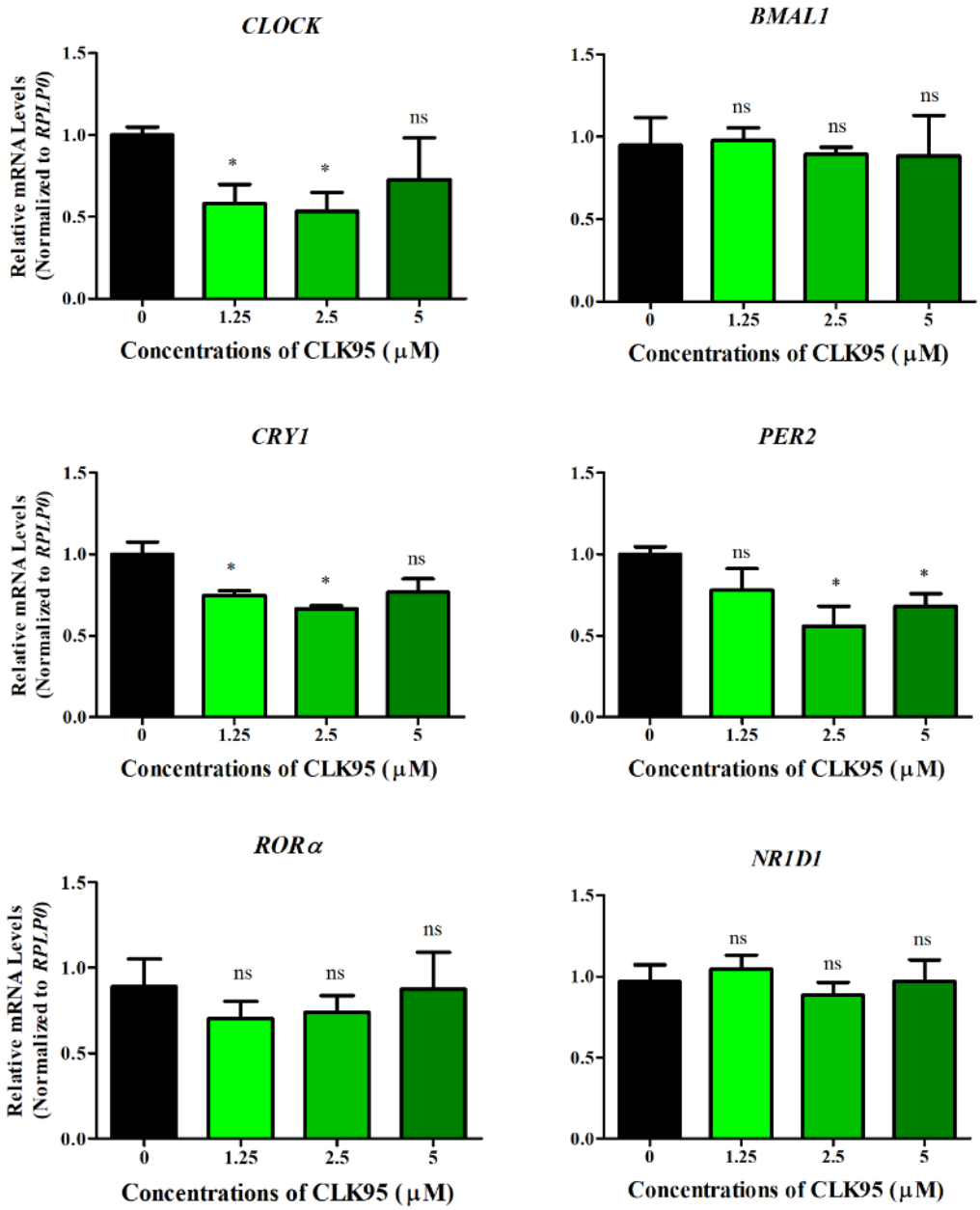

Figure 4. 11 The effect of CLK95 on transcription levels of core-clock and clock-control genes in unsynchronized U2OS Bmal1:dLuc cells. Quantification of mRNA levels was done using $2^{-\Delta\Delta Ct}$ method. Normalizations were done using *RPLP0* levels. Data represent mean ± SEM; n=3, independent experiments. *p-value <0.05.

4.8. In vivo Effect of CLK95 on Mice Liver

Results from *in vitro* experiments suggested that CLK95 binds specifically to CLOCK:BMAL1 complex which enhances the interaction between these two and leads complex to localize in nucleus. To test whether similar results were observed *in vivo*, a series of experiments were performed on mice liver. Livers were obtained from the mice who were sacrificed four hours after CLK95 treatment. CLK95 was subjected to C57BL/6J mice intraperitoneally at the dose of 50mg/kg by Yasemin Kübra Akyel (PhD candidate from Istanbul University). No signs of toxicity were observed in mice at this dose of the molecule. First, total protein levels were checked after CLK95 treatment in mice. Results revealed that CLOCK and BMAL1 levels were increased while no significant change was observed in CRY1 levels (**Fig. 4.12A, B**). When subcellular fractionation was performed on mice liver, increased CLOCK and BMAL1 levels were seen (**Fig. 4.13A, B**). All these data from *in vivo* experiments are in agreement with *in vitro* result. However, when mRNA levels were checked in mice liver, no significant change was observed in any of core-clock and their controlling genes (**Fig. 4.14.**). Considering the short exposure time of mice to the molecule, the effect may not have been seen as the time required for CLK95 to exert its effect on transcriptional levels was not sufficient.

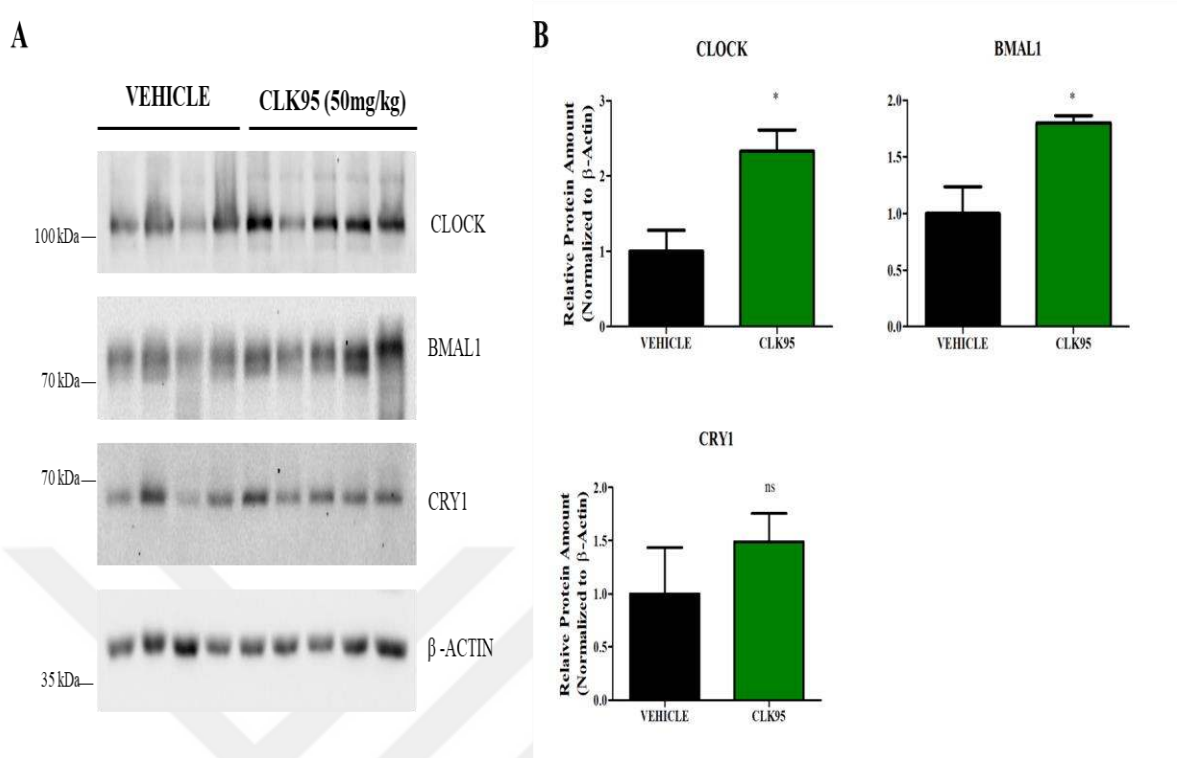

Figure 4. 12 Effect of CLK95 on core-clock proteins in mice liver. (A) Mice that treated with CLK95 (50mg/kg) or vehicle sacrificed, and total protein levels of core-clock proteins were immunoblotted. (B) Representation of quantification of core-clock proteins in mice liver. Protein levels were normalized against β-ACTIN levels. Data represent mean ± SEM; n=4 for control mice and n=5 for mice treated with CLK95. *p-value <0.05.

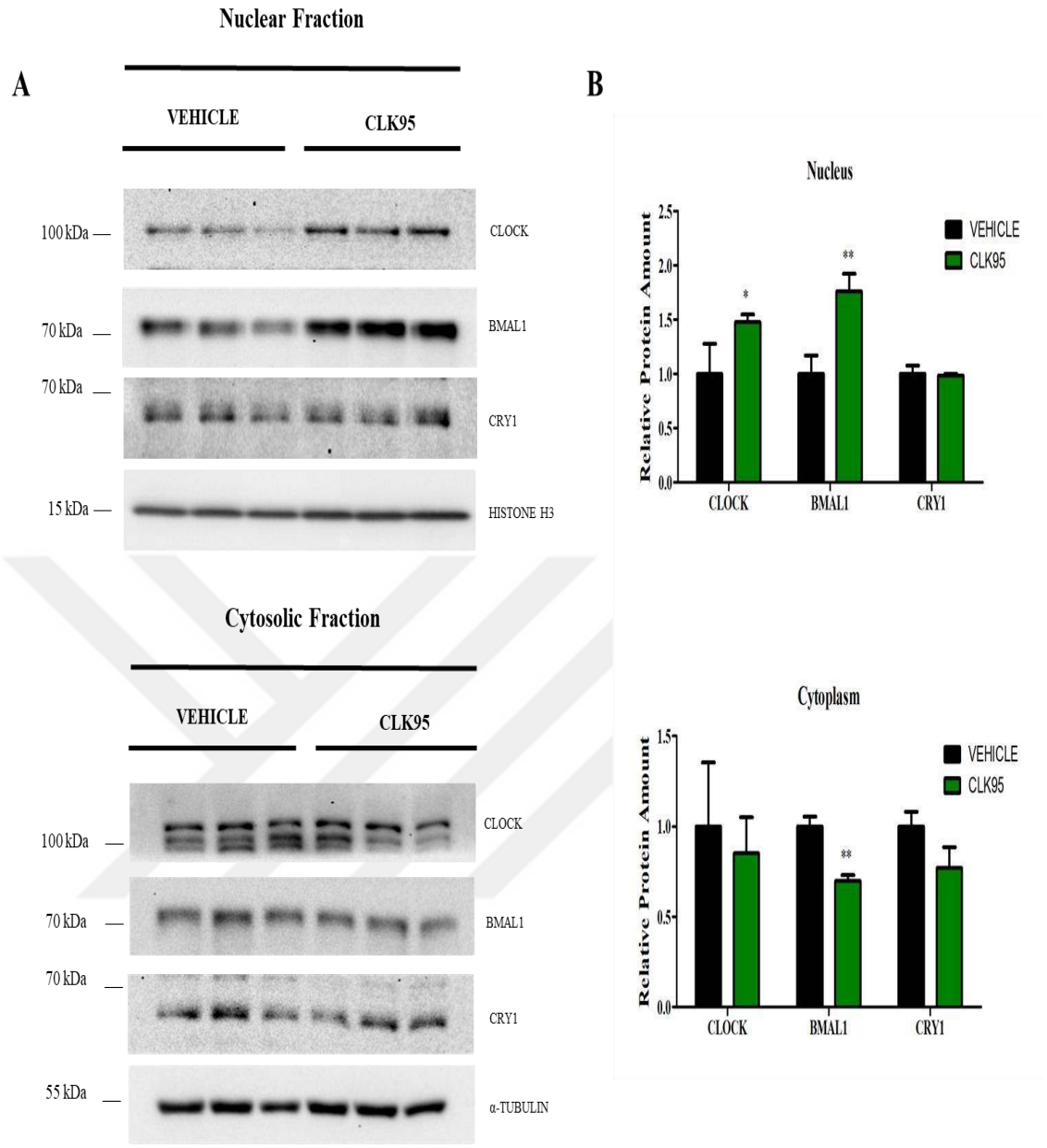

Figure 4. 13 Effect of CLK95 on subcellular localization of core-clock proteins in mice liver. (A) Mice that subjected to CLK95 (50mg/kg) or vehicle sacrificed and, livers of animals were harvested. After subcellular fractionation was performed, levels of core-clock proteins were immunoblotted in nuclear and cytosolic fractions. (B) Quantification of core-clock proteins in nucleus and cytoplasm. Protein levels were normalized against HISTONE H3 or α-TUBULIN levels. Data represent mean ± SEM; n=3 for control mice and n=3 for mice treated with CLK95. *p-value <0.05; **p-value <0.01.

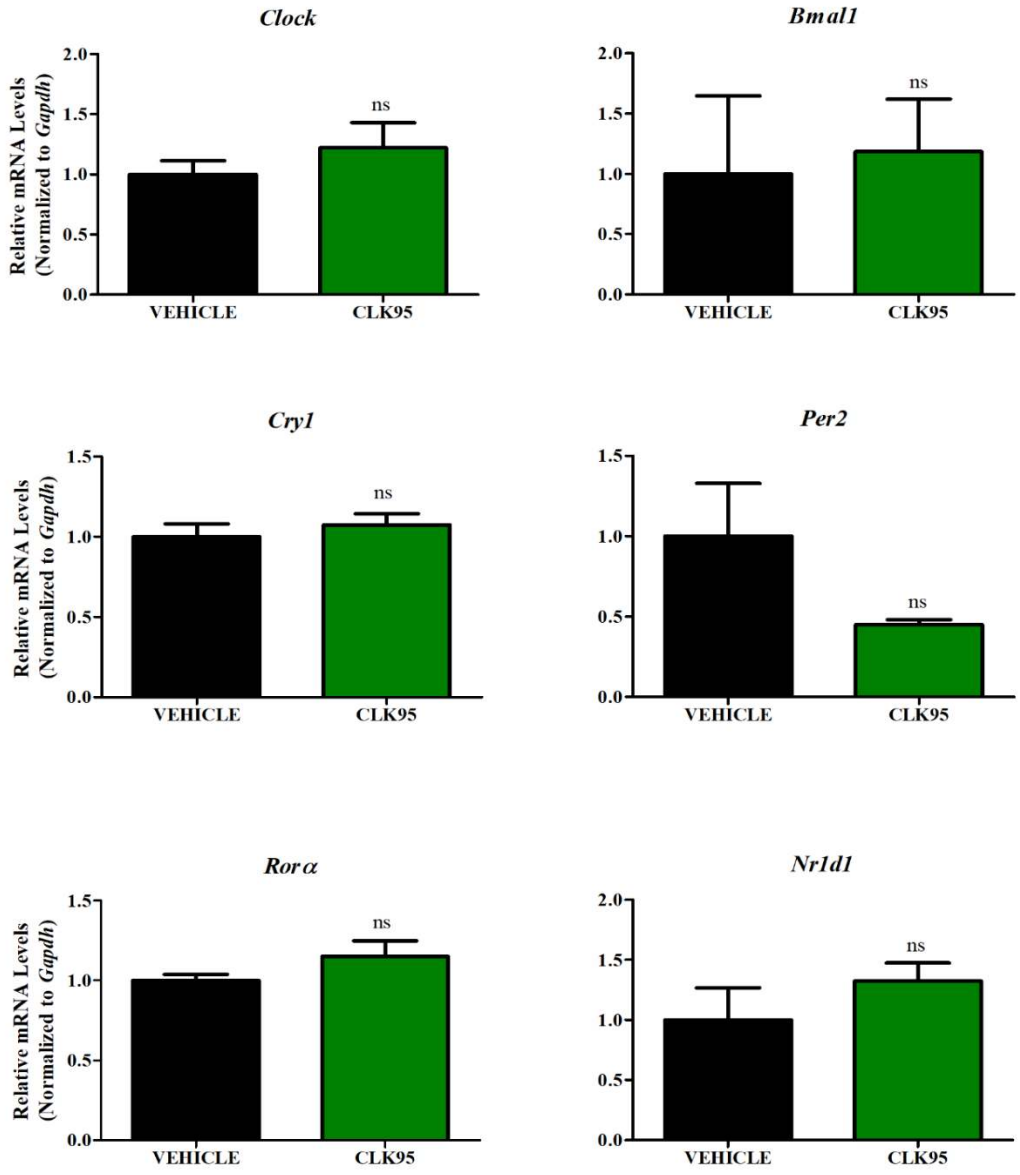

Figure 4. 14 Effect of CLK95 on transcription levels of core-clock and clock-controlled genes in mice liver. (A) 4 hours after molecule administration (50mg/kg), mice were sacrificed, and livers of animals were harvested. mRNA levels were assessed using $2^{-\Delta\Delta Ct}$ method. Normalizations were done using *Gapdh* levels. Data represent mean ± SEM; n=3 for control mice and n=3 for mice treated with CLK95. *p-value <0.05.

Chapter 5:
DISCUSSION

The finding of the small molecules against core clock proteins are great interest in the field. Having such molecules enable to decipher circadian rhythm mechanism at cellular level and have potential to be drugs for the treatment of diseases arose from disrupted circadian clock. To this end several studies have been initiated to develop molecules that have potential to change circadian rhythm [151]. These efforts yielded several molecules mainly regulating the activity of axillary clock proteins such as REV-ERB and Casein kinase Iε. Only a few molecules have been discovered against core clock proteins [21, 133, 137]. In this work, I characterized a novel molecule, CLK95 identified by virtual screening [21], that binds to CLOCK:BMAL1 complex. Experimental works showed that it affects the nuclear shuttling of the CLOCK and BMAL1 by regulating their interaction.

CLK95 binds to the cavity between α1, and α2 helix of the bHLH domain, and PAS-A domain of CLOCK:BMAL1 complex with a high energy and such binding is confirmed by mutagenesis studies. Pull-down assays with bCLK95, verified that the molecule can physically bind to both CLOCK, BMAL1, and CLOCK:BMAL1 complex. This observation was further supported by the loss of dose specific effect of CLK95 on circadian rhythms of *Clock* knockout and *Bmal1* knockout MEF cells. Altogether, results implied that CLK95 exerts its effect to circadian rhythm through CLOCK and BMAL1.

Considering the complexity of the regulations on circadian rhythm, elucidating the exact molecular mechanism underlying CLK95's effect could be challenging. However, our results showed that CLK95 promotes the nuclear localization of CLOCK and BMAL1 due to its stabilizing effect on their interaction. It was already known that the stoichiometric relationships between core-clock proteins relates with the properties of circadian clock. In fact, the robustness of the rhythm heavily depends on the negative loop of the main TTFL. Lee et al. (2011) has shown that the enhancement of the positive loop leads to dampening of the circadian rhythm [93, 94]. In agreement with this, our data suggest that CLOCK:BMAL1 dimer concentration increases in the presence of CLK95 and, in turn, dampens the circadian rhythm. Therefore, to explain the molecular

effects of CLK95, we proposed a mechanism where the stoichiometric ratios of positive and negative loop shifted in favour of the positive loop which eventually led to dampening of the circadian rhythm. (**Fig 5.1.**)

Although the levels of CLOCK:BMAL1 were high in the nucleus, *PER2* and *CRY1* expressions were decreased. This phenomenon could be explained by the transcription-factor cycling concept [14]. In this concept transcription factors bind to DNA, recruits RNA polymerases, and then get degraded to activate transcription. Under treatment of CLK95, even though there is no change in the negative loop, it still becomes weaker compared to high level of positive loop. Thus, this inefficiency results with inhibition of transcription-factor cycling which in return effects the gene expression levels.

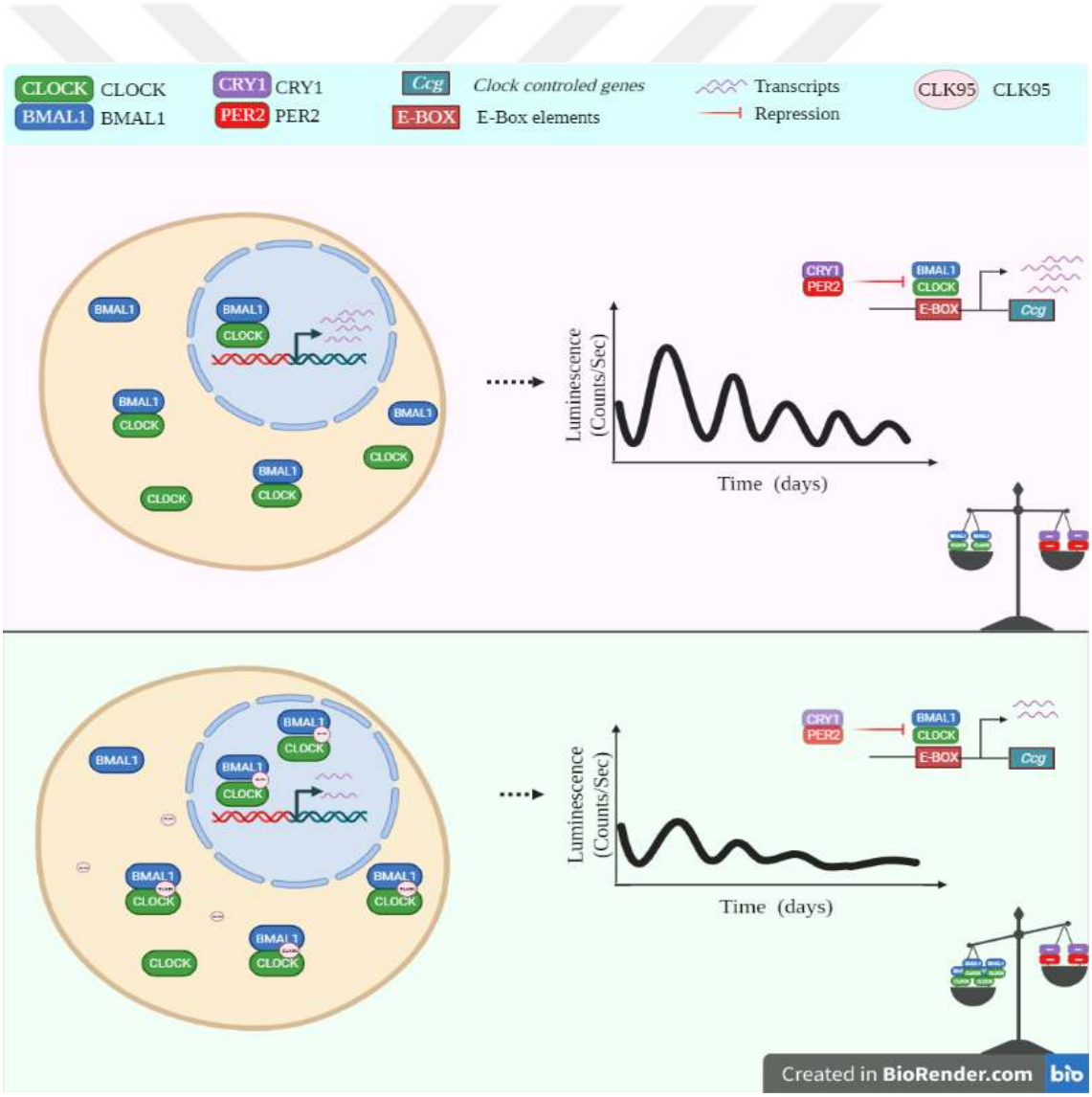

Figure 5. 1 Schematic representation of CLK95's working principle. CLK95 binds to CLOCK:BMAL1 complex. This binding increases the stability of the

CLOCK:BMAL1 interaction, thus promoting the nuclear accumulation of CLOCK and BMAL1. While CLOCK-BMAL1 levels increase, CRY-PER levels do not change which leads to a weaker negative loop. As the negative loop gets insufficient to repress CLOCK/BMAL1, transcription-factor cycling gets inhibited. Therefore, all these stoichiometric changes lead to dampening of the circadian rhythm with an increase on its period.

As CLK95 exerts opposite effects compared to CLK8 which is an amplitude enhancer that disrupts CLOCK:BMAL1 interaction, comparative studies using these molecules, can help us to better understand the molecular machinery of the circadian rhythm and can lead to finding of new targets for chronotherapy. Also, it was shown that the overexpression of *CLOCK* and *BMAL1* suppressed the cell growth by inhibiting cells to go into S-phase of the cell cycle [126]. This results can suggest that the CLK95 could be used as a chemotherapeutic agent due to its enhancing effect on CLOCK-BMAL1 levels. To confirm this hypothesis, effect of CLK95 on cancer cell growth must be determined.

Another use of this molecule could be in treatment of jet lag which is born from the desynchrony between internal clock and the time of the environment. Even though there are treatment methods such as exposure to bright light or melatonin intake, their effectiveness highly depends on the time they were used, and the time length of the new environment [152]. Because higher doses of CLK95 completely supresses the endogenous clock, resynchronization of the internal clock to the time of the new environment could be more efficiently operated. This possibility also needs to be confirmed by experimental set-ups. For example, locomotor activity of mice with jet lag behaviour could be monitored after CLK95 treatment.

VITA

Şafak İşin studied Molecular Biology and Genetics in Istanbul University and was graduated with a Bachelor of Science degree in 2018. Şafak continued his graduate education in Koç University, Molecular Biology and Genetics department. He was a research assistant in the Biotechnology and Circadian Clock Laboratory under the supervision of Prof. İ. Halil Kavaklı. He was also a teaching assistant during his graduate studies in Koç University, from September 2018 until August 2021. He graduated with a Master of Science degree in August 2021. During his M.Sc., Şafak studied on "Stabilization of the CLOCK:BMAL1 Heterodimer Decreases Circadian Rhythm Amplitude".

APPENDICES

APPENDIX A: SUPPLEMENTARY DATA

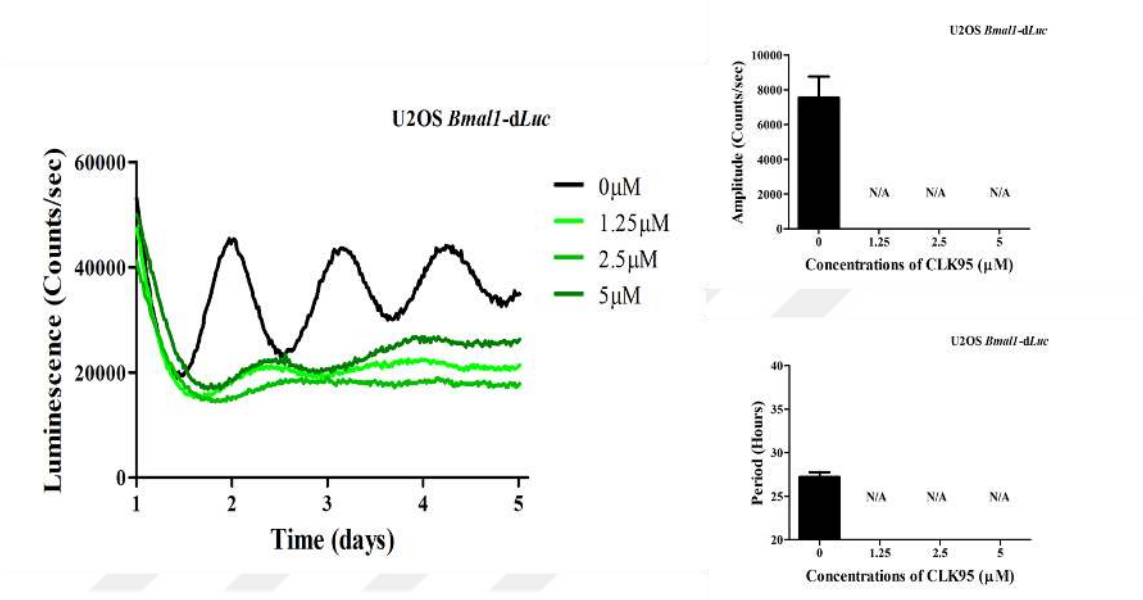

Figure A. 1 Effect of higher doses of CLK95 on U2OS Bmal1-dLuc cells. After cell synchronization, U2OS *Bmal1*-d*Luc* cells were treated with indicated higher doses of CLK95 and put into Synergy H1 microplate reader to monitor the circadian oscillations. Data were recorded for 5 days with 10 minutes or 24 minutes intervals, respectively. The data of the first day were omitted. Data represent the mean ± SEM; n=3 independent study.

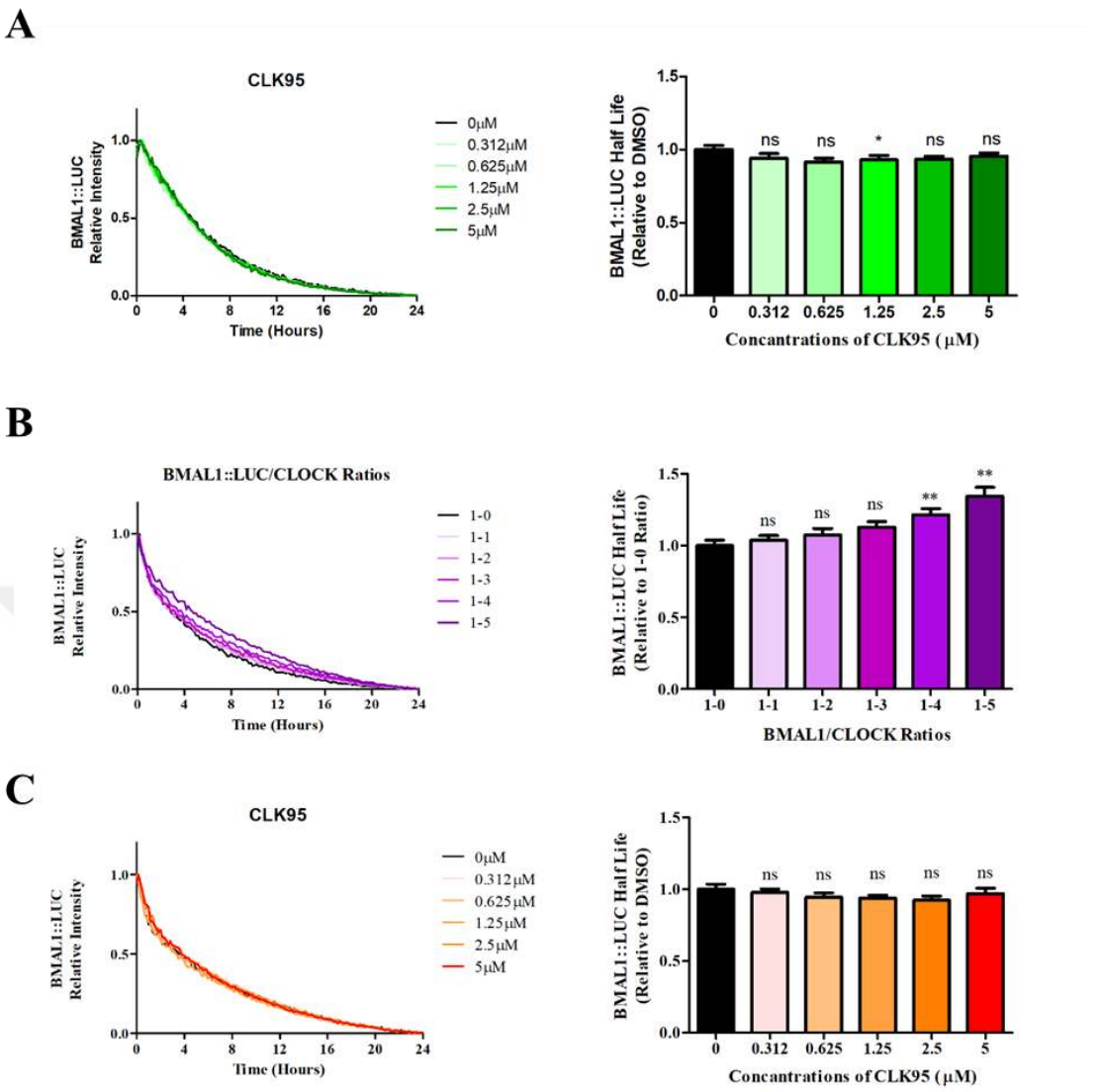

Figure A. 2 CLK95 does not affects the half-life of BMAL1. (A) Effect of CLK95 on BMAL1::LUC half-life was assessed in HEK 293T cells. 24 hours after cells were transfected with pcDNA4-*Bmal1-Luc* plasmid, treatment with indicated doses of CLK95 was performed. Luminescence signals were recorded until it reached to plateau. Data are mean ± SEM; n=3, independent experiments. (B) Several BMAL1 to CLOCK ratios were tested to select the best ratio for exploring CLK95's effect on BMAL1::LUC half-life when CLOCK is also overexpressed. Luminescence signals were recorded until it reached to plateau. Data are mean ± SEM; n=3, independent experiments. *p-value <0.05; **p-value <0.01. (C) Assessment of effect of CLK95 on BMAL1::LUC half-life in HEK 293T cells transfected with 1:5 ratio of pcDNA4-*Bmal1-Luc* and pCMV-Sport6-*CLOCK*, respectively. Cells were treated with indicated doses of the CLK95 (0.25%DMSO) and Luminescence signals were recorded until it reached to plateau. Data are mean ± SEM; n=3, independent experiments.

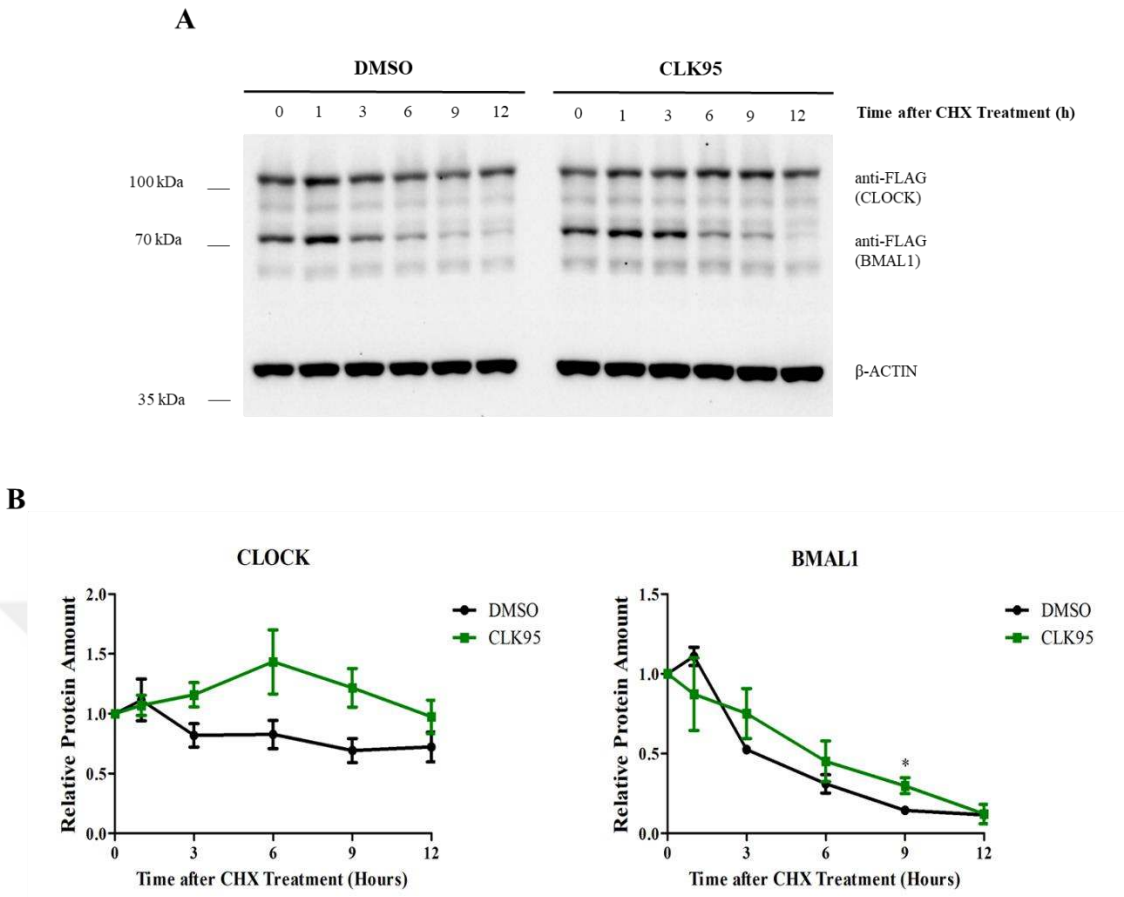

Figure A. 3 Assessment of the effect of CLK95 on CLOCK and BMAL1 by CHX-chase assay. (A) Treatment with 5µM CLK95 (0.25% DMSO) was performed to HEK 293T cells which were transfected with Flag-tagged pCMV-Spot6-*CLOCK* and Flag-tagged pcDNA4-*Bmal1*. Cells were harvested at indicated times after CHX treatment (100µg/mL) and western blot was performed. (B) Quantification of relative CLOCK and BMAL1 levels at indicated times. Protein amounts were normalized against β-ACTIN levels. Data represents mean ± SEM; n=3, independent experiments. *p-value <0.05; **p-value <0.01; *** p-value<0.001.

APPENDIX B: PRIMER SEQUENCES

Table A. 1 Primer Sequences

Real-Time Primers	
Gene	**Primer Sequence**
hsCLOCK	F: 5'-GGCACCACCCATAATAGGGTA-3' R: 5'-TGTTGCCCCTTAGTCAGGAAC-3'
hsBMAL1	F: 5′-TGCCACCAATCCATACACAG-3′ R: 5′-TTCCCTCGGTCACATCCTAC-3′
hsCRY1	F: 5'-ACAGGTGGCGATTTTTGCTTC-3' R: 5'-TCCAAAGGGCTCAGAATCATACT-3'
hsPER2	F: 5'-GCGTGTTCCACAGTTTCACC-3' R: 5'-GGCTTTTCCGGACACTGACA-3'
hsNR1D1	F: 5'-TGGACTCCAACAACAACACAG-3' R: 5'-GTGGGAAGTAGGTGGGACAG-3'
hsRORα	F: 5'-ACTACACACCAGCATCAGGC-3' R: 5'-GCAGGTTTCCAGATGCGATT-3'
hsRPLP0	F: 5'-TGTGGGAGCAGACAATGTGG-3' R: 5'-CATTCCCCCGGATATGAGGC-3'
mClock	F: 5'-ATGGTGTTTACCGTAAGCTGTAG-3' R: 5'-CTCGCGTTACCAGGAAGCAT-3'
mBmal1	F: 5'-GACTACCAAGAAAGTATGGACACA-3' R: 5'-TTTGTCCCGACGCCTCTTTT-3'
mCry1	F: 5'-ACTGAGGCACTTACACGTTTGG-3' R: 5'-GCAGGGAGTTTGCATTCATTC-3'
mPer2	F: 5'-GAGCGCCACCAAGTGACGG-3' R: 5'-GGTGGGACTTGGGGAGAAGT-3'
mNr1d1	F: 5'-GGCCATCACAACCTCCAGT-3' R: 5'-GGAGCCACTAGAGCCAATGT-3'
mRorα	F: 5'-ATTAGGATGTGCCGTGCCTT-3' R: 5'-GCGATTTCGTCTTCGGTCAG-3'
mGapdh	F: 5'-AACTTTGGCATTGTGGAAGG-3' R: 5'-ACACATTGGGGGTAGGAACA-3'
Site-Directed Mutagenesis Primers	
Gene	**Primer Sequence**
mBmal1	F: 5'-GAAAACTTTGGCAGGTGCCACCAACCC-3' R: 5'-GGCACCTGCCAAAGTTTTCATGTGCTG-3'

APPENDIX C: PROTEIN MARKERS

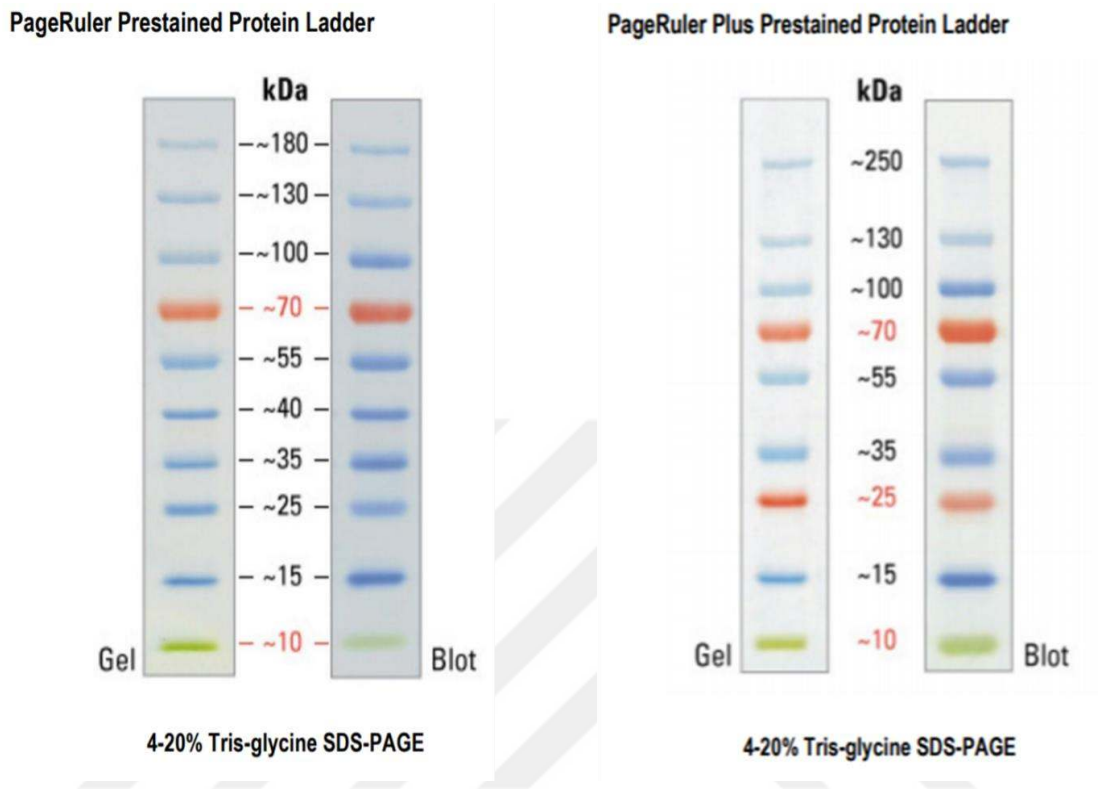

Figure A.4. Protein markers that were used in this study. (From Thermo Scientific)

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