MOLECULAR ANALYSES OF THE EFFECTS OF CLOCK GENE SNPS ON THE BIOLOGICAL CLOCK

by

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This is

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to my beloved family…

ABSTRACT

The circadian clock is a biological rhythm most organisms exhibit oscillating with a period of around 24 hours, coordinated with the day and night cycle of the earth. This biological rhythm produces a daily oscillation of physiological, mental and behavioral states. The circadian clock is based on a complex molecular mechanism of transcriptional and translational feedback loops. The direct effect of this rhythm on the physiological and behavioral states is of great interest, whereas a disorder in molecular clock of mammalians has been shown to be associated with many diseases including but not limited to diabetes, cancer, schizophrenia, metabolic syndrome, autism and depression. Understanding the molecular mechanism of clock further may shed a light on the causes of these diseases and their treatments.

Four core clock proteins generate rhythmicity in mammals. BMAL1 and CLOCK act as transcriptional activators, binding E-box elements on DNA and activating the expression of clock controlled genes, whereas CRYs and PERs repress BMAL1 and CLOCK proteins lead to a decrease in transactivation mediated by BMAL1:CLOCK heterodimer in clock controlled gene expression. SNPs in these critical clock genes may give rise to the molecular clock mechanism to dysfunction leading to the diseases associated with the circadian clock.

In this thesis, SNPs of Bmal1, Clock and Cry1 genes were selected from the 1000 Genome Project to evaluate their effects on circadian rhythmicity using both molecular and biochemical approaches. The SNPs are located on the functional domains of the proteins and were selected based on their SIFT (Sorts Intolerant From Tolerant) and PolyPhen (Polymorphism Phenotyping) scores. The effects of mutations on BMAL1 and CLOCK proteins were tested by measuring the transactivation capacities on mPER1 promoter while the effect of mutations on CRY1 were measured by investigating any significant decrease on BMAL1:CLOCK transactivation capacity on mPER1 promoter. It is found that the Arg84Cys BMAL1 mutant located within the bHLH domain, result in decreased transactivation, whereas the Phe122Ser CLOCK, located within the bHLH domain result in increased transactivation of the E-box mediated gene transcription. 3 variants of CRY1, Gly144Val, Arg293His and Arg348Cys show reduced repression activity on BMAL1:CLOCK driven transcription.

To see the effect of the mutant proteins on circadian rhythm lentivirus harboring appropriated wild type and mutant cDNA were infected into the U2OS cell line. Then circadian rhythms were measured by luminometric assay. Arg84Cys variant of Bmal1 overexpression showed a

different oscillation than the wild type when overexpressed. Cry1 wild type, Gly144Val and Arg348Cys gave arrhythmic phenotypes when overexpressed, whereas Arg293His did not have any effect on the oscillation. Phe122Ser CLOCK mutant overexpression caused a slight increase in period length compared with wild type overexpression.

In this thesis we are showing five core circadian clock gene alleles to have significant effects on biological rhythmicity. As many metabolic events and chronic disorders are related with the circadian mechanism, we propose that in the future these alleles may prove to be associated with disorders in humans, helping with their diagnosis and treatments.

ÖZET

Sirkadiyan saat birçok organizmada bulunan ve gün içerisindeki gece gündüz döngüsü ile koordine olarak yaklaşık 24 saatlik salınım gösteren biyolojik bir ritimdir. Bu biyolojik ritim organizmalarda gün içerisindeki fizyolojik, ruhsal ve davranışsal salınımları oluşturur. Sirkadiyan saatler, transkripsiyonal translasyonel geri besleme döngüleri içeren komplex bir mekanizma üzerine kuruludurlar. Bu ritmin diyabet, kanser, şizofreni, metabolik sendrom, otizm ve depresyon gibi birçok hastalık ile ilişkisi gösterilmiş olduğu için, fizyolojik ve davranışsal durumlar üzerindeki etkisi merak konusudur. Sirkadiyan saatin moleküler mekanizmasını anlayabilmek bu hastalıkların sebep ve tedavilerine ışık tutabilecektir.

Memelilerde sirkadiyan saatlerin moleküler mekanizmasının temelinde 4 adet protein bulunmaktadır. BMAL1 ve CLOCK proteinleri saat kontrolü altındaki genler için transkripsiyonel aktivatörler olarak işlev görürken, CRYlar ve PERler ise BMAL1 ve CLOCK proteinlerine karşı birer baskılayıcı görevi görerek saat kontrolündeki genlerin ekspresyonunu azaltırlar. Bu kritik saat genlerindeki tek nükleotid polimorfizmleri moleküler saat mekanizmasının aksamasına sebebiyet vererek sirkadiyan saat ile ilişkilendirilebilen hastalıklara yol açabilirler.

Bu tez çalışmasında, Bmal1, Clock ve Cry1 genleri için 1000 genome projesinde bulunmuş olan tek nükleotid polimorfizmleri, protein üzerindeki ilgili alanları, SIFT ve PolyPhen skorları dikkate alınarak seçilmiştir. BMAL1 ve CLOCK proteinlerinin transaktivasyonel kapasiteleri, CRY1 proteinlerinin ise baskılama kapasiteleri, lusiferaz analizleri ile test edilmiştir. Lusiferaz taraması sonucu BMAL1 bHLH bölgesinde bulunan Arg84Cys varyasyonunun transaktivasyonel kapasiteyi düşürdüğü, CLOCK bHLH bölgesinde bulunan Phe122Ser varyasyonunun ise transaktivasyonel kapasiteyi yükselttiği bulunmuştur. PHR bölgesinde bulunan 3 adet CRY1 varyasyonun; Gly144Val, Arg293His ve Arg348Cys, baskılama kapasitesi üzerinde önemli miktarda bir azalmaya sebebiyet verdiği keşfedilmiştir.

Ümit vadeden tek nükleotid polimorfizmleri kinetik lusiferaz analizlerinde kullanılarak biyolojik ritim üzerindeki etkileri incelenmiştir. BMAL1 Arg84Cys varyasyonunun hücre içerisindeki aşırı ekspresyonu sokak türünün aşırı ekspresyonundan farklı bir salınım göstermiştir. CRY1 sokak türü, Gly144Val ve Arg348Cys varyasyonları aşırı ekspresyonda aritmik fenotipler verirken Arg293His varyasyonun salınım üzerinde herhangi bir etkisi olmamıştır.

Bu tez çalışmasında insanlarda bulunan beş adet sirkadiyan saat gen alelinin biyolojik ritim üzerindeki etkisini göstermekteyiz. Birçok metabolik olay ve kronik hastalıklar sirkadiyan saat ile ilişkili olduğundan, bu alellerin ileride belirli hastalıklar ile doğrudan ilişkilendirilip teşhis ve tedavisinde önemli rol oynayacağını düşünmekteyiz.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BMAL1	Aryl hydrocarbon receptor nuclear translocator-like
CaMK	Calcium/calmodulin-dependent protein kinase
CK2	Casein kinase 2
CK2-α	Casein kinase 2 alpha
CKI-δ	Casein kinase 1 delta
CKI-ε	Casein kinase 1 epsilon
CLOCK	Circadian locomotor output cycles kaput
CRE	Calcium/cAMP response element
CREB	Ca^{+2}/cAMP-responsive element-binding protein
CRY	Cryptochrome
DBP	D-box binding protein
DMEM	Dulbecco's modified Eagle's Medium
FBXL3	F-box and leucine-rich repeat protein 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LB	Luria broth
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PER	Period
REV-ERBα	Nuclear receptor subfamily 1, group D, member 1
RORE	ROR-responsive element
RORα	RAR-related orphan receptor A
SCN	Suprachiasmatic nucleus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SUMO	Small ubiquitin-related modifier protein

CHAPTER 1

INTRODUCTION

Most organisms exhibit daily oscillations in their physiological, mental and behavioral states due to a biological timing system called circadian rhythm. This biological clock, responding primarily to the light and dark cycles during the day, follows a 24 hour cycle. This provides temporal homeostasis with the external environment.

The mammalian circadian clock is based on a feedback loop system. CLOCK and BMAL1 are two core clock components that function as transcriptional activators. CLOCK/BMAL1 transactivator complex functions by binding to the E-Box elements on DNA. CRY and PER proteins in the feedback loop act as inhibitors to this BMAL1/CLOCK transactivator complex and represses their activation. Single nucleotide polymorphisms found on these proteins have been shown to be associated with many physiological disorders, such as metabolic disorders and infertility, as well as some mood disorders, such as circadian mood oscillations and characteristics of temperament.

Circadian rhythm and its possible effects on human physiology and behavior is one of the hot topics in molecular biology today. Many disorders today are being screened to find a correspondence within the biological rhythm. In this thesis work we decided to screen some single nucleotide polymorphisms on BMAL1, CLOCK and CRY genes and their effects on biological clock to find out whether these polymorphisms do cause a change in the circadian rhythm. For this purpose we have selected some polymorphisms already found by the 1000 genome project, according to their SIFT (sorts intolerant from tolerant) and Polyphen (Polymorphism Phenotyping) scores, taking the localizations of these SNPs on the protein domains. Finally 5, 8 and 5 SNPs were chosen for Bmal1, Clock and Cry1 genes respectively. We screened the selected SNPs for both their effect on E-Box mediated transcription and their interaction with each other. Here we show 1 SNP form both Bmal1 and Clock genes change their activation rates, and 3 Cry1 SNPs change the repression levels of the transactivation. We

also show that the same 3 SNPs on Cry1 levels have altered the proteins ability to interact with BMAL1 protein. The biological significance of this change is however yet to be validated.

In Chapter 2, detailed information regarding the biological rhythm, its components and the molecular mechanisms underlying the feedback transcriptional loop are given.

In Chapter 3, the materials and experimental procedures used during the thesis work are explained in detail. Selection of SNPs and their generation by site-directed mutagenesis, dual luciferase assay, mammalian two hybrid assay, biochemical validation of the protein expression and lentiviral transduction of mammalian cells are included in this chapter.

In Chapter 4, the results from the experiments contained in this thesis are shown.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Biological Rhythms

Different rhythms have been affecting life-forms for long decades, helping them have an evolutionary advantage over other competitive life-forms [1]. Some of these rhythms can be classified as geophysical cycles. Geophysical cycles are usually a response to environmental temporal cues such as light and temperature. Daily rhythms (∼24 hours ± 30 seconds) govern the daily biological changes in the living creatures. These changes may include body temperature, hormone secretion, urine production and daily feeding patterns [2 - 3]. Seasonal cycles have a period of 365.24 days governing changes in physiological, psychological and behavioral aspects through the seasons. This cycle is especially important for the coordination of seasonal events such as migration, hibernation and reproduction [4-6], . The seasonal cycles, even though still not very clearly understood, also seem to have a significant effect on the wellbeing of the organisms. The phase of the moon also affects the living world creating a lunar cycle that circulates in 29.53 days, as well as due to its gravitational force a tidal cycle of approximately 12.8 hours. Lunar rhythms are mostly effective on nocturnal animals due to changes in illuminance at night when sunlight is not directly available. It was also shown by Law S.P. in 1986 [7] that there was a synchrony between the lunar cycle and menstrual cycle in humans. Tidal cycles arise from tidal variables such as temperature, hydrostatic pressure and salinity and is a powerful environmental cue for aquatic life. The crab *Carcinus maenas* showed changes in its locomotor acivity when salinity was changed under laboratory conditions [8].

Biological rhythms also be classified in a different way, concerning three main rhythmic activites with different periods: circadian, ultradian and infradian rhythms. Circadian rhythms have a period of around 24 hours generated by an internal clock mechanism. This circadian rhythm, ubiquitous in many organisms such as plants, animals, fungi, algae and bacteria, regulates and alters the organism's physiological and psychological status throughout the day primarily affected by the dark and light conditions during the day [3]. Ultradian rhythms gives rise to cycles which have a shorter period than circadian rhythm. This period may range from seconds to hours, fine-tuning the blood circulation, thermoregulation, urination and bowel

activity as well as very short periodic events such blinking [4], [9]. Infradian rhtyhms have a period longer than 24 hours. The period length of the infradian rhythms may diverse from days to seasons making it a huge oscillation with a lower frequency. The best known examples of infradian rhythms are the menstrual cycle of humans, seasonal hibernation of animals as well as seasonal migrations of certain life-forms [4].

2.2 Biological Rhythms

The significance of biological rhythms has been understood decades ago and a new scientific discipline, chronobiology, has emerged to research and understand both the mechanism underlying these rhythms and their influences on the biological life [10]. One of the most amazing findings of chronobiologists is the daily rhythm in living organisms. Physiological and behavioral states of organisms are under the influence of a precise internal biological clock mechanism that can synchronize various physiological and behavioral states with different timespans through the day. This has been shown to be related to the circadian clock, originated from the Latin words circa and diem, meaning about a day [11]. Circadian rhythm has been shown to be conserved through many species due to the evolutionary advantages to the organisms. Cyanobacteria for example is able to carry out photosynthesis during day and nitrogen fixation at night [12]. This separation of two different events into two distinct time windows allows the algae to execute two biochemically incompatible processes for survival. Malarial patients exhibit fever only during the night due to malarial plasmodia bursting out of the red blood cells, when the local mosquitos are active, attracting more mosquitos in order to spread even further [13], [14]. In *Arabidopsis* circadian rhythm allows the delay of flowering until the days are longer, providing a fitness advantage for those growing on higher latitudes where day length increases rapidly, avoiding the freezing weather [15].

2.2.1 History of Circadian Rhythm

A rhythm in biological systems have been known for centuries but not studied carefully and were based on mere observations of nature. However in 1729 Jean Jacques d'Ortous De Marian, a French chronobiologist, demonstrated the existence of a circadian rhythmicity in *Mimosa pudica*. De Marian experimented with the plants that are known to open and close their leaves during the day. Under constant dark conditions De Marian found out that the plant's leaves

were still opening and closing regardless of any external cue. Since then it has been noted that nearly all life-forms on earth has an internal timing mechanism [17], [18].

In 1962 Michel Siffre a French scientist spent two months in a subterranean cave, isolated from any environmental cues such as light. During these two months his sleep/wake cycle was recorded. The findings proved that humans had an internal biological clock as well, also that it was slightly longer than the internal clock experienced on the surface of the earth, around 24 hours and 30 minutes [19]. Following Siffre, in 1965 Jürgen Aschoff reported his findings from over 150 isolation experiments conducted in an underground bunker. Through these isolation experiments Aschoff measured the isolated subject's body temperature, activity pattern, time estimation and urination intervals. With his findings he also introduced the term zeitgebers [20].

2.2.2 Biological Importance

Biological rhythms can be found in whole organisms as well as individual cells. The circadian rhythm is significantly important for life-forms as the rhythm allows the most efficient use of biological activities. There is a significant increase in survivability when the organisms are able to synchronize better with their environment. Circadian rhythm functions as a clock mechanism that enables the separation of conflicting processes, such as sleeping, feeding and mating [24]. It has also been shown that many physiological and behavioral processes such as energy metabolism, cell division, endocrine secretion, blood viscosity, body temperature and locomotor activity [25-30] are under the influence of fine-tuning clock mechanism. The clock mechanism is extensively important for human physiological, psychological and behavioral well-being, perturbations of this mechanism has been shown to be associated with health disorders such as insomnia, hypersomnia and jetlag as well as metabolic syndrome, diabetes, hyperglycemia, insulin sensitivity and cancer [31]. As more and more disorders are being associated with circadian rhythm perturbations, understanding the core clock mechanism will shed a light on associated diseases and their treatment.

2.2.3 Characteristics and Entrainment

An oscillation can be defined by three main characteristics: periodicity, robustness and entrainment. Periodicity is the tendency of an oscillation to recur at regular intervals. Circadian clock has a persistence (free-running) period length of around 24 hours, and this period length is conserved under constant conditions. The circadian rhythm also exists even when there is no

external sensory input. Robustness refers to the ability of maintaining the periodic oscillation even under intrinsic or extrinsic perturbations. Circadian rhythms are able to compensate for temperature changes, maintaining its intrinsic period, phase and amplitude despite external temperature fluctuations. This allows the circadian rhythm to be maintained under different temperature conditions without any perturbations. The third characteristic of oscillations, entrainment, suggests that the oscillations period and phase can be aligned to the period and phase of an external rhythm. The circadian clock can be entrained in different ways, light being the primary entrainer synchronizing the circadian rhythm with the light dark conditions throughout the day [29], [32-33]. Including the light, the entrainers of circadian rhythm are called *Zeitgebers* (cf German Zeit or "time", and Geber or "giver"). These factors that align the circadian rhythm with external cues include but not limited to feeding [29], exercise [34], social interaction [35], temperature cycles [36] and sleep deprivation [37].

Circadian system works as a hierarchical mechanism in mammals. Mammalian brain functions as the key to synchronize the whole circadian rhythm in the organism. Individual cells of tissues possess their own internal circadian clock and they need to be synchronized with other cells in the same tissue for the tissue to function as necessary. Different tissues also need to be aligned with the rhythm in other tissues for better functionality. When a cell is aligned to the external time, the endogenous clock starts to produce oscillating output signals to pass the clock information to other cells, and tissues [38]. Brain plays a major part in this synchronization. The most important zeitgeber, light, is perceived by eyes as an input signal for synchronization which is conveyed to the suprachiasmatic nuclei (SCN) where the input signal entrains the SCN according to the environmental time [27]. With the input for synchronization the behavioral and physiological activities of the organism are also entrained synchronizing their phase with the external time. Tissue and cellular level clocks are synchronized through the external zeitgebers as well. It can be seen that these zeitgebers function as an input, processed by a pacemaker, the circadian clock, and organizing various periodic physiological effects as the output [38-39].

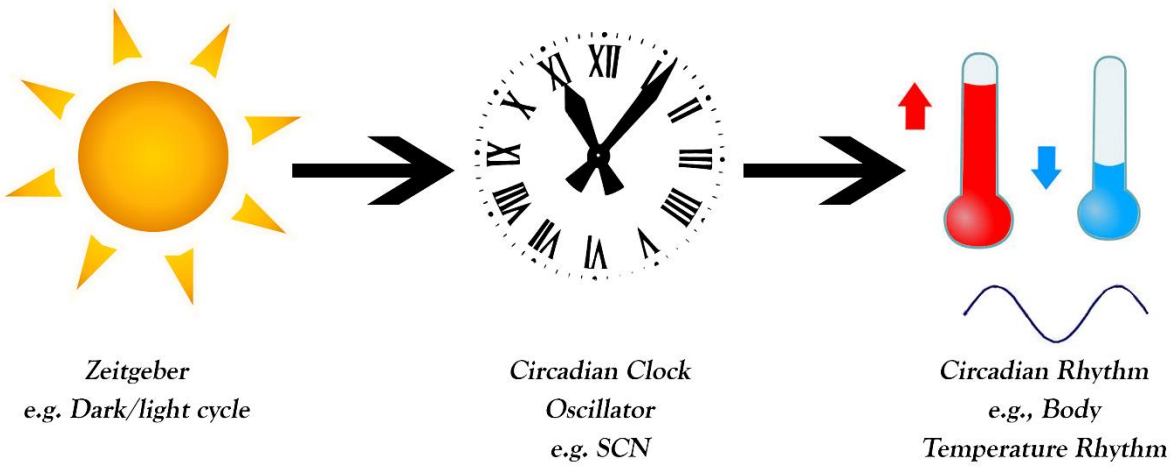


Figure 2. 1.Schematic representation of hierarchical manner in circadian rhythms

2.3 Discovery of Novel Clock Components

Through time the molecular mechanism of the circadian rhythm started to be investigated thoroughly. Phenotype-driven or forward mutagenesis and reverse genetics, behavioral phenotyping and molecular cloning were used to find core clock components. In 1971 Ronald J. Konopka and Seymour Benzer found three mutations in *Drosophila melanogaster*, while screening for clock genes. These three mutations either shortened or lengthened the period of the flies' locomotor activity as well as their eclosion rhythm. These three mutations were mapped to the same locus of a single gene named "per" for period. These mutations gave rise to three different rhythmicity phenotypes other than the natural 24 hour period: a longer period of 28 hours, a shorter period of 19 hours and an arrhythmic phenotype showing no significant circadian pattern. [21]. The second clock gene Clock (Circadian Locomotor Output Cycles Kaput) was discovered by Joseph S. Takahashi and his group in 1994 through a genetic screening of mice treated with ENU. A mutation lengthening the circadian period and abolishing persistence of rhythmicity was mapped to the Clock gene [22]. In 1997 Hogenesch characterized ARNTL1 (Aryl hydrocarbon receptor nuclear translocator-like protein 1), a basic helix-loop-helix-PAS superfamily protein, known as BMAL1 and MOP3 to be a core clock component [23].

2.4 Molecular Mechanism of the Mammalian Circadian Clock

The circadian clock mechanism in mammals consists of interacting positive and negative autoregulatory transcriptional-translational feedback loops. The first loop, core loop, have two basic helix-loop-helix-Period-Arnt-Single-minded (bHLH-PAS) family transcription factors, BMAL1 and CLOCK, as well as repressor proteins, CRY1, CRY2, PER1 and PER2. BMAL1 and CLOCK heterodimerize in the cytoplasm to form the BMAL1/CLOCK transactivator complex and translocates in the nucleus where they promote the expression of aforementioned Per and Cry family genes through binding to E-Box (5'-CACGTG-3') or E'-Box (5'-CACGTT-3') elements [40-42]. This increases the levels of PER and CRY levels in the cell [43-45]. After reaching a high level CRY and PER proteins dimerize in the cytoplasm translocating into the nucleus [46-48]. In the nucleus PER/CRY dimer functions as a repressor, inhibiting BMAL1/CLOCK transactivation, and decreases the expression rates of themselves as well as other clock-controlled genes. Afterwards CRY and PER levels are reduced by ubiquitin ligase complexes FBXL3 and beta-TrCP1. This core loop takes approximately 24 hours to reach the primary protein levels and start a new cycle, creating an oscillation of core-clock and clock-controlled gene transcription and translation [49]. Even though it is a very simple and straightforward model, this transcriptional-translational feedback loop is not enough for an accurate and robust clock oscillation. [50].

The second loop, stabilizing loop, helps to fine tune the first feedback loop. In this second feedback loop BMAL1/CLOCK complex binds to the E-Box elements found in the promooters of retinoic acid-related orphan receptors, Rorα and Rev-erbα, activating their transcription [3, 51]. Bmal1 promoter contains retinoic acid-related orphan receptor response elements (ROREs), to which REV-ERBα and RORα competes to bind. REV-ERBα represses Bmal1 transcription whereas RORα functions as an activator. This second transcriptional-translational feedback loop regulates BMAL1 levels both positively and negatively helping to sustain a precise circadian oscillation.

The oscillation of circadian clock, however, is not only regulated by the two feedback loops mentioned above, but also through post-translational modifications of clock proteins. The function, precision and speed of circadian clock is regulated by various phosphatases and kinases [52]. As degradation of PER and CRY is necessary to terminate the repression phase to ensure the oscillation, the molecular clock relies on the precise degradation of these repressor proteins. CKIε and CKIδ phosphorylate PER proteins which makes them a target for βTrCP

ubiquitination and degradation by the 26S proteasome [53, 54]. CRY proteins are polyubiquitinated by FBXL3 after phosphorylation by AMPK (AMP activated protein kinase), targeting them for proteosomal degradation [55]. CRY2 on the other hand is phosphorylated by a sequential DYRK1A/GSK-3β cascade [56]. BMAL1 is phosphorylated by CK2α through Ser90. This phosphorylation causes intracellular localization of BMAL1. SUMOylation, a post-translational modification, also plays a part in BMAL1 degradation. SUMO (small ubiquitin-related modifier protein) binds to lysine residues on BMAL1 interacting with CLOCK. This effects the turnover of BMAL1 directly [57-58]. BMAL1 acetylation is also crucial for the sustainability of circadian oscillations. [59]. CLOCK also functions as a histone acetyltransferase [60]. For the activation of genes, regulated by the CLOCK/BMAL1 transactivator complex such as DBP, acetylation of H3 and H4 histones in the promoter regions is required [61]. CRY interaction with CLOCK/BMAL1 complex for repression is also achieved by BMAL1 acetylation by CLOCK [62]. CRY repression on BMAL1:CLOCK was revealed to happen even in the absence of PER activity [63-64].

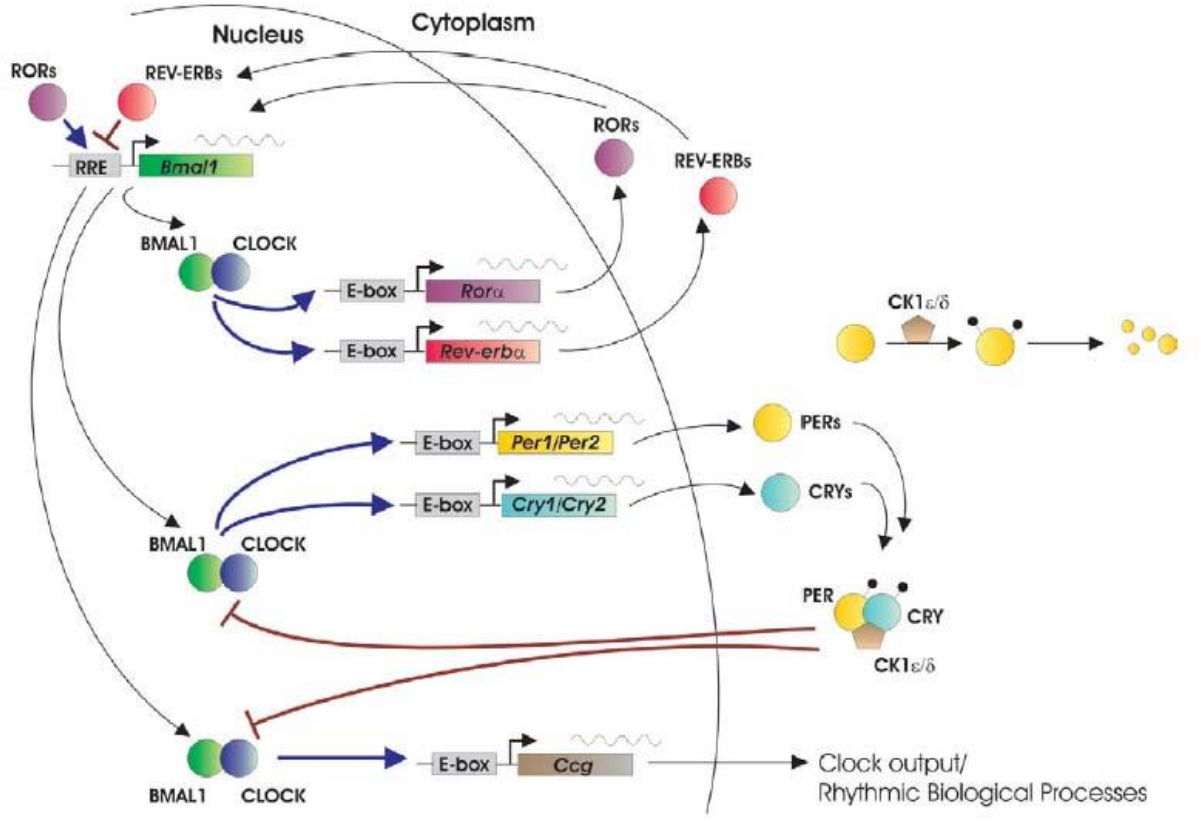


Figure 2. 2. Schematic representation of molecular clock and its components. Adapted from [50]

2.5 Structures of core clock components

In the recent years crystal structures of the core clock proteins were discovered. It can be seen from these crystal structures that all the core clock components share similar highly conserved domains [65].

Both CLOCK and BMAL1, the transactivators of circadian clock mechanism share similar domains, an N-terminal bHLH (basic helix-loop-helix) domain, two tandem PAS domains (PAS-A and PAS-B) and TAD (trans-activating domain) [66]. bHLH domains found on both activators allow them to bind short DNA motifs on specific promoters called E-Boxes required for transactivation and also function as a heterodimerization surface for both proteins [66-68]. PAS (Per-ARNT-Sim) serve as interaction motifs for CLOCK/BMAL1 heterodimerization. The PAS domains consists of ∼130 amino acids, which form 5 antiparallel β-sheets and several α-helices flanking them. The cavity formed by these secondary structures allows the FAD binding [66]. PAS-A and PAS-B domains directly interacts with their corresponding domains of each other. PAS-A of CLOCK is also shown to interact with bHLH domain of its own, but due to a significant difference in the arrangement of domains in the two trans-acting proteins, BMAL1 PAS-A domain does not have a direct effect with its bHLH domain. The exposed CLOCK PAS domains are negatively charged opposed to the exposed BMAL1 PAS domains, which are positively charged or neutral. This difference gives rise to different interactions with PER1, PER2, CRY1 and CRY2 proteins [69-71]. PAS-B domain of BMAL1 contacts with PAS-B domain of CLOCK, burying hydrophobic residues on both subunits. PAS-B domain of CLOCK and C-terminal region of BMAL1 has also been shown to interfere with CRY repression [72-74]. CLOCK shares homology with acetyl-coenzyme A binding motifs which allows it to exhibit histone acetyl transferase activity [60].

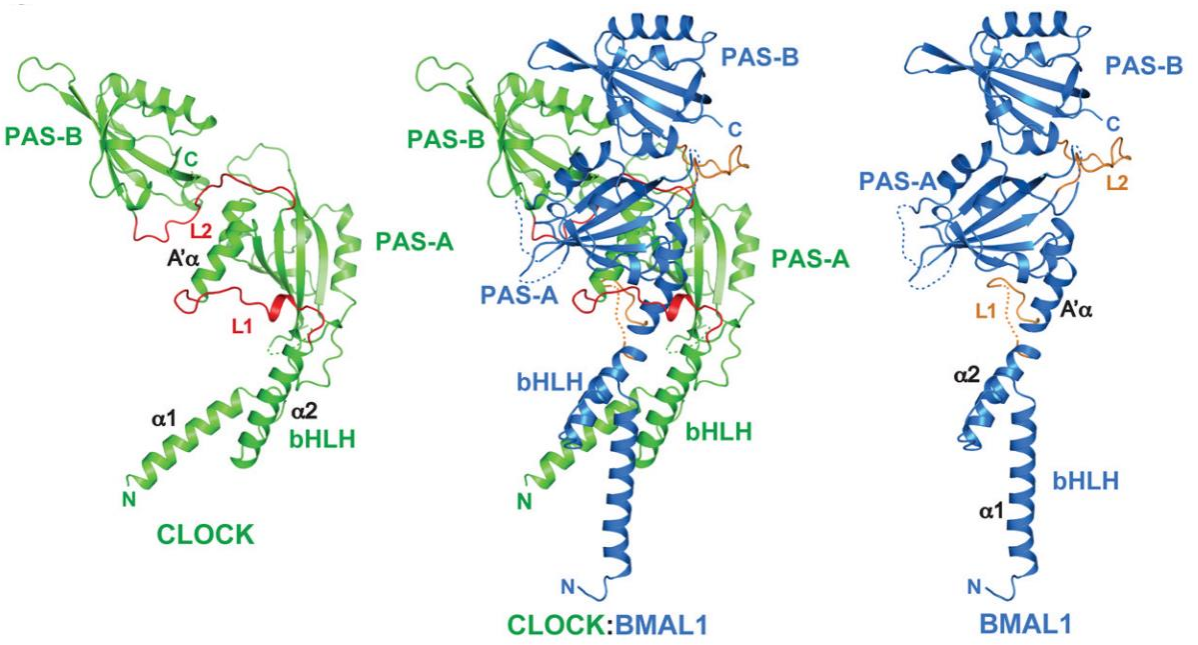


Figure 2. 3.Crystal structure of mBMAL1:mCLOCK complex. Adapted from [66]

Mammalian CRYs contain 2 major domains: PHR (photolyase homology region) domain and an unstructured C-terminal domain. The C-terminal tail together with coiled-coil helix of the PHR allows interactions with C-terminal BMAL1 residues [48, 72, 75-76]. C-terminal of PER1 and PER2, and PAS domains of CLOCK and FBXL3 also interact with CRYs through interaction with this PHR domain [55, 66, 77-78]. Phosphorylation of Ser71 by 5' AMPK destabilizes CRY1, weakening PER2 binding and increasing FBXL3 affinity [55]. FBXL3 interacts with the helix α22, C-terminal lid and the FAD binding pocket of CRY1 [79]. Cys412, C-terminal lid, forms a disulfide bridge with Cys363 on α14 helix. α11 helix, especially Tyr287/Gly288 were found to be critical for transcriptional repression activity of CRY1 [80-81]. Met309, Glu214, Glu216, Ala217, Leu218, and Cys259, were shown to play an important part in transcriptional repression [81]. PER2 interaction of CRY1 is stabilized by a zinc ion interacting with Cys414 (lid) and His473 residues of CRY1 and Cys1210, Cys1213 residues of PER2 [82].

Figure 2.4. Crystal structure of mCRY1. Adapted from [179].

2.6 The Central Circadian Pacemaker and Entrainment

As mentioned before the circadian clock in mammalians act as a 3 component system including a circadian clockwork or a pacemaker that functions as the bridge between inputs and outputs. This clockwork can be found in different tissues whereas they need to be synchronized through a central pacemaker, SCN (suprachiasmatic nucleus), located in the hypothalamus of mammals. The most effective zeitgeber, light, is perceived by the eye and signaled to SCN [29, 83]. Upon receiving the signal from the eye via retinohypothalamic tract (RHT), SCN sends out neuronal and hormonal signals to different regions of the brain and peripheral tissues synchronizing them to the environmental light/dark cycles [27, 84]. SCN was reported to be the possible main pacemaker in a study where SCN lesions of rats were disrupted. It was shown that drinking behavior and locomotor activity of rats that have disrupted SCN showed a similar free-running circadian to the blind rats compared with the rats with effective SCN [85].

There are many environmental stimuli that effects circadian rhythm such as feeding and temperature [3], but the strongest zeitgeber for mammalian circadian rhythmicity is light [29]. Light perception can differ from organism to organism, including eyes, photoreceptors, pineal glands and retina [86]. In mammalians light is perceived through rod and cone photoreceptors

located in the inner retina of eyes and the information is sent to SCN via RHT [87]. Upon receiving the input from eyes, CaMK (calcium/calmodulin dependent protein kinase), MAPK (mitogen-activated protein kinase) and PKA (protein kinase A) are activated in SCN, which in turn activates CREB (Ca+2/cAMP-responsive element-binding protein) via phosphorylation [88-89]. Upon activation, CREB binds to CRE (calcium/cAMP response element) and activates the expression of Per genes functioning in the negative loop of the molecular clock. Once light is not perceived (subjective night) Per expression decreases again [27, 90].

2.7 Peripheral Clocks and Entrainment

Core clock components are expressed in peripheral tissues creating a circadian rhythmicity. These tissues all express their own core clock components, but they are all synchronized to light/dark cycles and each other. This synchronization is possible through metabolic and neuroendocrine cues depending on the SCN, the pacemaker [91]. Microarray analyses and oligonucleotide arrays showed 8-10% of expressed genes in liver and heart were under circadian regulation and 10 % of these genes were similar to core clock signaling [92-93].

Peripheral clocks can be synchronized through cues from SCN as mentioned previously. However they can also be entrained through other environmental cues. One example is the external temperature fluctuations. A slight rhythm change in the external temperature is able to reset the peripheral clocks [39]. HSF1 (heat shock factor 1), which has an oscillatory pattern, was proposed to be a possible protein for the mechanism [94-95] and the humoral and neural pathways between the body and hypothalamic thermal center was suggested to play a role in temperature entrainment [96]. Decreased food availability has also been shown to reset peripheral clocks without effecting SCN [97]. Glucocorticoid hormone was also revealed to be regulating the circadian rhythmicity in peripheral tissues by directly interacting with the core clock genes' regulatory regions containing glucocorticoid-response elements even in the presence of disrupted SCN tissue [98-100].

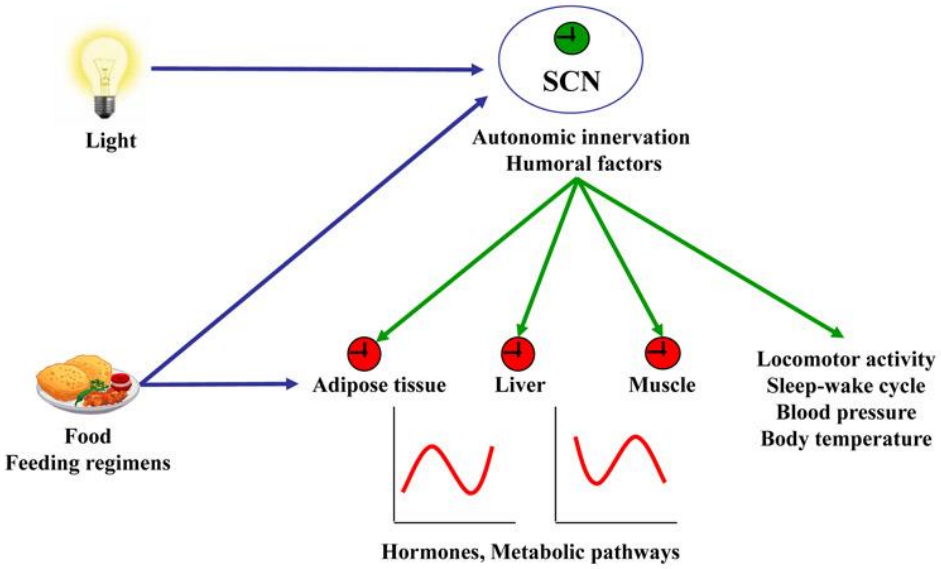


Figure 2. 5.Schematic representation of resetting central and peripheral clocks.

2.8 Circadian Regulation of Metabolism

Metabolism should be aligned with the environmental cues such as the sleep/wake cycle and feeding times. Alignment with these external changes that effect the circadian rhythmicity are crucial, whereas disturbances may cause metabolic diseases [101]. A study in 2011 has revealed shift workers having a higher risk of metabolic diseases, such as obesity and diabetes [102]. Oscillations in metabolic hormone levels suggest that there is a direct correlation between metabolism and circadian rhythm. Insulin, glucagon, adiponectin, leptin, and ghrelin exhibit circadian oscillation as well as metabolic enzymes and transport systems such as lactate dehydrogenase, glycogen phosphorylase, cytochrome oxidase, and acetyl-CoA carboxylase [103-110].

Human glucose homeostasis is directly correlated with the circadian clock. A study based on the plasma profiles of glucose and insulin in SCN-lesioned rats revealed that SCN directly controls basal glucose concentrations [111]. In another study ClockΔ19 mutant mice showed decreased oscillation in hepatic glycogen levels as well as expression levels of the rate-limiting enzyme of glycogenesis, Gys2 (glycogen synthase 2) [112]. Another rate-limiting enzyme for

gluconeogenesis PEPCK (phosphoenolpyruvate carboxylase) is regulated by liver clock as shown in a study using liver-specific knockout BMAL1 mice. In the study *L-Bmal1*−/− mice lost the rhythmic expression of hepatic glucose regulatory genes giving rise to exaggerated glucose clearance [113]. The pineal hormone melatonin also plays a big role in glucose homeostasis. Melatonin modulates circadian rhythms in mammalian peripheral tissue helping them synchronize with SCN [114]. Melatonin receptor knockout mice continuously express PER1 and secretes increased levels of insulin from pancreatic islets [115]. CRY1 and CRY2 interact with the glucocorticoid receptor and affect the transcriptional response to glucocorticoids in mice. Loss of Cry1 or Cry2 greatly increases PEPCK1 levels resulting in glucose intolerance [116]. A 28 hour free-running rhythm was observed in mice overexpressing a CRY1 mutant Cys414Ala as well as symptoms of diabetes [117]. REV-ERBs and RORs functioning in the feedback loop of molecular clock regulate PEPCK expression, whereas the loss of REV-ERBs lead to high blood glucose levels in the fasting phase, and RORα stimulates G6Pase (Glucose-6-phosphatase) [118].

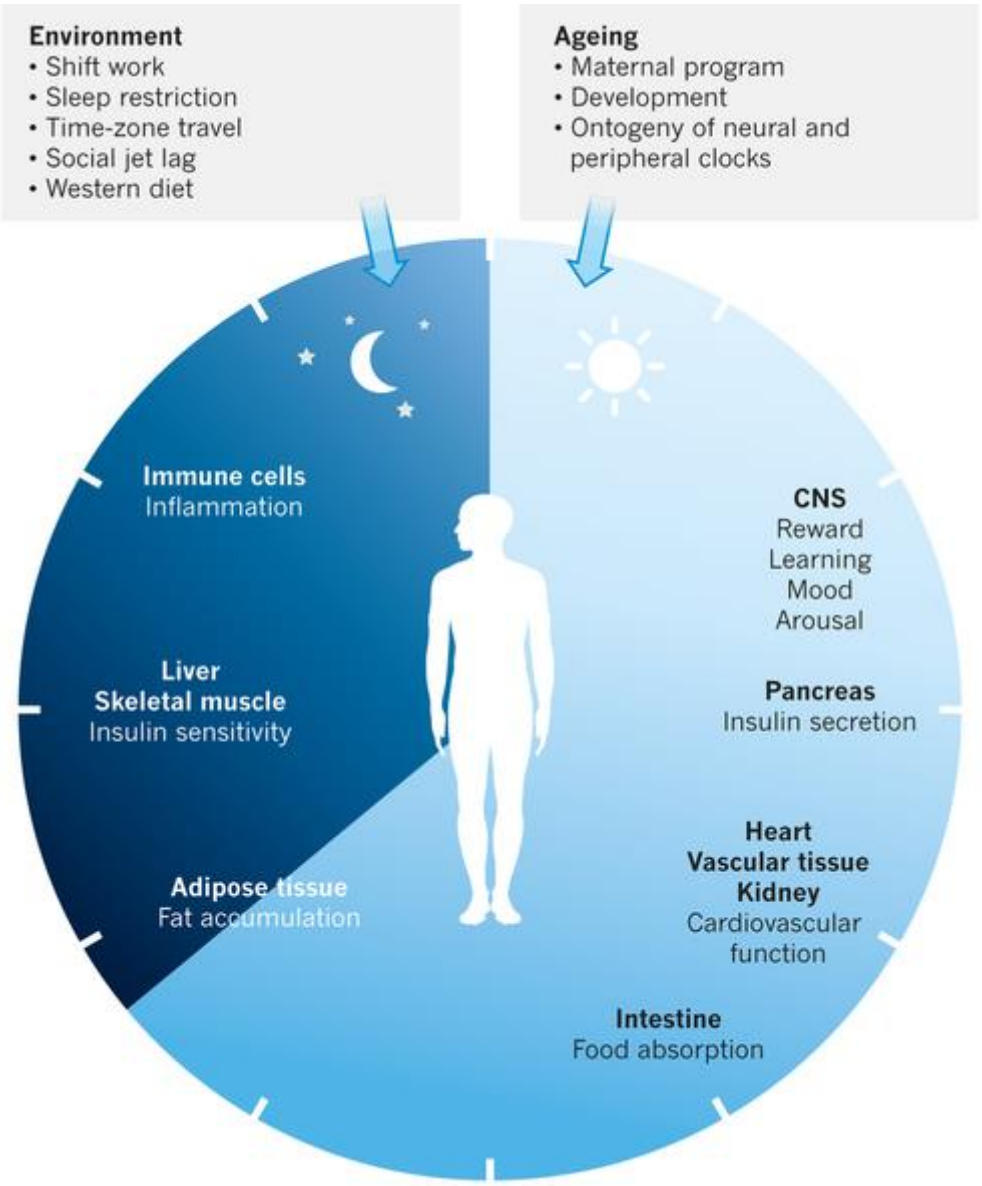


Figure 2. 6. Effect of ageing and environmental disruption on circadian control of metabolic processes. Adapted from [17]

2.9 Circadian Rhythm Sleep Disorders

As the circadian rhythm regulates the sleep/wake cycle in humans, disruptions of the clock may cause circadian rhythm sleep disorders (CRSDs). CRSDs are chronic patterns of sleep/wake rhythm disturbances caused by alterations in the circadian clockwork or desynchronization between the endogenous circadian rhythm and the sleep/wake cycles [119]. The inability to sleep and wake up at socially acceptable times is called DSPD (delayed sleep-phase disorder). The patients with DSPD have more than 2 hours of delay in the major sleep period. 3 major

biological factors, including increased sensitivity to the phase-delay response of evening light, decreased response to the phase-advancing effect of light in the mornings [120], and a longer circadian period. G320T, C319A and G294A SNPs (single nucleotide polymorphisms) and a variable tandem repeat polymorphism (-318 ½ VNTR) in the PER3 promoter were associated with DSPD [121]. ASPD (advanced sleep-phase disorder) on the other hand is when a patient have a chronic inability to stay awake until the socially acceptable sleep time followed by an earlier than desired wake-up time. ASPD is less common than DSPD and is likely correlated with age, similar to the phase advance of circadian pacemaker with aging [122]. One of the major causes of FASPS (familial advanced sleep-phase disorder) has been reported to be the T44A mutation in the human CKIδ (creatinine kinase 1 delta) gene causing a shorter circadian period phenotype [123]. Another circadian rhythm disorder is ISWRD (irregular sleep-wake rhythm disorder) characterized by multiple sleep and wake patterns during the 24 hour cycle. ISWRD disorder is particularly observed in older adults with dementia, and it is suggested to be caused by the altered PER3 expression in older people [124]. Some sleep/wake cycles cannot be synchronized to the 24-hour environment causing a non-entrained rhythm disorder known as N24SWD (non-24-hour sleep-wake disorder). As expected this disorder is very common in blind people (approximately 50%). The disorder is believed to be caused by a long circadian period that cannot be entrained [125], whereas a mutation in CKIε (creatinine kinase 1 ε) is believed to be the associated with the disorder [126].

2.10 Circadian Rhythm and Chronic Diseases

Following the dramatic changes in industrialization and social development, diseases such as cancer, diabetes, cardiovascular diseases and chronic respiratory disease are increasing rapidly among humans. This dramatic increase has raised the question of whether changes in lifestyles of people can directly influence the development of health problems due to the disruption of circadian rhythms.

As revised previously circadian rhythm affects very crucial metabolic event in the body such as glucose homeostasis and lipid metabolism. Disruptions in the circadian rhythmicity may be caused both by misalignment to the external environment and/or due to the molecular mechanism of circadian clock not functioning properly.

In the recent years there have been many studies trying to find a connection between the circadian clock and disorders [4, 127-128]. Better understanding of the clock might give promising results to medical research on treatment for specific chronic diseases.

2.10.1 Cancer

There have been a lot of studies suggesting a direct correlation between night-shifts and occurrence of breast cancer [129-131]. Constant exposure to light has increased the breast cancer incidences in mice supporting the idea of being subjected to light conditions during night, thus disrupting the alignment between molecular clock and the environmental clock, may be the cause of this increased cancer occurrence [132-133]. The disruption of circadian rhythm causes a change in the melatonin levels which was shown to be associated with the development of breast cancer [134]. Another study on the airline pilots showed an increase in the risk of prostate cancer which is also believed to be caused by melatonin levels [135]. Other cancer types such as endometrial cancer and non-Hodgkin's lymphoma were also observed to be more common among night-shift workers [136-137].

In 2005 Zhu Yong and his team found a variation of Per3 genotype to be associated with an increased risk of breast cancer among premenopausal women [138]. Later the same group also found out that breast cancer risk was increased in women with NPAS2 Ala394Thr variant [139]. Another research based on Chinese populations revealed variants in CRY2, NPAS2, PER1 and PER3 gave rise to a significant increase to prostate cancer risk [140]. Interestingly the results showed a 4 fold increase in prostate cancer risk in men with diabetes suggesting a possible link between CRY2, diabetes and prostate cancer. It was also observed that in prostate cancer cells CLOCK and PER levels were down-regulated as opposed to BMAL1 levels, which were upregulated [141]. Other studies have also revealed mPER2 and BMAL1 proteins may play a role in tumor suppression by driving the cells to apoptotic cell death [142-143], as opposed to the Cry knockout was revealed to increase the risk of cancer in mice [144]. Epigenetics was also found to play a possible role in cancer development, whereas in a study conducted by Shou-Jen Kuo, 37 out of 53 breast cancer cell lines showed hypermethylated PER1, PER2, CRY1, or BMAL1 promoters [145]. Even though there are many studies suggesting a direct link between circadian rhythm disruption and cancer there are other studies contradicting this idea [146].

2.10.2 Metabolic Syndrome and Obesity

Metabolic syndrome is a collection of several medical conditions including central obesity, hyperglycemia, elevated fasting plasma glucose and hypertension. Metabolic syndrome is a big concern in the recent society because 25% of the human population suffers from it which also makes the people with syndrome have a higher risk for type 2 diabetes, heart attacks and strokes [147]. Similar to cancer, prevalence of type 2 diabetes has increased significantly in the recent years, suggesting that it may be related to circadian rhythm dysfunctions. This idea is further supported by studies showing the interplay between metabolism and circadian clock [25-30]. A major finding concerning diabetes was the realization of insulin secretion and basal blood glucose levels being under circadian control [148]. Three independent studies based on CLOCK protein polymorphisms showed circadian rhythm was effective on obesity, nonalcoholic fatty liver disease and metabolic syndrome risks [149-151]. NPAS2 and PER2 was later shown to be associated with hypertension and high fasting blood glucose, respectively [152]. A polymorphism in Bmal1 promoter in spontaneously hypertensive rat strain is associated with type 2 diabetes and hypertension [153]. ClockΔ19 mutant was revealed to develop a metabolic syndrome, including hyperglycemia, hyperlipidemia, hyperleptinemia and hepatic steatosis and in mice [154].

2.10.3 Cardiovascular Diseases

CVDs (cardiovascular diseases) include vascular diseases associated with heart, brain and blood vessels. It is the biggest cause of deaths worldwide with more than 17 million deaths worldwide per year [155]. Disruptions in circadian rhythm are believed to be lead to cardiovascular diseases [156-158]. In 1968 a study conducted by Masahiro Kaneko human peripheral blood flow levels was revealed to show circadian variations [159], followed by other studies showing a circadian variability in vascular function [160-161]. Environmental factors affecting circadian rhythm are believed to play a big role in the development of cardiovascular diseases [162-164] due to the fact that a mismatch of oxygen supply and demand occurring in the morning when the body shifts from sleep state to wake state. [165-166].

In the recent years there have been studies using clock mutant mice to associate CVDs with molecular clock. Bmal-1 knockout and Clock mutant mice exhibit endothelial dysfunction, where variation in sensitivity of aorta to vasoactive agents is altered, while the response to nitric oxide, remains unaltered [167-168]. A very similar model is also observed in Per2 mutant mice

as well showing significantly lower norepinephrine and epinephrine levels in plasma throughout the day with a low mean arterial pressure independent circadian time [169]. CRY deficient mice exhibited suppressed catecholamine-mediated vasoconstriction response to an α-1-adrenergic receptor agonist response to α-adrenoceptor response [170]. Bmal1 was also associated with CVDs whereas upon deletion it caused COMT (catechol-O-methyl transferase) in aorta had altered expression and Kinninogen expression was dysregulated [171-172]. Further research concerning circadian clock genes and cardiovascular diseases may shed a light on how a disruption of the circadian rhythm increases the risk for cardiovascular diseases.

2.11 1000 Genome Project and Core Clock Protein SNPs

1000 Genome Project, started in 2008, aims to characterize human genome sequence variations deeply in order to find the relationship between genotypes and phenotypes [173]. For this purpose next-generation sequencing was used and 1092 individual's genome was sequenced [174]. One of the main reasons behind this project is to find the genetic variants with frequencies higher than 1% in the population. This data can be used by researchers who aim to find regions associated with a certain disease. Normally researchers sequence certain individuals that are with and without a disease and look for common variations in genes, including SNPs and structural variants, of individuals with the disease. With the data gathered from the 1000 Genome Project these researchers are able to combine their data with additional variants giving them the ability to localize disease-associated regions more precisely.

The complete human genome consists around 3 billion DNA base pairs, including 20000 protein coding genes. To sequence the genome completely the samples taken from individuals are broken into small pieces and sequenced thoroughly. The sequenced pieces are then aligned to the human reference genome sequence (Build 35) [175] and joined to each other. This sequencing is done 28 times so that the whole genome may be covered.

The 1000 Genome Project also contains a browser (browser.1000genomes.org) based on the Ensembl release 73, containing 1092 human genome samples [174]. A researcher can search for a specific gene to see either its specific region in more detail or check for the variants sequenced for this gene such as missense variants, coding sequence variants, intron variants and 5' or 3' variants.

For Bmal1, Clock and Cry1 genes the browser contains 548, 363 and 98 missense variants respectively. The locations of these variations, allele frequencies, SIFT and PolyPhen scores and their sequences are also available on the browser.

CHAPTER 3

MATERIALS AND METHODS

3.1. Preparation of Chemically Competent DH5α, Stbl3, DB3.1 and Transformation

E.coli strains were streaked on an agar plate and grown overnight at 37^0C. The following day a single colony was picked and grown in 5 ml LB medium overnight. The following day, after 16 hours, the bacteria culture was inoculated to fresh 500 ml LB medium. The new culture was incubated at 37^0C, shaking at 200 rpm. Every 20 minutes OD value was taken using a spectrophotometer and the culture was grown for around 3.5 – 4 hours until OD_{600} reached 0,9. The culture was then cooled to 4^0C in the cold room, transferred to pre-chilled 50 ml centrifuge tubes and centrifuged at 4^0C, 4000 rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 250 ml of ice cold 0.1 M $CaCl_2$. The washing steps were repeated 2 more times using 50 and 25 ml of 0.1 M $CaCl_2$. After the final washing step the pellet was resuspended in a 10 ml solution containing 80 mM $Cacl_2$, 50 mM $MgCl_2$ and 25% glycerol. The suspension was aliquoted to pre-chilled 1.5 ml microcentrifuge tubes. Aliquots were frozen in liquid nitrogen and stored at -80^0C.

For transformation 100 ng of plasmids were inoculated to 50 µl of competent *E.coli* cells on ice and incubated for 30 minutes. The cells were then heat shocked at 42^0C for 60 – 90 seconds and transferred back to ice for 2 minutes. The cells were then grown for 1 hour in LB medium. After 1 hour the grown culture were spread on an LB agar plate containing the antibiotic of choice.

3.2. Primer Design and Site-Directed Polymerase Chain Reaction (PCR)

For site-directed mutagenesis quick-change method was used. The primers were designed to have around 30 base pairs with the designated base changed in the middle of the primer. All the primer sequences for site-directed mutagenesis can be found Appendix A. Both primers designed for a SNP were used for PCR reaction with the plasmids containing the genes in question. The PCR reaction mixtures contained 0.3 mM dNTP, 5 µl of 10X Phusion GC Buffer (Thermo Scientific), 3% DMSO, 1 µM of each primer, 50 ng of the template plasmid and 1 unit

of Phusion DNA polymerase in a 50 µl of final volume reached with ddH_2O. The reaction conditions were set to 98^0C for 30 seconds, 55^0C for 30 seconds and 68^0C for 5 minutes, 18 cycles. PCR products were visualized in 1% agarose gel. The samples with correct sized bands were digested with 1 unit of DpnI FastDigest enzyme (Thermo Scientific) for 1 hour at 37^0C and then transformed to *E.coli* DH5α cells.

3.3. Gene Cloning and Transformation

All the variants of the genes were created on Bmal1-Sport6, Clock-Sport6 and Cry1-pBind vectors using site-directed mutagenesis using the methodology in section 3.2. These variants were then cloned to different vectors for different purposes. Bmal1 variants were cloned into pAct, and Clock variants were cloned into pBind vectors for Mammalian Two Hybrid Assays. For both of the constructs we first performed PCR reactions to amplify each of the variants with a specific primer for pAct and pBind vectors including 5' NotI and 3' EcorV restriction sites. The reaction mixture included 50 ng of template, 10 µL of 5X Phusion HF Buffer (Thermo Scientific), 0.3 mM dNTP, 0.5 µM forward primer, 0.5 µM reverse primer, 0.5 units of Phusion DNA polymerase and enough ddH_2O to make 50 µl. The amplification was checked on 1% agarose gel and cleaned using PCR Cleanup Kit (Thermo Scientific). 1 µg of PCR product and 1 µg of vector was digested by NotI and EcorV FastDigest enzymes (Thermo Scientific). The reaction mixture of the digestion protocol included 1 µg of template DNA or PCR product, 2 µl of 10X FastDigest Buffer, 1 µl NotI FastDigest and 1 µl of EcoRV FastDigest enzymes. Enough ddH_2O was added to make the final volume 20 µl. 1 µl of FastAP was also included into the vector digestion mixture to prevent self-ligation. Both of the mixtures were left to incubate at 37^oC for 1 hour and were cleaned using PCR Cleanup Kit (Thermo Scientific). The amount of remaining DNA after the cleanup was determined by Thermo Scientific NanoDrop™ ND-2000 1-position Spectrophotometer. The digested fragments were then mixed in a 1:3 (vector:insert) ratio to total in 1000 ng of DNA per ligation reaction. The ligation reaction mixture included 100 ng of vector:insert mixture, 4 µL of 5X FastLigase Buffer (Life Technologies) and 1 µL T4-FastLigase enzyme (Life Technologies). The mixture was then incubated at room temperature for 30 minutes. 10 µl of the mixture was then transformed into 100 µl DH5α cells using the methods mentioned above. Cells were spread onto agar plates containing 100 µg/ml ampicillin and were left to grow overnight. The following day 4 colonies from each variant plate were picked for colony PCR using the same protocol above. Positive colonies were inoculated into 5 ml of LB medium containing 100 µg/ml ampicillin, overnight.

The following day miniprep was performed using GeneJET Plasmid Miniprep Kit (Life Technologies).

Selected variants, namely Gly144Val, Arg293His, Arg348Cys for Cry1, Arg84Cys for Bmal1 and Phe122Ser for Clock, and their wild types were cloned into pENTR1A no ccDB vector for gateway cloning into pLenti CMV Hygro DEST lentiviral expression vector. Cry1 wild type and variants were cloned directly by digesting the Cry1-pBind vector with SalI and NotI digestion enzymes in a mixture containing 2 µg of template pLenti CMV Hygro Dest lentiviral vector or Cry1 – pBind vector, 2 µl of 10X FastDigest Buffer, 1 µl SalI FastDigest and 1 µl of NotI FastDigest enzymes. Enough ddH_2O was added to make the final volume 20 µl. pENTR1A digestion mixture also included FastAP (Thermo Scientific) to prevent self-ligation. The digested mixture of Cry1 – pBind was then loaded on a 0.8% agarose gel and ran until the bands separated well from each other. The bands containing Cry1 and variants were purified using PCR Cleanup Kit (Thermo Scientific). Ligation protocol was the same as mentioned above for pAct and pBind clonings. Bmal1 and Clock wild types and variants were on the other hand amplified by PCR, using specific primers for pENTR1A vector and then digested using NotI and EcoRV enzymes using the same protocol above, where we used EcoRV instead of SalI; followed by ligation to pENTR1A in the same way as Cry1 ligation. 5 µl of ligation mixtures were transformed to 100 µl of DH5α cells using the same methodology mentioned above, spread onto 100 µg/ml ampicillin containing plates. After grown overnight 4 colonies from each plate were picked for colony PCR. Positive colonies were inoculated into 5 ml of LB medium containing 100 µg/ml ampicillin, overnight. The following day miniprep was performed using GeneJET Plasmid Miniprep Kit (Life Technologies).

3.4. Cell Culture

For luciferase and mammalian two-hybrid assays HEK293T cell line and for kinetic luminescence assays U2OS cell line were used. The cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% FBS (Sigma-Aldrich), 100µg/ml streptomycin, 100µg/ml penicillin and 2 mM L-Glutamine (Sigma-Aldrich). Cells were grown at 37^0C in a humidified cell culture inhibitor supplied with 5% CO_2. Confluent cells were passaged by aspiring the media they were grown in, washing with 5 ml of PBS, removing the FBS and adding 1 ml of Trypsin, incubating at 37^0C for 1 minute and then resuspending the cells in 4 ml of fresh medium. 1 ml of the resuspended cells were then transferred to a new 10 cm plate containing 9 ml of fresh medium.

3.5. Luciferase Assay

HEK293T cells were passaged to a 10cm plate and were left to grown for around 24 hours until the plate was 70 – 80% confluent. A mixture of 5ng of Bmal1 – Sport6, 120 ng of Clock – Sport6, 50 ng of mPer1Luc, 1 ng of PGL4, 124 ng of Sport6 and 0.9 µl of PEI in 50 µl of DMEM was prepared for each well of a 96-well plate. The mixture was left in RT for 20 minutes. The medium in which the HEK293T cells were grown was aspired, the cells were washed with PBS, and resuspended in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% FBS (Sigma-Aldrich), 200µg/ml streptomycin, 200µg/ml penicillin and 4 mM L-Glutamine (Sigma-Aldrich); enough to make the final suspension around 80000 cells/ml. The cells were counted using a hemocytometer. After the required cell dilution was reached 50 µl of cells were aliquoted to each well of a 96 well plate. 50 µl of prepared plasmid – PEI mixture was added on top of each well, 3 wells per sample. The plate was then incubated at 37^0C in a humidified cell culture incubator supplied with 5% CO_2.

After 24 hours the incubated plate was taken out and cooled to RT. Luciferase buffer containing 75 mM HEPES (pH: 8.0), 4 mM $MgSO_4$, 20 mM DTT, 100 µM EDTA (pH: 8.0), 530 µM ATP, 270 µM CoA and 470 µM D-Luciferin was added to each cell in the dark room and the luciferase reading were taken using Fluoroskan Ascent FL (Thermo Scientific) after 5 minutes of incubation. Following the luciferase readings renilla buffer containing 7.5 mM Na_4PP_i - decahydrate, 125 µM APMBT, 200 mM Na_2SO_4 and 0.25 µM Coelenterazine-h was added to each well. After 10 minutes of incubation at RT renilla readings were taken again using Fluoroskan Ascent FL (Thermo Scientific). The luciferase readings of each well was normalized by renilla readings by dividing the luciferase values to their respective renilla values.

For CRY1 repression assays the same procedure was followed, except 2.5 ng of Cry1 – pBind was added to the mixture decreasing the amount of Sport6 vector used by 2.5 ng.

3.6. Protein Expression and Western Blot

HEK293T cells were passaged to 6-well plates each well containing around 200000 cells in 2 ml of 1X medium. After 24 hours when the cells reached 70 – 80% confluency, a mixture containing 2µg of plasmid and 6 µl of PEI in 200µl DMEM was prepared and left for incubation at RT for 20 minutes followed by dropwise addition onto the cells. The cells were left to

incubate for 36 hours at 37^0C in a humidified incubator supplied with 5% CO_2. After 36 hours the cells were collected on ice using 150 µl of ice cold SDS sample buffer containing 1 M Tris-HCl (pH 6.8), 0,5 M EDTA, 10% SDS, 1% β-mercaptoethanol, 8% glycerol and 1X Protein Inhibitor Complex (PIC). The collected cells were incubated on ice for an additional 10 minutes followed by 15 seconds of sonication. The sonicated samples were then centrifuged at 13000 g, 4^0C for 10 minutes. After the centrifugation the supernatant was collected and their concentrations were calculated using Bradford assay. 1µl of x% Bromophenol blue was added to each sample followed by incubation of the samples at 95^0C for 10 minutes. After the incubation the samples were cooled down on ice, centrifuged at 16000 g at RT. The soluble phases were used for SDS-PAGE using a 10% gel. After the electrophoresis gel contents were transferred to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, and USA), blocked by 4% milk in 0.015% TBS-T for 1 hour, and incubated in primary and secondary antibodies for 1 hour respectively. The membranes were visualized in a dark room using ECL (Enhanced Chemiluminescence) buffer containing 700 µl 1.5 M Tris HCl (pH 8.8), 50 µl of 250 mM luminol, 25 µl of 90mM coumaric acid, 3 µl 30% H_2O_2 and ddH_2O up to a total volume of 10 ml. BMAL1 overexpression was visualized using BMAL1 antibody (Abcam), CLOCK was visualized using CLOCK antibody (Abcam), and CRY1 was visualized using GAL4 antibody - DBD (SantaCruz). Anti-β-ACTIN antibody (Abcam) was used for loading control.

3.7. Mammalian Two Hybrid Assay

HEK293T cells were passaged to a 10cm plate and were left to grown for around 24 hours until the plate was 70 – 80% confluent. A mixture of 50 ng Bmal1 – pAct, 50 ng of Clock – pBind, 50 ng of PG5Luc, 1 ng of PGL4, 149 ng of Sport6 and 0,9 µl of PEI in 50 µl of DMEM was prepared for each well of a 96-well plate. The mixture was left in RT for 20 minutes. The medium in which the HEK293T cells were grown was aspired, the cells were washed with PBS, and resuspended in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% FBS (Sigma-Aldrich), 200µg/ml streptomycin, 200µg/ml penicillin and 4 mM L-Glutamine (Sigma-Aldrich); enough to make the final suspension around 80000 cells/ml. The cells were counted using a hemocytometer. After the required cell dilution was reached 50 µl of cells were aliquoted to each well of a 96 well plate. 50 µl of prepared plasmid – PEI mixture was added on top of each well. The plate was then incubated at 37^0C in a humidified cell culture incubator supplied with 5% CO_2.

After 24 hours the incubated plate was taken out and cooled to RT. Luciferase buffer containing 75 mM HEPES (pH: 8.0), 4 mM $MgSO_4$, 20 mM DTT, 100 µM EDTA (pH: 8.0), 530 µM ATP, 270 µM CoA and 470 µM D-Luciferin was added to each cell in the dark room and the luciferase reading were taken using Fluoroskan Ascent FL (Thermo Scientific) after 5 minutes of incubation. Following the luciferase readings renilla buffer containing 7.5 mM Na_4PP_i - decahydrate, 125 µM APMBT, 200 mM Na_2SO_4 and 0.25 µM Coelenterazine-h was added to each well. After 10 minutes of incubation at RT renilla readings were taken again using Fluoroskan Ascent FL (Thermo Scientific). The luciferase readings of each well was normalized by renilla readings by dividing the luciferase values to their respective renilla values.

3.8. Lentivirus preparation

For promising variants of Bmal1, Clock and Cry1, lentiviruses were prepared for overexpression in U2OS cell line. For this purpose the wild types and the variants of the proteins were cloned to pENTR1A vector as mentioned above. Afterwards the genes were transferred to pLenti CMV Hygro DEST vector using Gateway LR CLONASE enzyme mix II (Invitrogen). For lentivirus production HEK 293 T cells were splitted to 100mm culture dishes with a density of $2.5x10^6$ cells per plate using 10 ml of high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% FBS (Sigma-Aldrich), 200µg/ml streptomycin, 200µg/ml penicillin and 4 mM L-Glutamine (Sigma-Aldrich).

The following day a mixture containing 10 µg of lentiviral expression vector (pLenti CMV Hygro DEST) containing the gene of interest, 1 µg of VsVg and 9 µg of 8.2 Δvpr were mixed with enough DMEM to make a mixture of 380 µl. 20 µl of PEI was added to the mixture, the mixture was mixed gently and incubated at room temperature for 10 minutes. After the incubation 5 ml of medium from the 100mm plate was aspirated and the prepared mixture was added on top of the remaining medium dropwise. The cells were then incubated overnight.

The following morning (~16 hours) 3 ml of fresh medium was added to the plates. After 48 hours of the transfection the remaining medium was aspirated from the plates and 8ml of fresh medium was added to the plates. After 72 hours from the transfection primary lentivirus was collected to 50ml falcon tubes, and fresh medium was added to the plates. At 96 hours post-transfection secondary lentivirus was collected to the same falcons as the primary lentivirus. The lentivirus was then filtered through a 0.45 micron filter, aliquoted and kept at -80 ^oC.

3.9. Lentivirus transduction

On the day prior to transduction U2OS cell line containing P(Bmal1) – Luc reporter was split to 35mm plates with a density of $1x10^5$ cells per plate using 1.5 ml of high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% FBS (Sigma-Aldrich), 200µg/ml streptomycin, 200µg/ml penicillin and 4 mM L-Glutamine (Sigma-Aldrich). On the day of transduction a mixture containing 750µl of filtered virus, 250 µl of fresh medium and 1 µl of 8mg/ml Protamine sulfate (Sigma-Aldrich) was prepared. The medium in the plates were aspirated and the cells were left to grow in the new prepared medium containing lentivirus. At 16 hours post-transduction the medium was aspirated and 1.5 ml of fresh medium was added to each plate.

3.10. Kinetic Luminescence

Kinetic Luminescence assay was done performed after the transduced U2OS cells reached 100% confluency. The medium on top of the 35 mm plates were aspirated and 2 ml of freshly prepared lumicycle medium containing DMEM powder (sigma D-2902, 10X 1L), 0.35 gr sodium bi-carbonate (tissue culture grade, sigma S5761), 3.5 gr D(+) glucose powder (tissue culture grade, sigma G7021), 10 mL 1 M HEPES buffer (Gibco 15140-122), 2.5 mL Pen/Strep, 50 mL 5% FBS and up to L sterile milliQ water was added to the plates in dark conditions. To eliminate air passage, the inner circumference of the lid is coated with autoclaved grease oil. The plates were then put into ACTIMETRICS LumiCycle 32 for analysis. Luciferase readings were taken for the following 7 days and the data was analyzed discarding the first day. The results were analyzed using the waveclock statistical software.

3.11. Selection of SNPs

The SNPs for Bmal1, Clock and Cry1 genes were selected from 1000 Genome Project database (http://browser.1000genomes.org/index.html). The SNPs are selected from the missense variants database based on their aminoacid locations in the proteins and their SIFT and PolyPhen scores. For the SNP selection we used the scores readily available on the 1000 Genome Project database, choosing the variants with the lowest SIFT (0 or close to 0) and highest PolyPhen (1 or close to 1) scores.

3.12. RNA isolation and cDNA preparation

RNA was isolated from the U2OS cells transduced with lentivirus containing the wild type and variants of Bmal1, Clock and Cry1 genes. Following transduction the cells were grown enough to reach a 100% confluency. The medium was aspirated and 1 ml of Trizol was added to the plates under the hoods incubating the plates for the following 5 minutes at room temperature. The cells were then collected into 1.5 ml microcentrifuge tubes. 200 µl of chloroform was added to each tube and the tubes were left to incubate for 2 minutes at room temperature. Following the incubation the tubes were centrifuged at 12.000 g for 15 minutes at 4ºC. After the first centrifugation the aqueous phase of the solution was mixed with 500 µl of pre-chilled 100% isopropanol, mixed gently by inversion for 15 times and left to incubate at room temperature for 10 minutes. The samples were centrifuged again for 10 minutes at 12000 g at 4ºC. The supernatant was discarded and the remaining pellet was washed with 75% EtOH, followed by centrifugation at 12500 g for 15 minutes at 4ºC. The EtOH is discarded and the tube is left to air dry to get rid of the remaining EtOH. Once EtOH completely evaporated the pellet was resuspended in 50 µl of DEPC treated water. To analyze the quality of the isolated RNA, 8µL of the solution is run on 1% agarose gel. The concentration of the RNA mix was estimated using Nanodrop2000 (Thermo Scientific). 1500 ng of the RNA isolated was taken for DNase treatment and the rest was stored at -80 ºC for future use. For DNase treatment 1 µl of DNase1 (Thermo Scientific) and 1 µl of 10X DNase buffer (Thermo Scientific) with enough DEPC treated water to make 10 µl of solution was added to the 1500 ng of RNA mix and incubated at 37ºC for 30 minutes. After the incubation 1 µl of EDTA was added to the solution for enzyme inactivation. After the EDTA addition the sample was incubated at 65ºC for 10 minutes. Following the incubation for the first strand cDNA synthesis the sample 1 µl of Oligo(dT) $_{18}$ (Thermo Scientific) was added and incubated at 65°C for 10 minutes. For the synthesis of cDNA, 4µl of 5X Reverse transcription buffer (Thermo Scientific), 10µl of 10mM dNTP mix, 1µl of Ribolock RNase inhibitor, 1µl of Revert Aid Reverse Transcriptase and 1 µl of DEPC treated water was added to the sample. The mixture was then incubated at 42°C for 1 hour followed by incubation at 70°C for 15 minutes. Obtained cDNA was diluted with 80µL DNase and RNase free water for real-time PCR.

3.11. Real-Time PCR

Real time PCR was mainly used in the project to see the overexpression levels of proteins. cDNAs used for this purpose were produced using the methodology mentioned above in chapter 3.10. For qPCR analysis TIANGEN SuperReal PreMix SYBR Green is used. qPCR

analysis is performed in reaction mixture with ingredients as follows: 10µL of 2X SYBR Green, 2µL of primer mix (5pmole of both forward and reverse primers), 2µL of template cDNA and 6µL of MilliQ water. List of the primer sequences can be found in appendix 1. The cycling protocol is performed on LightCycler 2.0 Instrument (Roche). The cycling protocol is performed as 95°C 10 seconds denaturation, 57 to 62°C for 20 seconds annealing (the degree is determined by primer type) and 72°C for 30 seconds of elongation. The quality of the PCR product is analyzed by Tm curves and agarose gel imaging. Normalization of the values is made with GAPDH values.

CHAPTER 4

RESULTS

4.1 Selection of Core Clock Protein SNPs

1000 genome project database contains a great number of SNPs for core clock components. Using this database we selected missense variants for 3 core clock proteins, BMAL1, CLOCK and CRY1. SNPs, which would cause a gain-of-function or a loss-of-function were selected in the coding region of those 3 clock genes. For this purpose followings were used to select the SNPs: the location of amino acid change in the protein, SIFT and PolyPhen scores. SIFT scoring calculates the degree of conservation of specific amino acid residues among closely related sequences, using PSI-BLAST. SNPs with low SIFT scores, suggest amino acid substitution will affect protein function. PolyPhen score is based on values calculated by parameters obtained from three different data sources: 1) the substitution position of amino acids, 2) PSIC scores derived from multiple alignment and 3) structural information. In this study SNPs that have a PolyPhen score of 1 or close to 1 were selected to ensure SNPs are located in the important regions of the proteins. In addition those SNPs were mapped into 3D structures of the BMAL1, CLOCK and CRY1 to see their locations within the protein. BMAL1 and CLOCK contains 4 major domains, bHLH, PAS-A, PAS-B and TAD; whereas CRY1 contains a PHR domain and a C-Terminal domain. The selected SNPs were chosen from these sites as they would play an important role in these domains giving rise to possible alterations in protein function. Considering all three properties mentioned above we chose 5, 8 and 6 missense variants for Bmal1, Clock and Cry1 genes, respectively.

Arg84Cys and Asp110Tyr variants of Bmal1 (Figure 4.1.1 A, B) are found on the basic helix-loop-helix (bHLH) domain which was shown to interact with the DNA [66], Leu196Gln (Figure 4.1.1 C) is found on PAS-A domain which directly interacts with the Clock PAS-A domain. Lys405Thr and Ala434Gly (Figure 4.1.1 D, E) variants are found on the PAS-B domain of Bmal1 which plays a role of Bmal1 – Clock interaction [66] (Table 1).

Clock variants were also mainly selected from the 3 domains mentioned above (bHLH, PAS-A and PAS-B). Variants Arg84Gln and Phe122Ser (Figure 4.1.2 A, B) are on bHLH domain, whereas Asp128Gly and Ser208Cys (Figure 4.1.2 C, D) are from the PAS-A domain.

Gly235Glu and Leu395Ile were selected from PAS-B domain, and His542Leu and Gln772His were selected from the TAD domain at the C-terminal (Table 2).

Cry1 consists of two main domains: an N-Terminal PHR (photolyase homology domain) domain and a C-Terminal domain [181-182]. CRY1 PHR domain was shown to interact with PER1 and PER2 C-Terminal domains [182-183], FBXL3 and FBXL21 [55, 184]. PHR domain and C-Terminal domain also play an important part in BMAL1:CLOCK repression. [76]. C-Terminal domain of CRY1 harbor a nuclear localization signal as well as a coiled-coil domain critical for CRY1 localization into nucleus, and mCRY1 C-tail domain was also shown to be essential for mPER2 and BMAL1 interactions [76-182].

Table 1. Selected Bmal1 SNPs from 1000 Genome Database

Variant	Allele	SIFT	POLYPHEN	Domain
Arg84Cys	C/T	0	0,999	bHLH
Asp110Tyr	G/T	0	1	bHLH
Leu196Gln	T/A	0	1	PAS-A
Lys405Thr	A/C	0,01	0,9	PAS-B
Ala434Gly	A/G	0,01	0,847	PAS-B

Table 2. Selected Clock SNPs from 1000 Genome Database

Variant	Allele	SIFT	POLYPHEN	Domain
Arg82Gln	C/T	1	0,086	bHLH
Phe122Ser	A/G	0,01	0,901	bHLH
Asp128Gly	T/C	0,07	0,988	PAS-A
Ser208Cys	G/C	0,01	0,566	PAS-A
Gly235Glu	C/T	0,33	0,216	PAS-B
Leu395Ile	G/T	0,09	0,184	PAS-B
His542Leu	T/A	0,02	0,098	TAD
Gln772His	T/A	0,25	0,628	TAD

Table 3. Selected Cry1 SNPs from 1000 Genome Database

Variant	Allele	SIFT	POLYPHEN	Domain
Leu56Arg	A/C	0	1	PHR
Gly144Val	C/A	0	0,901	PHR
Arg293His	C/T	0	0,994	PHR
Arg348Cys	G/A	0,01	0,993	PHR
Cys363Phe	C/A	0	1	PHR
His411Tyr	G/A	0,01	0,992	PHR

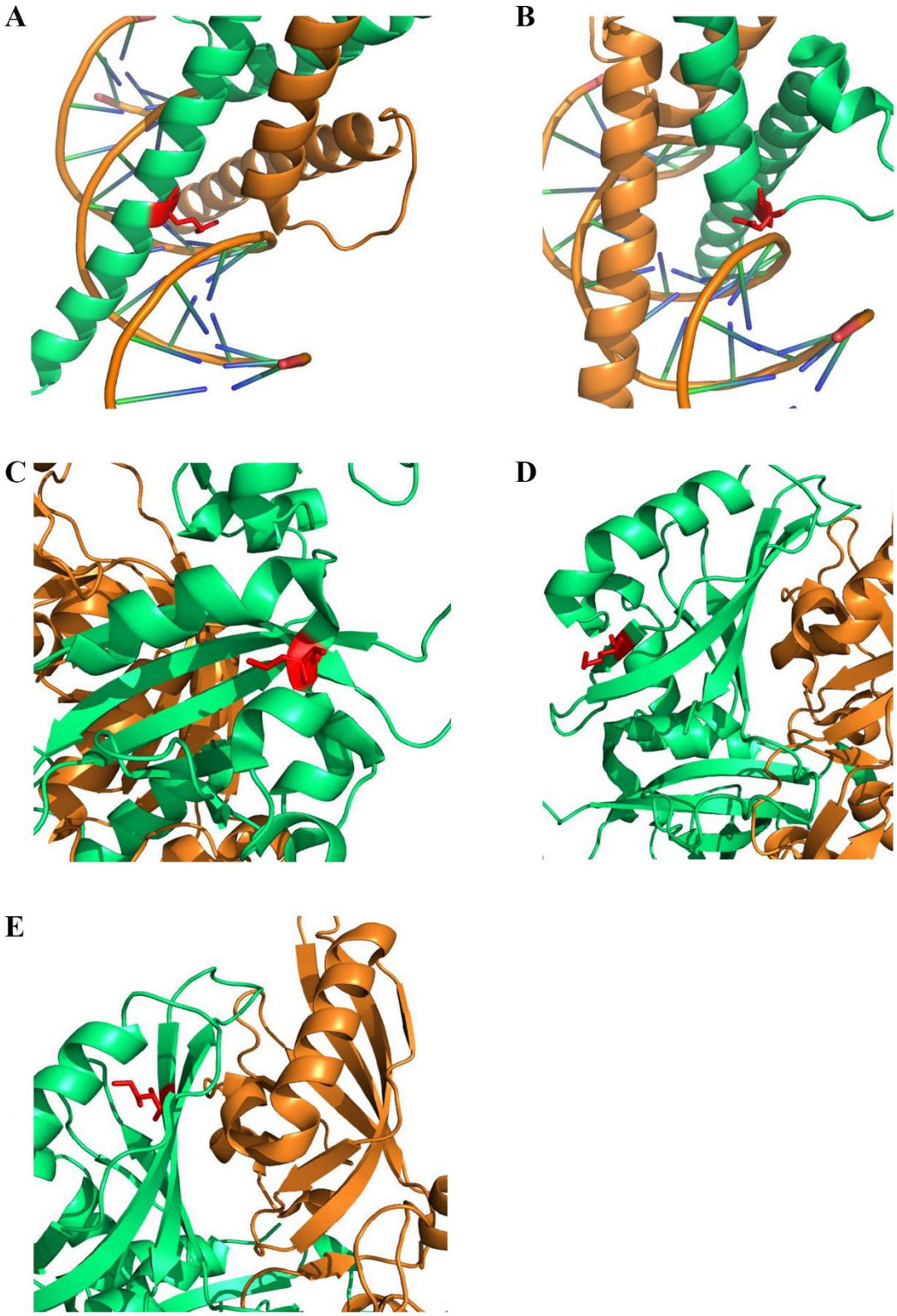


Figure 4.1. Locations of selected amino acids on BMAL1. BMAL1 is represented as green and CLOCK is represented as orange. Amino acids are shown as red. (A) Arg84Cys (B) Asp110Tyr (C) Leu196Gln (D) Lys405Thr (E) Ala434Gly.

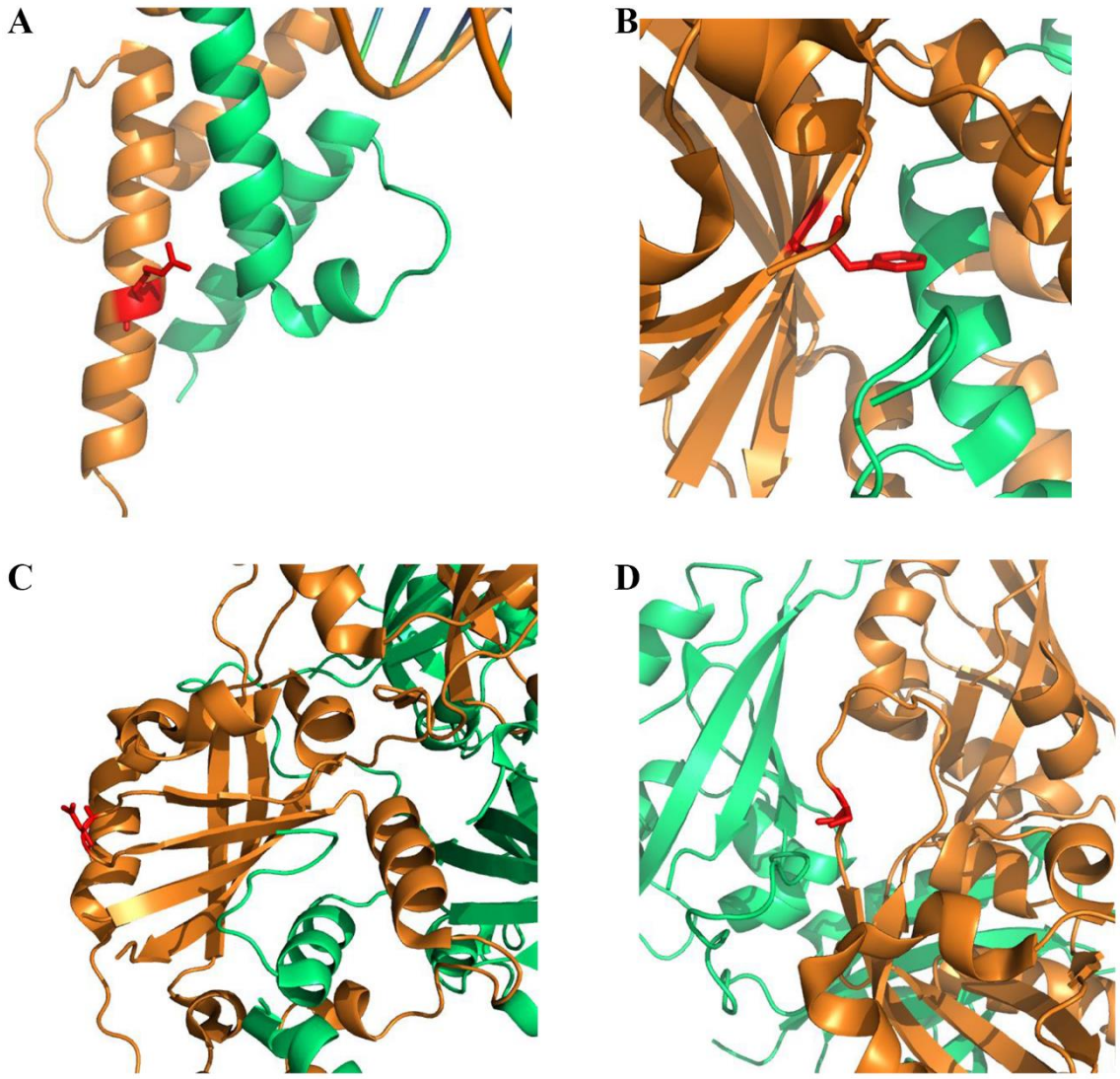


Figure 4.2.Locations of selected amino acids on CLOCK. BMAL1 is represented as green and CLOCK is represented as orange. Amino acids are shown as red. (A) Arg82Gln (B) Phe122Ser (C) Asp128Gly (D) Ser208Cys.

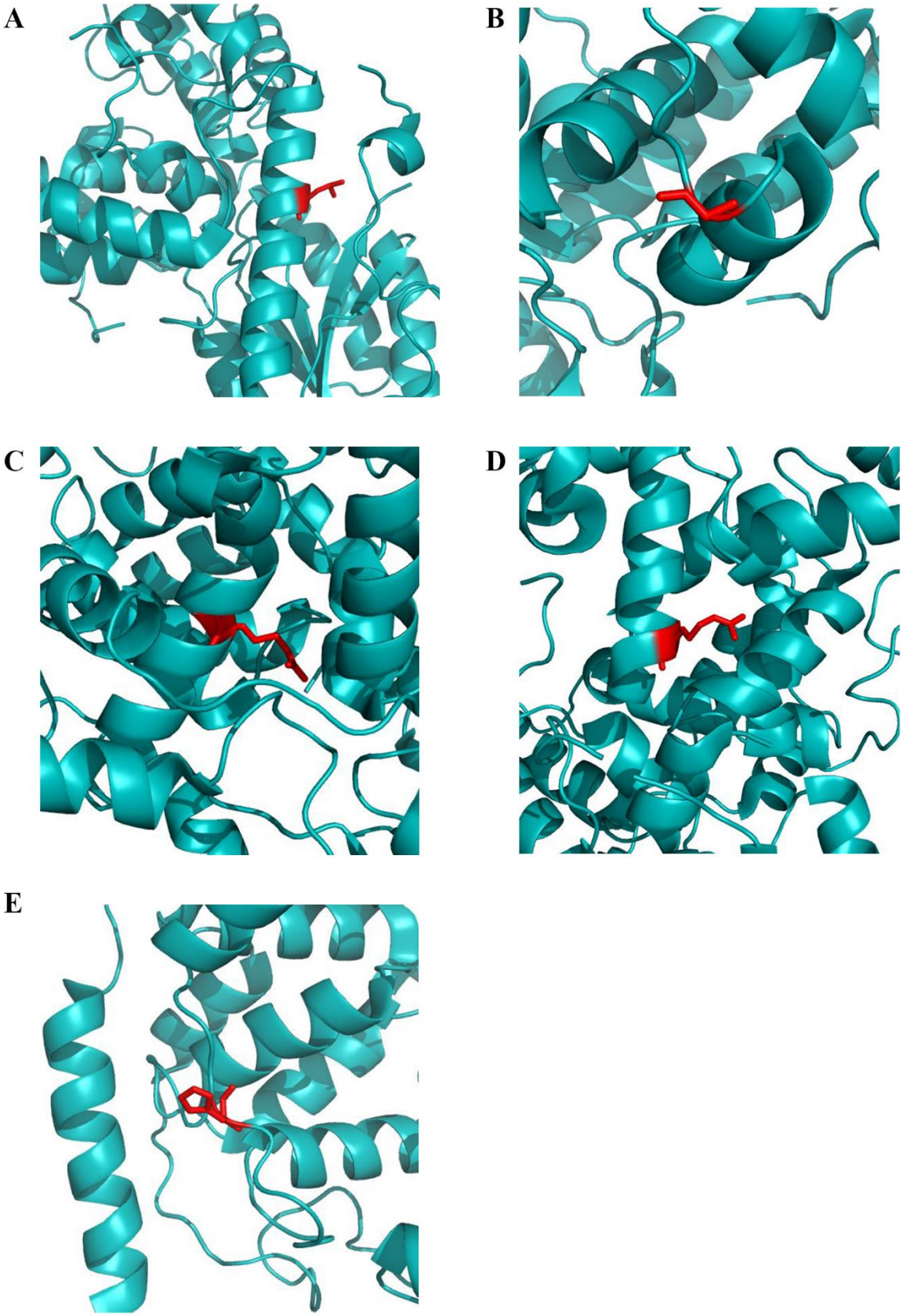


Figure 4.3. Locations of selected amino acids on CRY1. CRY1 is represented as cyan. Amino acids are shown as red. (A) Leu56Arg (B) Gly144Val (C) Arg293His (D) Arg348Cys (E) His411Tyr.

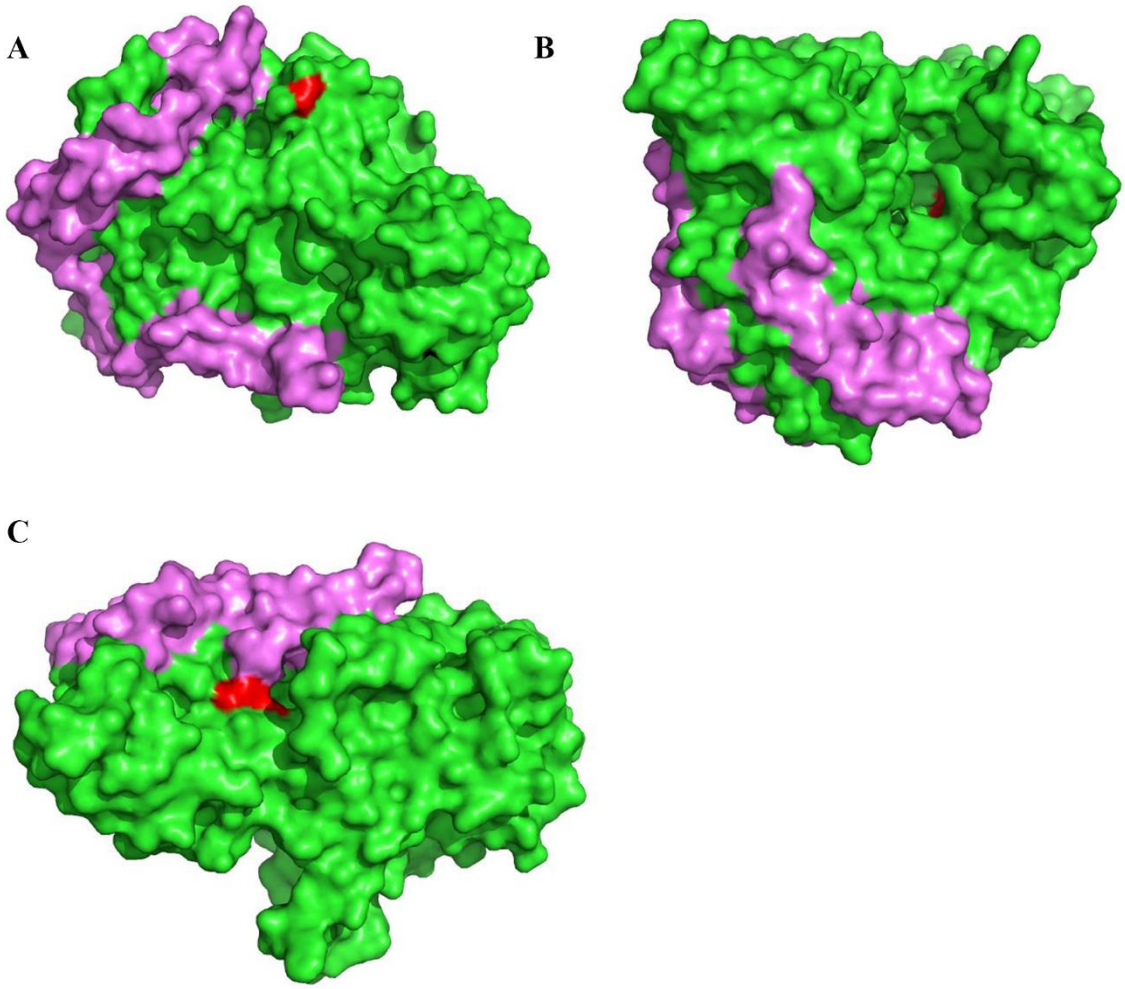


Figure 4.4.Visualization of (A) Gly144Val (B) Arg293His and (C) Arg348Cys on CRY1. CRY1 is represented as green and PER2 is represented as pink. Amino acids are shown as red.

4.2 Site-Directed Mutagenesis of Core Clock SNPs

To see the effect of the selected mutations on the activity core clock proteins Arg84, Asp110, Leu196, Val430 and Ile433 (Table 1) were replaced into Cys, Tyr, Gln, Gly, and Val in Bmal1 cDNA, by site-directed mutagenesis, respectively. With a similar approach mutations shown in Table 2 were introduced in Clock cDNA by PCR based site-directed mutagenesis. For Cry1 Leu56Arg, Gly144Val, Arg293His, Arg348Cys, Cys363Phe and His411Tyr variants were chosen and were successfully reproduced using site-directed mutagenesis. Cry1 mutations, Leu56Arg, Gly144Val, Arg293His, Arg348Cys, Cys363Phe and His411Tyr, were also introduced in the same fashion to Cry1 cDNA (Table 3). After mutagenesis the presence of mutations were confirmed with Sanger sequencing. All sequencing results can be found in

APPENDIX B. A representative sequencing chromatogram for Arg84Cys is shown in figure 4.2.1.

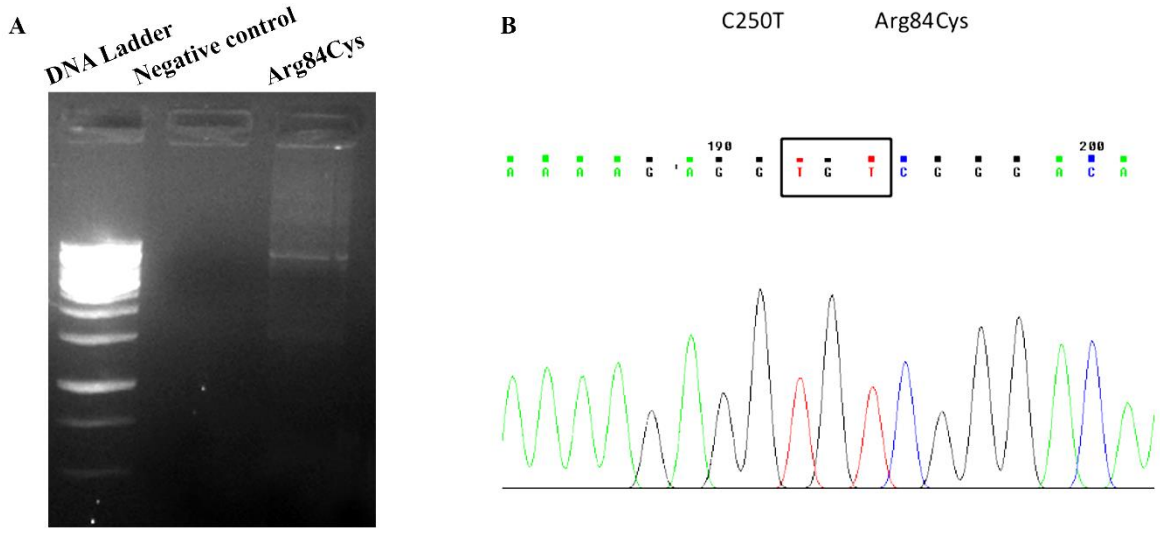


Figure 4. 5.(A) Site directed mutagenesis of Arg84Cys. Amino acid substitution is introduced using site directed mutagenesis. Negative control contains template DNA but no primers. The amplicons are digested using DpnI restriction enzyme and visualized on agarose gel. (B) Sequencing result for Arg84Cys of BMAL1 for verification of amino acid change.

All the selected variants for Bmal1 and Cry1 are on conserved aminoacids between mouse and human homologs. Chosen Bmal1 variants were produced on mBmal1 gene on Sport6 vector whereas those of Clock gene were produced on hClock gene Sport6 vector. Cry1 variants were produced on the available mCry1 gene available on pBind vector. For the sake of simplicity they will be regarded as Bmal1, Clock and Cry1 from now on.

Arg84Cys and Asp110Tyr variants of Bmal1 are found on the basic helix-loop-helix (bHLH) domain which was shown to interact with the DNA [66], Leu196Gln is found on PAS-A domain which directly interacts with the Clock PAS-A domain. Val431Gly variant is found on the PAS-B domain of Bmal1 which plays a role of Bmal1 – Clock interaction [66].

Clock variants were also mainly chosen from the 3 domains mentioned above (bHLH, PAS-A and PAS-B). Variant Arg84Gln is on bHLH domain, whereas Phe122Ser, Asp128Gly, Ile169Val and Ser208Cys are from the PAS-A domain. Gly235Glu and Leu395Ile were selected from PAS-B domain, and His542Leu and Gln772His were selected from the TAD domain at the C-terminal.

Cry1 protein contains a photolyase homology (PHR) domain which can bind FAD co-factors [82]. All of the selected gene variants of Cry1 were from this PHR region.

4.3 Effects of Core Clock Protein Variants on BMAL1/CLOCK Transactivation

Transcriptional regulation of circadian promoter activity has been studied in cell culture. BMAL1 and CLOCK proteins heterodimerize to form a transactivation complex and bind the three E-box promoter elements (5'-CACGTG-3') within a 2.0-kb mPer1 promoter fragment to activate transcription in cell transfection/luciferase reporter assays [176]. CRY proteins inhibit transcriptional activity by acting on BMAL1/CLOCK transactivation complex (Figure 4.3.1) [82].

In this work same methods was used to investigate the effect of the wild type and variants' activity in Per1-luc system. Any significant change between the wild types and the variants in the screens suggest a functional change in the variants. To eliminate transfection efficiency between the samples Renilla activities were measured. The luciferase values were normalized with Renilla values. Statistical analyses of the results are done using student's t-test ($p<0.05$).

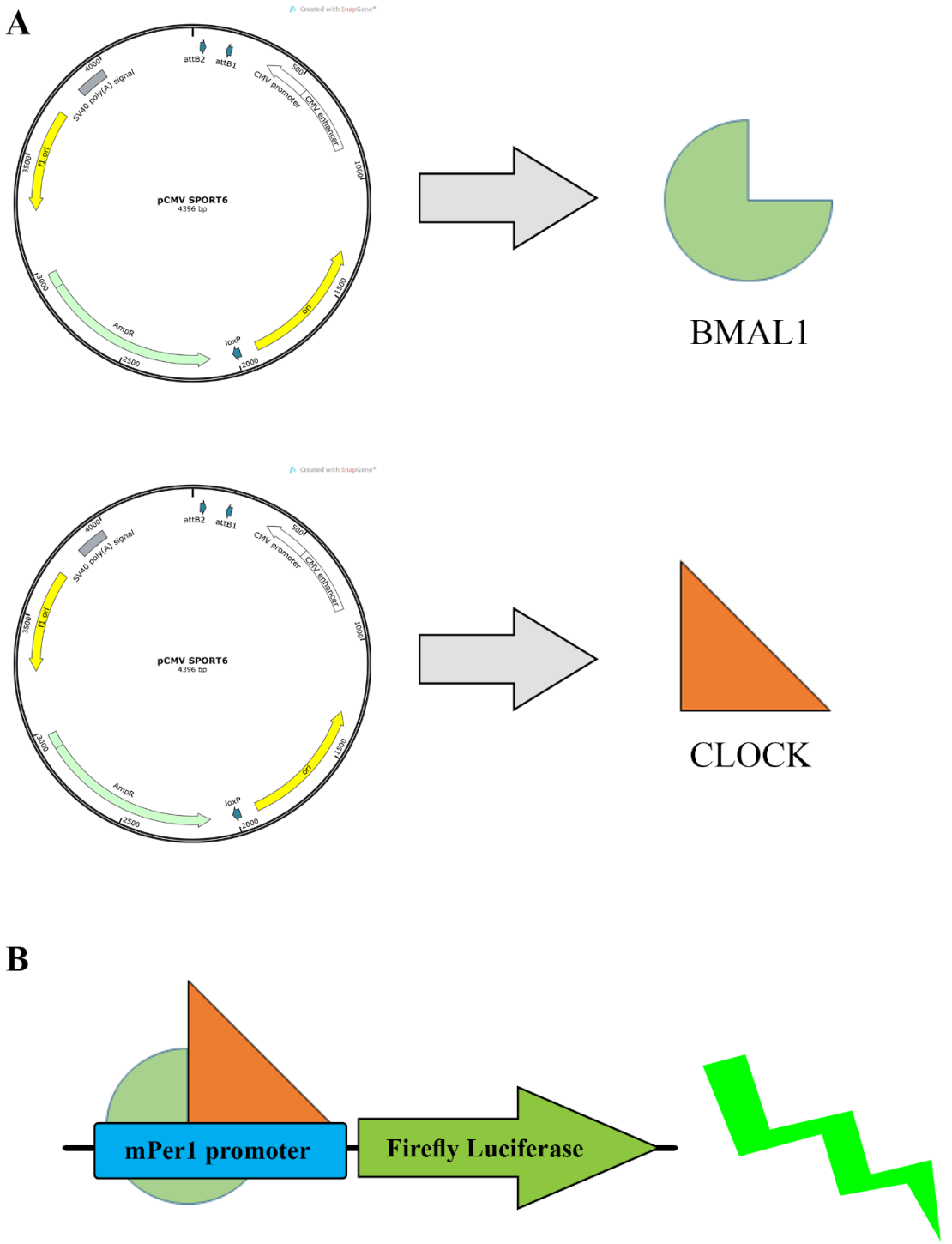


Figure 4. 6.Representation of luciferase assay. (A) Bmal1 and Clock are cloned into pCMV Sport6 vectors. The vectors are transfected to HEK293T cells to produce BMAL1 and CLOCK. (B) BMAL1 and CLOCK heterodimerize and bind to the E-box element of mPer1 promoter expressing firefly luciferase gene.

4.3.1 The effect of BMAL1 variants on mPer1 promoter transactivation

BMAL1 heterodimerizes with CLOCK in order to bind to E-box elements on mPER1 promoter. We analyzed BMAL1 variants together with CLOCK wild type protein to see if any of the selected variants cause an alteration in transactivational rate of BMAL1:CLOCK complex (Figure 4.3.1.1).

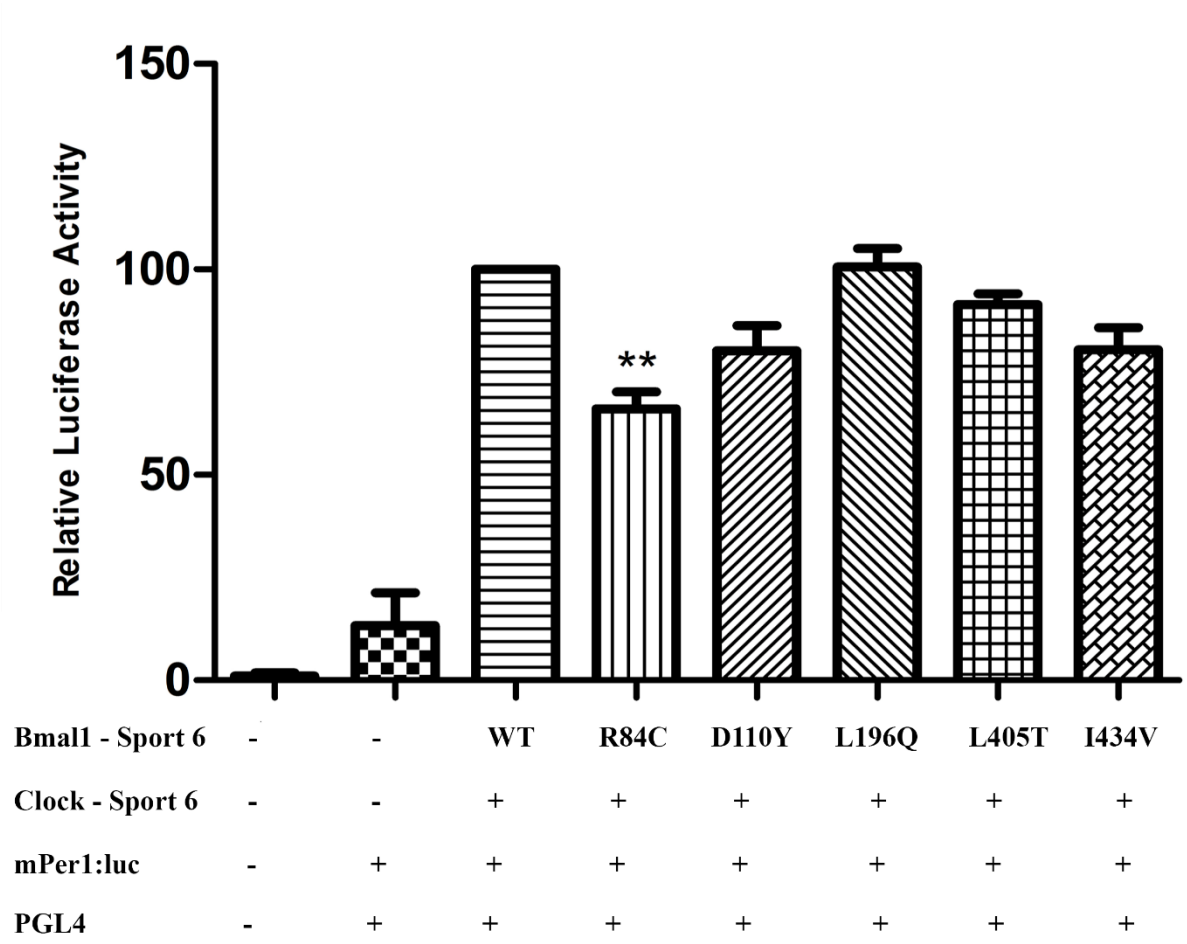


Figure 4. 7.Luciferase assay to test the transactivation rates of BMAL1 variants coupled with wild type CLOCK. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

Arg84Cys mutation found on the bHLH domain of BMAL1 showed a significant, 35% decrease in its capability to activate luciferase gene expression. bHLH domain functions as the DNA binding domain of BMAL1. Thus, it is possible that this substitution alters the DNA binding affinity of BMAL1:CLOCK complex.

CRY1 functions as a repressor to the BMAL1:CLOCK transactivator complex by binding to BMAL1 [66, 82]. It is possible that substitutions in BMAL1 amino acid sequence may have an

effect on CRY1 repressional capacity. We used BMAL1 variants together with wild type CRY1 in order to see alterations in CRY1 repression that may be caused by the BMAL1 variants (Figure 4.3.1.2).

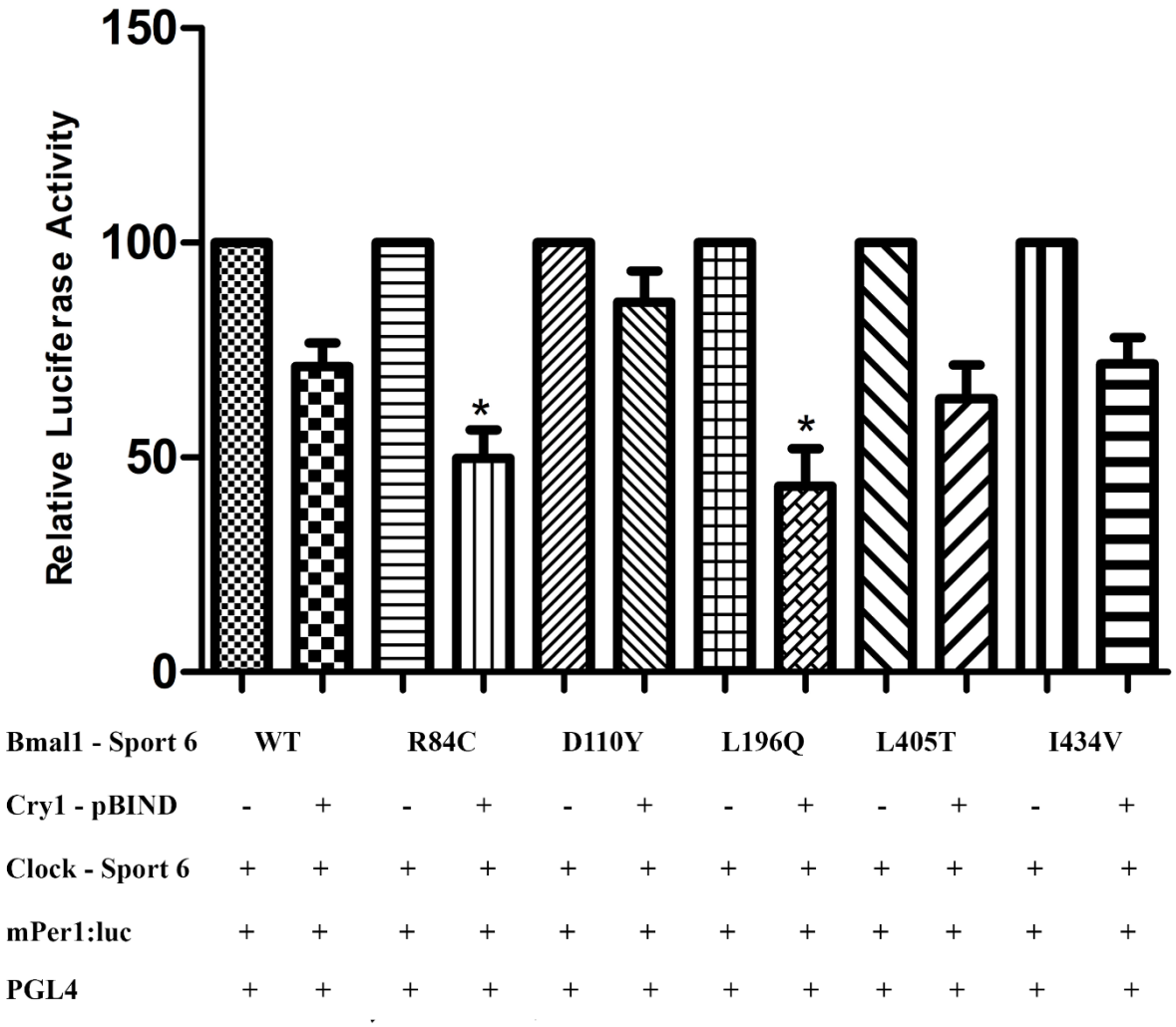


Figure 4. 8. Luciferase assay to test the effect of BMAL1 variants on the repression activity of CRY1. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

The statistical analysis of luciferase data indicates increased Cry1 repression on Arg84Cys and Leu196Gln variants compared to wild type.

4.3.2 The effect of CLOCK variants on mPer1 promoter transactivation

Together with BMAL1, CLOCK is the main activator of molecular clock system. Similar to BMAL1 it is probable that any alterations on CLOCK would cause an alteration in the expression of clock controlled genes. In order to find out whether our variants had an effect on BMAL1:CLOCK transactivational capacity, we used mPer1-luc reporter system (Figure 4.3.2.1).

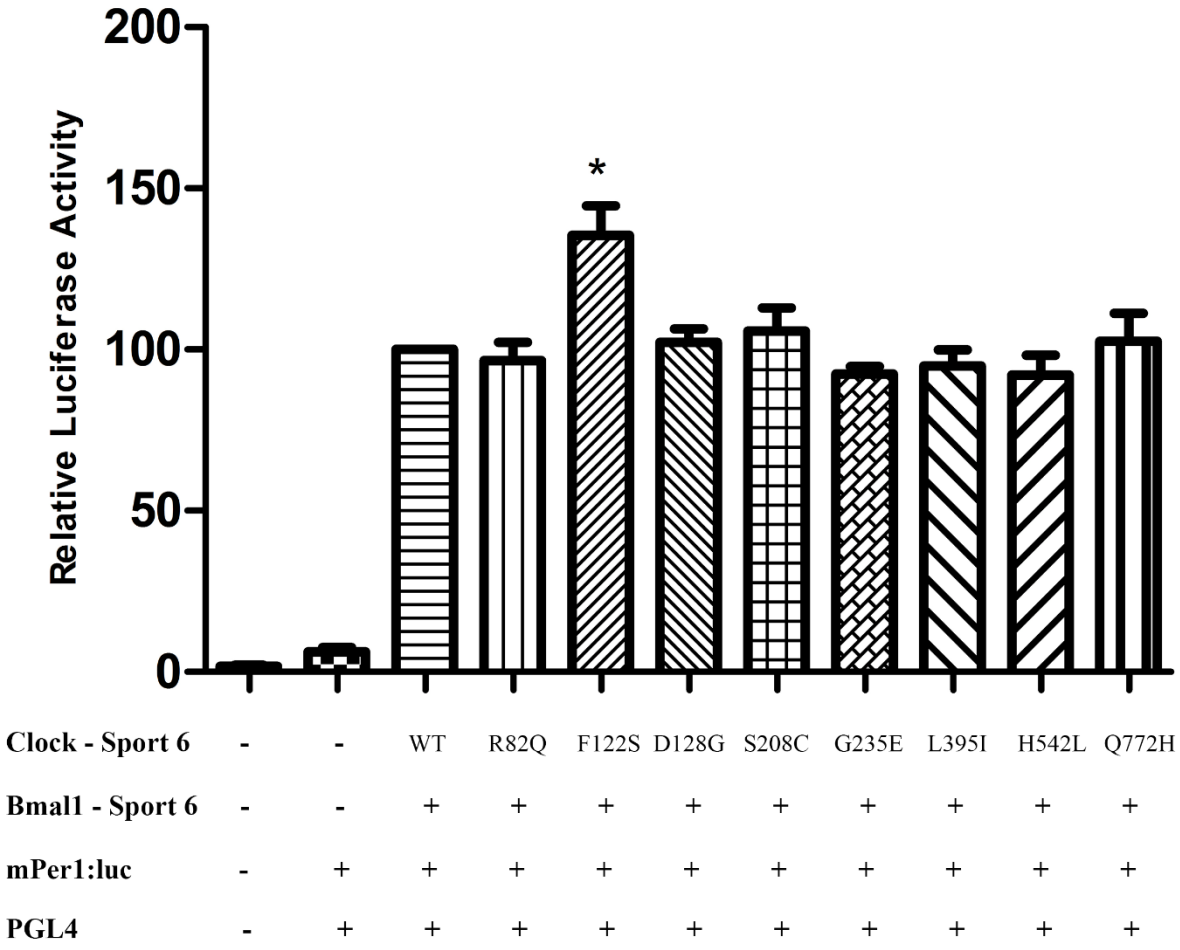


Figure 4. 9.Luciferase assay to test the transactivation rates of CLOCK variants coupled with wild type BMAL1. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

CLOCK variant Phe122Ser shows an increased luciferase reading in luciferase assays. This suggests an increase in CLOCK proteins transactivational capacity. Similar to BMAL1 Arg84Cys, Phe122Ser variation is in the bHLH domain of the protein, hinting a probable increase in the binding affinity of BMAL1:CLOCK complex to E-box elements.

CLOCK PAS-B domain has a direct effect on CRY1-BMAL1 interaction [66]. In order to see whether any of our selected CLOCK variants plays a role in the CRY1 repression rates we transfected the cells with expression plasmids containing wild type Bmal1 and Cry1 together with Clock variants (Figure 4.3.2.2).

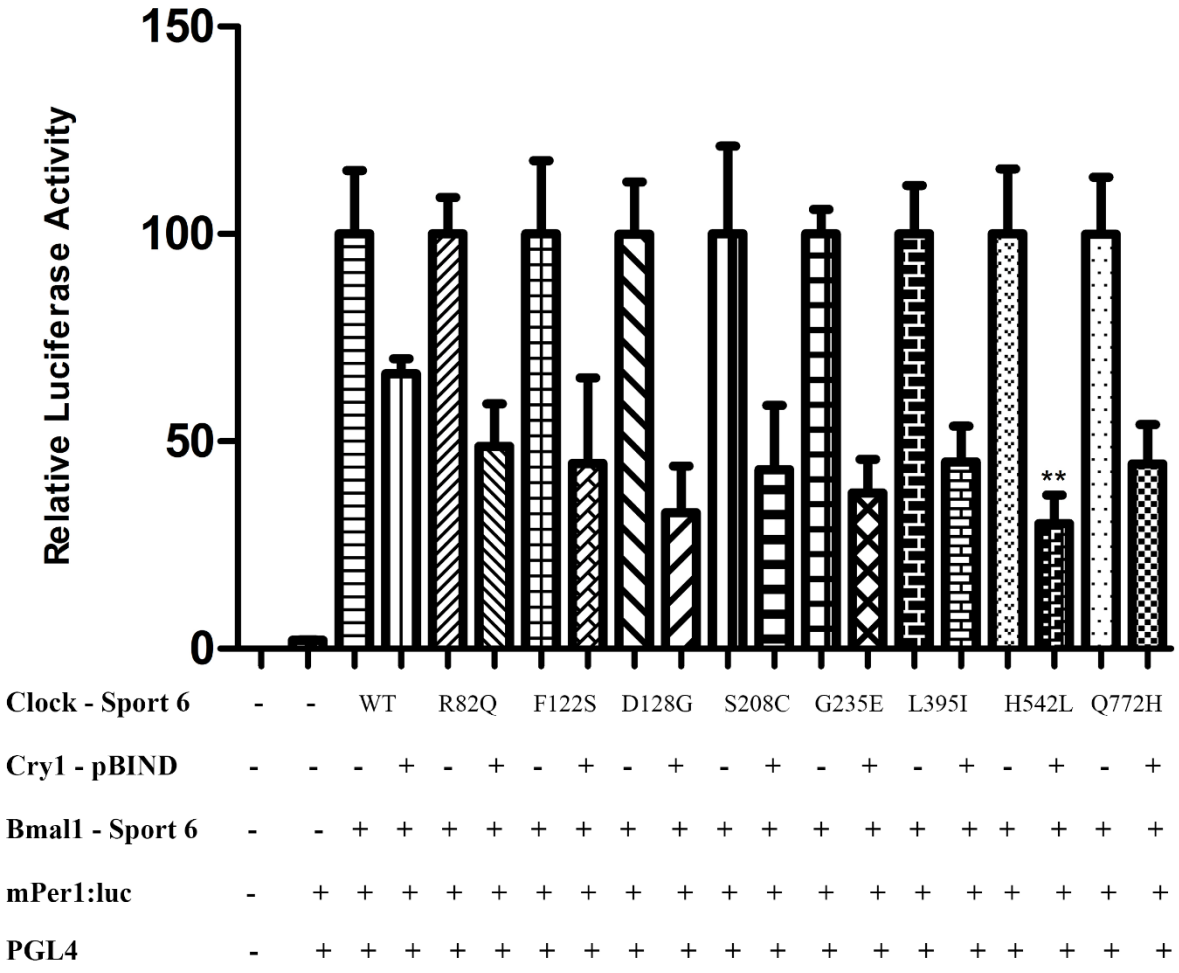
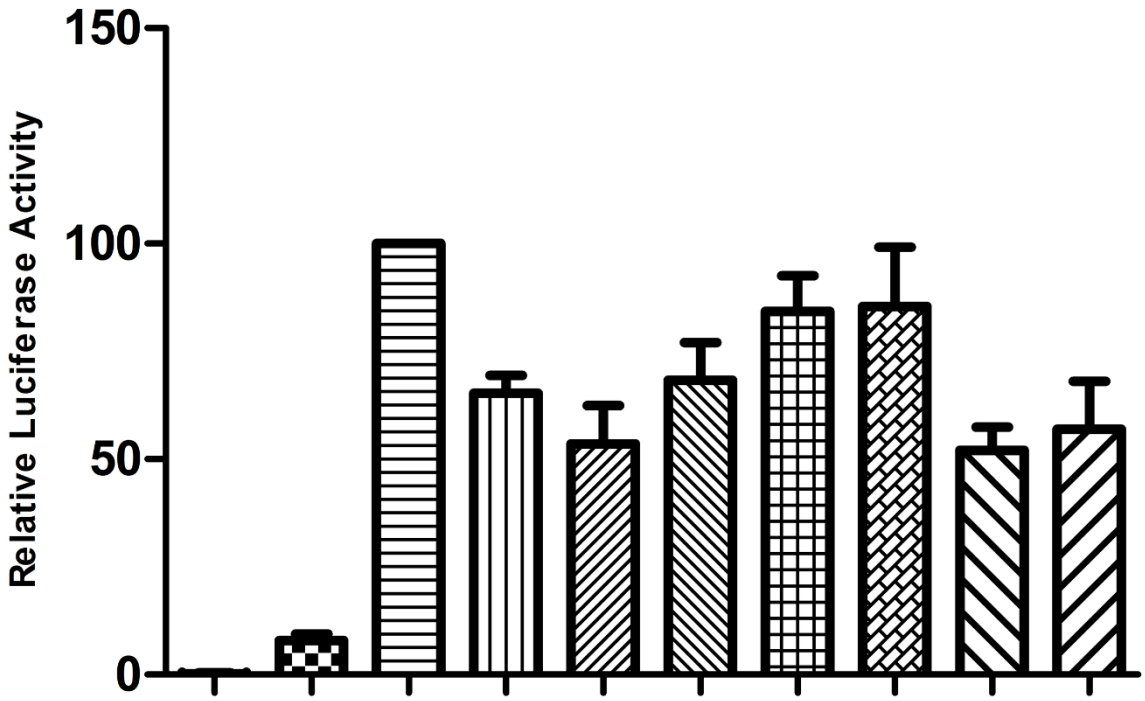


Figure 4. 10.Luciferase assay to test the effect of CLOCK variants on the repression activity of CRY1. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

Cry1 repression increases when coupled with Clock variant His542Leu. The statistical analysis also suggests that this decrease in transcriptional repression activity is significant compared to the repression on wild type CLOCK:BMAL1 transactivation.

4.3.3 The effect of CRY1 variants on mPer1 promoter transactivation

Cry1 functions as a repressor to BMAL1:CLOCK transactivation. In order to see whether Cry1 variants caused any functional changes to the repression activity, we transfected HEK293T cells with wild types of Bmal1 and Clock together with Cry1 wild type and variants. We normalized the luciferase readings from the cells without Cry1 repression to 100, and used wild type Cry1 transfected cells as the positive control (Figure 4.3.3.1).



Cry1 - pBIND	-	-	-	WT	L56R	G144V	R293H	R348C	C363F	H411Y
Bmal1 - Sport 6	-	-	+	+	+	+	+	+	+	+
Clock - Sport 6	-	-	+	+	+	+	+	+	+	+
mPer1:luc	-	+	+	+	+	+	+	+	+	+
PGL4	-	+	+	+	+	+	+	+	+	+

Figure 4. 11. Luciferase assay to test the repression activity of CRY1 variants on wild type BMAL1:CLOCK transactivation. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

When Cry1 wild type is present in the cells, luciferase expression decreases to around 60%, compared to the cells expressing only Bmal1 and Clock. This decrease in the luciferase expression is due to the repressional activity of Cry1. When the variants of Cry1 are introduced

instead of the wild type, there is no significant change in the luciferase readings according to the statistical analysis. However a decrease in the repressional capacity can be observed in Arg293His and Arg348Cys mutations.

4.4 Interactions of BMAL1, CLOCK and CRY1 Variants

The luciferase assay data showed some of the variants altered the BMAL1:CLOCK transactivation or CRY1 repression rates. We decided to use mammalian two-hybrid assay to assess whether these mutations affect the interaction between the core clock proteins or their DNA binding ability. Mammalian two-hybrid assay is an extremely powerful method that allows us to detect the interaction between two proteins in vivo. For this one of the genes is cloned into a pBind vector allowing the protein to be expressed fused to a yeast GAL4 binding domain (amino acids 1-147). The other gene is also cloned into a pACT vector, containing a different fusion protein, herpes simplex virus VP16 activation domain (amino acids 411-456). When co-transfected with a PG5luc vector, these two fused proteins will bind to a GAL4 binding site, upstream of a TATA box, on the Firefly luciferase gene promoter; allowing the expression of firefly luciferase if the two cloned proteins interact with each other (Figure 4.4.1). Better interaction between the proteins ensure more firefly luciferase expression [177]. To eliminate transfection efficiency between the samples Renilla activities were measured. The luciferase values were normalized with Renilla values. Statistical analyses of the results are done using student's t-test ($p<0.05$).

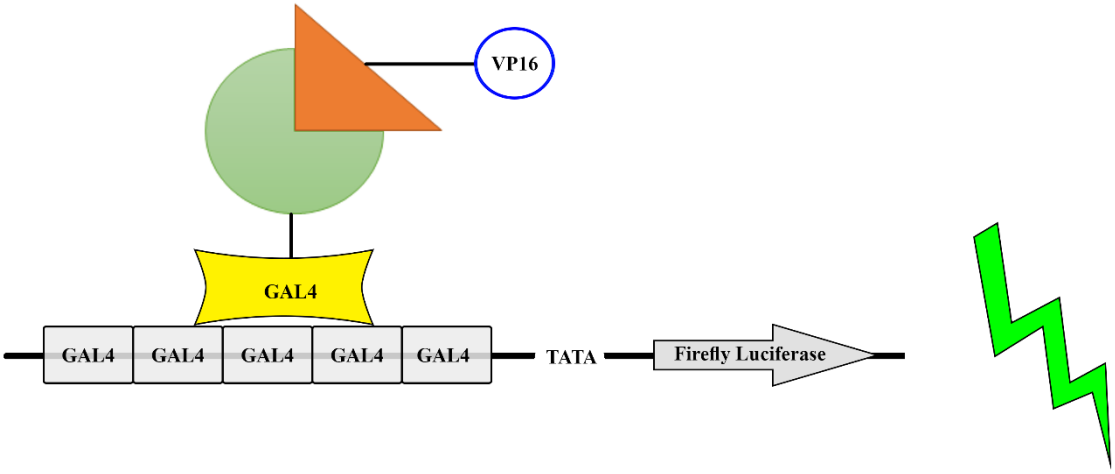


Figure 4. 12.Representation of mammalian two-hybrid assay. One protein is fused to VP16 and another is fused to GAL4. If the proteins interact with each other GAL4 and VP16 comes into close proximity allowing the expression of luciferase gene. As the efficiency of protein-protein interaction increases more luciferase is expressed.

It is known that BMAL1 and CLOCK proteins bind to each other forming a transactivational complex [66]. CRY1 was also shown to interact directly with BMAL1, but not CLOCK [82]. Even though CRY1 and CLOCK does not have a direct interaction, CLOCK PAS-B domain is shown to play a big role in BMAL1-CRY1 interaction [66]. Taking these information into consideration we used mammalian two hybrid assay to examine the interactions between BMAL1-CLOCK and BMAL1-CRY1. We also investigated the interaction between BMAL1-CRY1 in the presence of different CLOCK variants. For these experiments Bmal1 variants were cloned into pACT and Clock variants were cloned into pBIND vectors. All of the luciferase readings from the variants' interactions were compared with those of the wild types.

4.4.1 BMAL1 – CLOCK interactions

First we wanted to see whether the decrease in transactivational efficiency of Arg84Cys was due to its interaction with CLOCK. For this purpose we infected HEK293T cells with Bmal1 variants on pACT vector and wild type Clock on pBIND vector together with the reporter plasmid PG5luc (Figure 4.4.1.1).

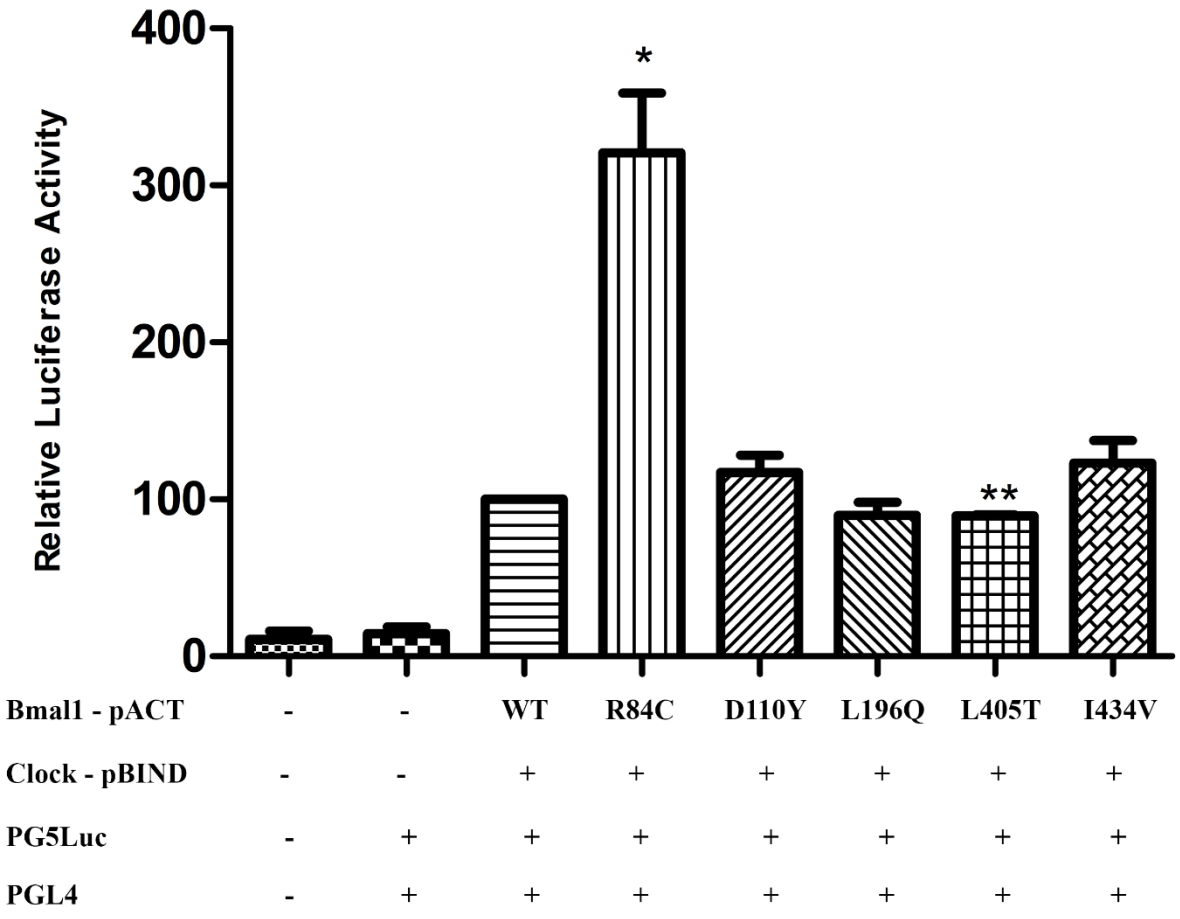


Figure 4. 13.Mammalian two hybrid assay to test the interaction between BMAL1 variants and wild type CLOCK. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

According to mammalian two-hybrid assay, BMAL1 Arg84Cys shows a significant 3-fold increase in CLOCK interaction compared to the wild type BMAL1. Arg84Cys is a bHLH domain residue which is not as significantly important for binding as PAS domains. However this result suggests that there may be a structural change in the protein 3D structure causing an increase in protein-protein interaction, however, resulting in a decrease in transactivational efficiency.

4.4.2 BMAL1 – CRY1 interactions

CRY1 is known to bind BMAL1 however the 3D structure of this complex as well as the BMAL1 binding domain of CRY1 is unknown. We wanted to test our CRY1 variants for their BMAL1 binding efficiency. For this purpose we followed a similar fashion to the one mentioned above however we used CRY1 variants instead of BMAL1 variants and BMAL1 wild type rather than that of CLOCK (Figure 4.4.2.1).

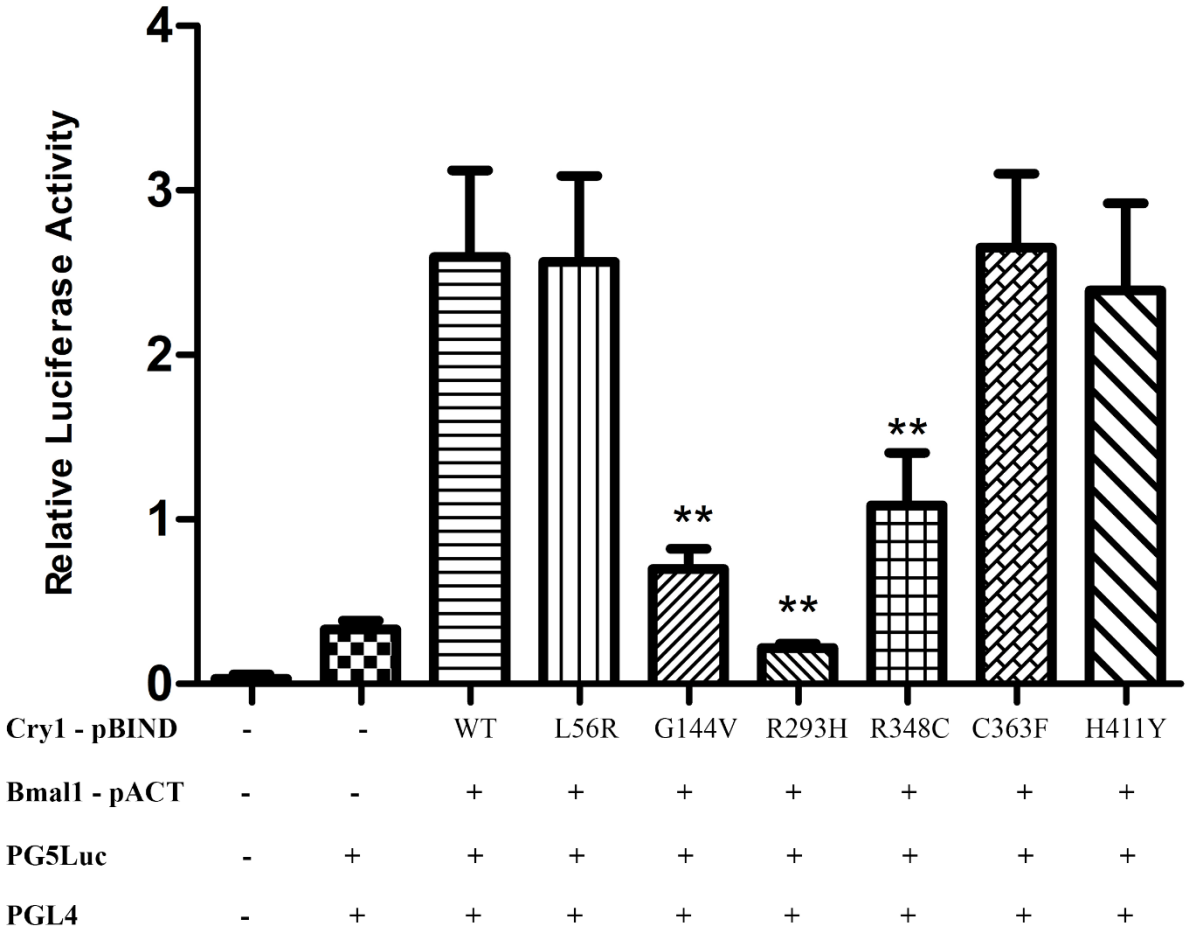


Figure 4. 14.Mammalian two hybrid assay to test the interactions between CRY1 variants and wild type BMAL1. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

The results show a significant decrease in BMAL1 binding efficiency of Gly144Val, Arg293His and Arg348Cys variants compared to the wild type CRY1. We observe no interaction between Arg293His variant and BMAL1 where as Gly144Val and Arg348Cys variants show 75% and 60% decreased interactions with BMAL1 respectively.

4.4.3 Effect of CLOCK on BMAL1 – CRY1 interaction

CLOCK does not directly interact with CRY1, however it is known that PAS-B domain of CLOCK plays an active role in the interaction of BMAL1 and CRY1. We used mammalian two-hybrid assay to observe whether our CLOCK variants would cause a difference in BMAL1 and CRY1 interaction. For this we used wild type CRY1 and BMAL1 fused to GAL4 and VP16, respectively, together with CLOCK variants on Sport6 vector (Figure 4.4.3.1).

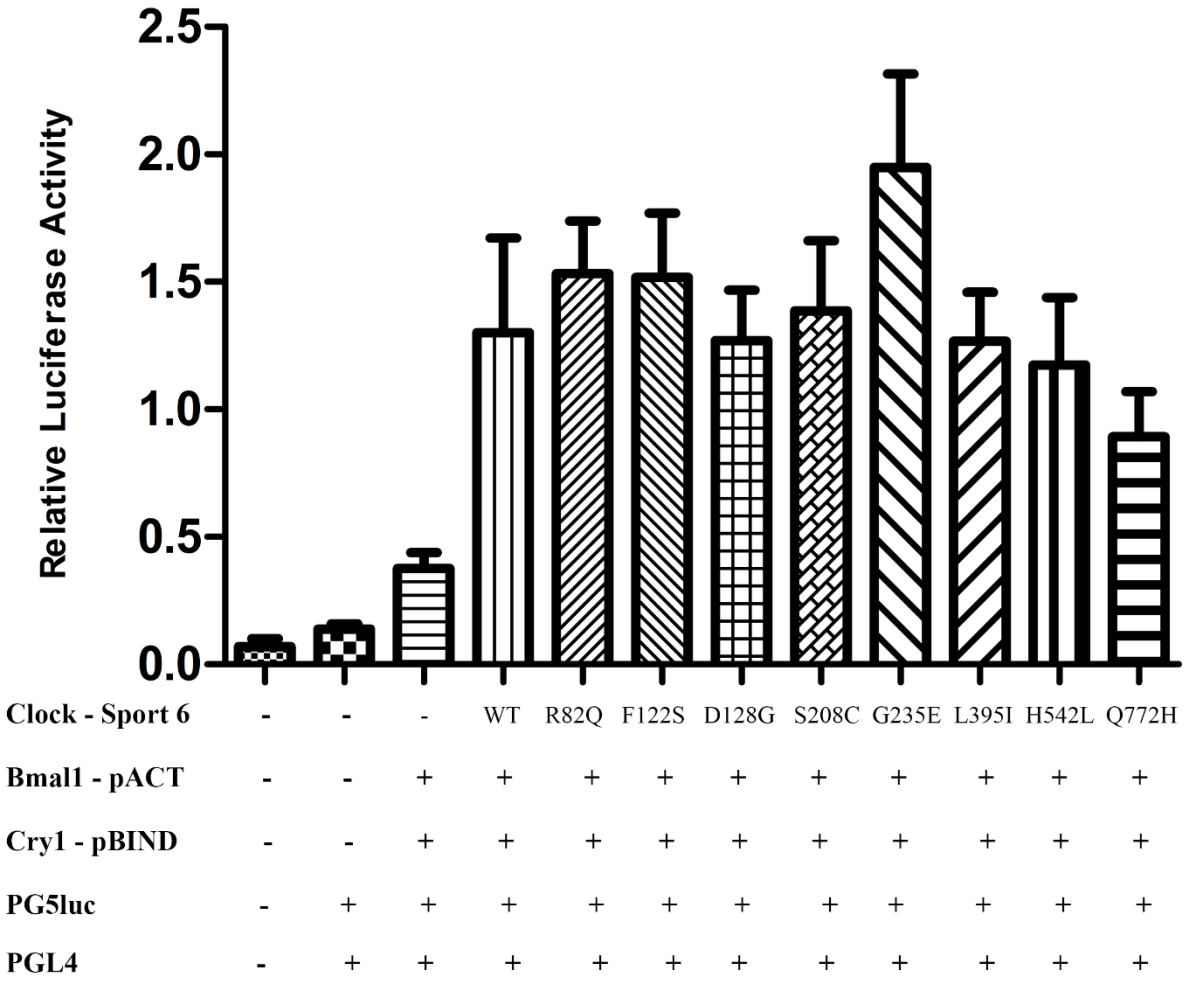


Figure 4. 15.Mammalian two hybrid assay to test the interaction between wild type CRY1 and wild type BMAL1 in the presence of different CLOCK variants. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

The results showed there was no statistically significant effect of our CLOCK variants on CRY1-BMAL1 interaction.

4.4.4 CRY1 – PER2 interaction

We wanted to see whether the decrease in CRY1-BMAL1 interaction was due to a structural change in CRY1 3D structure or due to amino acid substitutions effecting CRY1 binding to BMAL1. For this purpose we also conducted a mammalian two-hybrid assay of CRY1 with another protein it binds to mPER2 [82]. We used mPER2 wild type together with Cry1 variants (Figure 4.4.4.1).

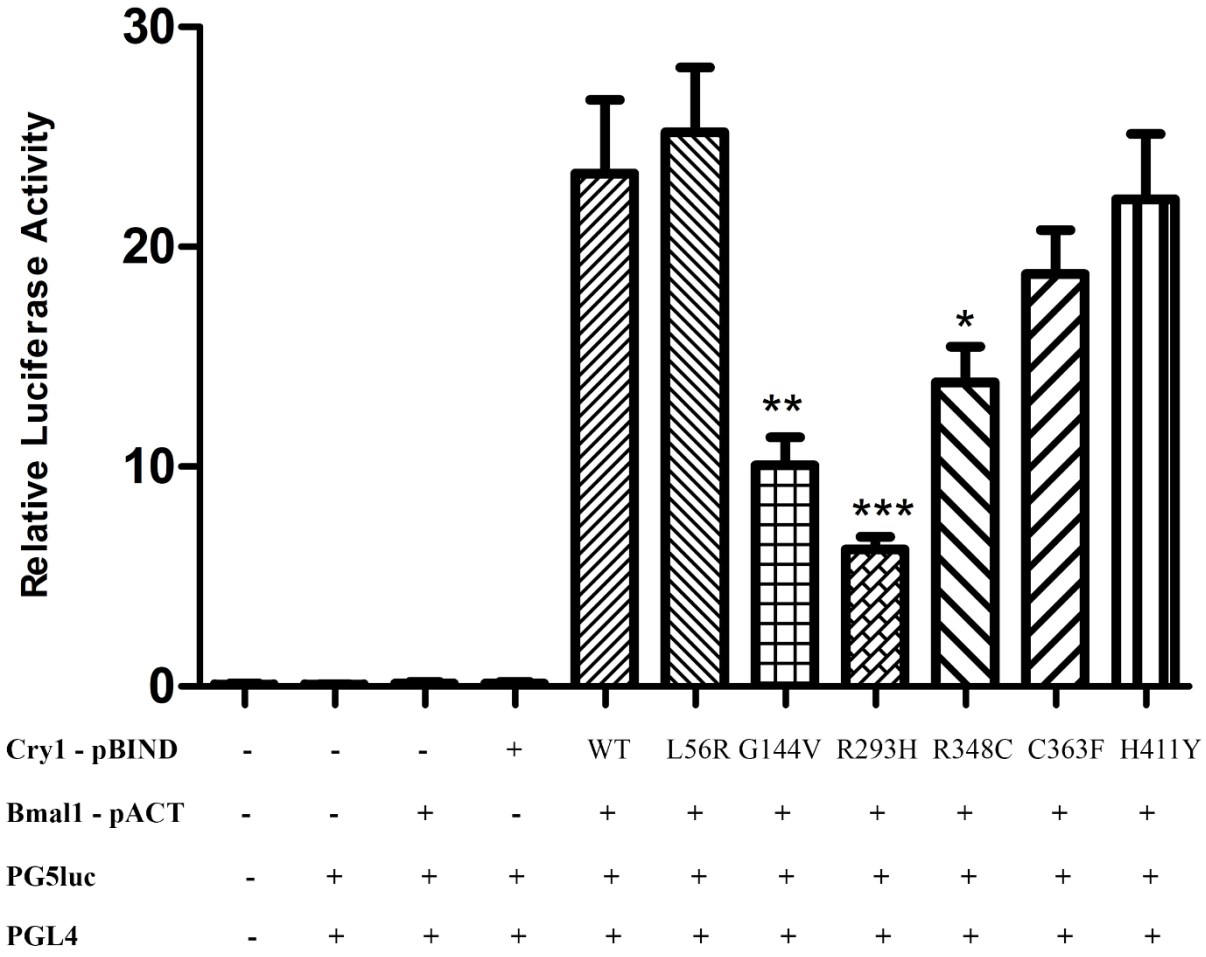


Figure 4. 16.Mammalian two hybrid assay to test the interactions between wild type CRY1 and wild type mPER2. The experiment is repeated 3 times and mean values are plotted on the graph. All values are normalized with Renilla.

Similar to BMAL1 – CRY1 interaction Gly144Val, Arg293His and Arg348Cys interacts less with mPER2 compared to wild type CRY1. However even though Arg293His shows no interaction with BMAL1 there is an interaction with mPER2. Gly144Val and Arg348His also show better interaction with mPER2 compared to their interactions with BMAL1.

4.5 Western Blotting of Proteins

Overexpressed proteins were checked using western blotting technique to see whether there were any problems during their synthesis. To analyze their expressions, we transfected all the wild type genes and their variants to HEK293T cells for overexpression. For transfection we used Bmal1 and Clock genes, and their variants on pCMV-Sport6 vectors, as well as Cry1 gene and its variants on pBind vector. The western blots were performed as mentioned in the materials and methods section. Beta-actin was used as the loading control in each case.

All of the wild types and their variants showed strong bands on the membrane implying no problems with expression (Figure 4.5.1).

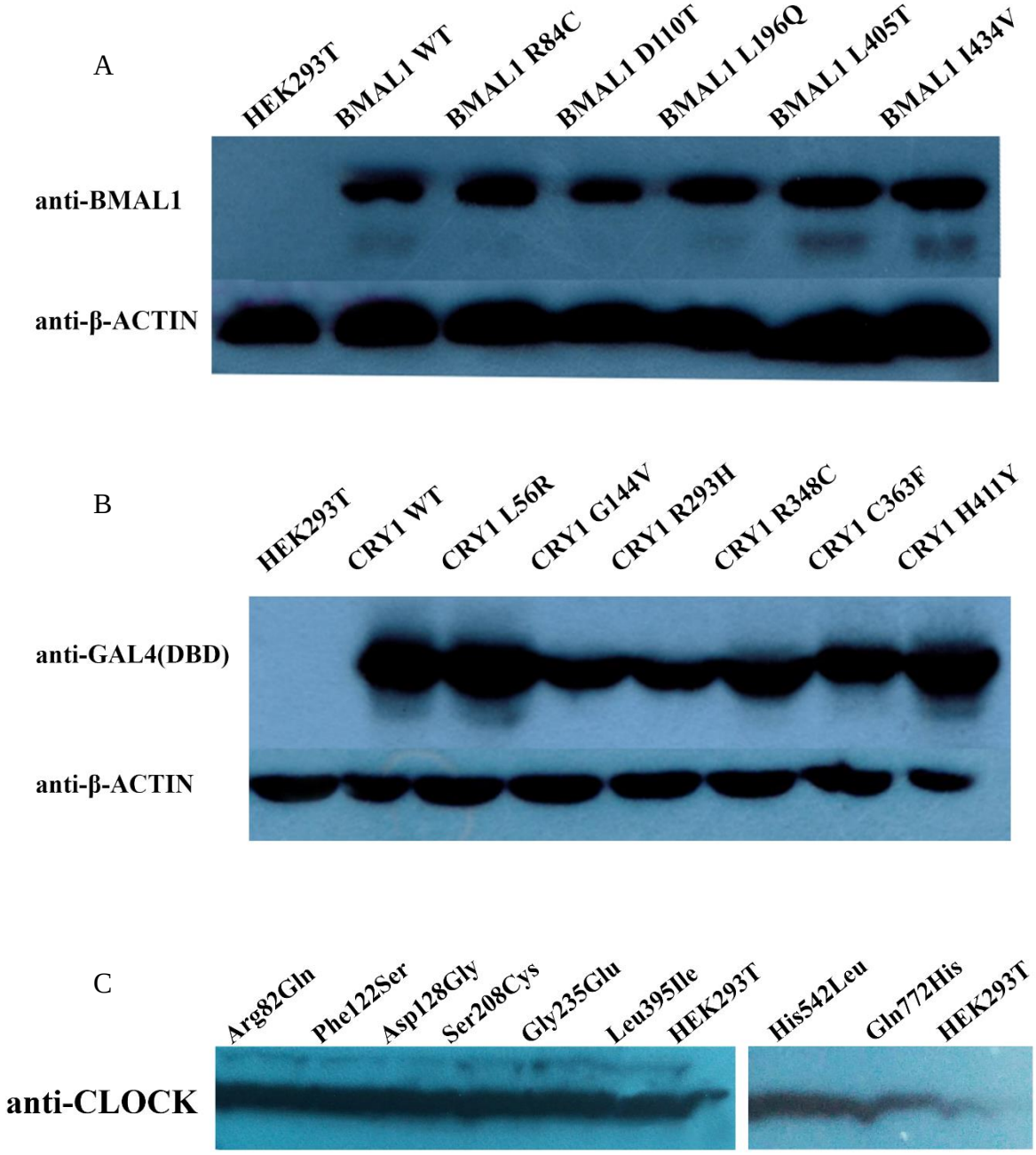


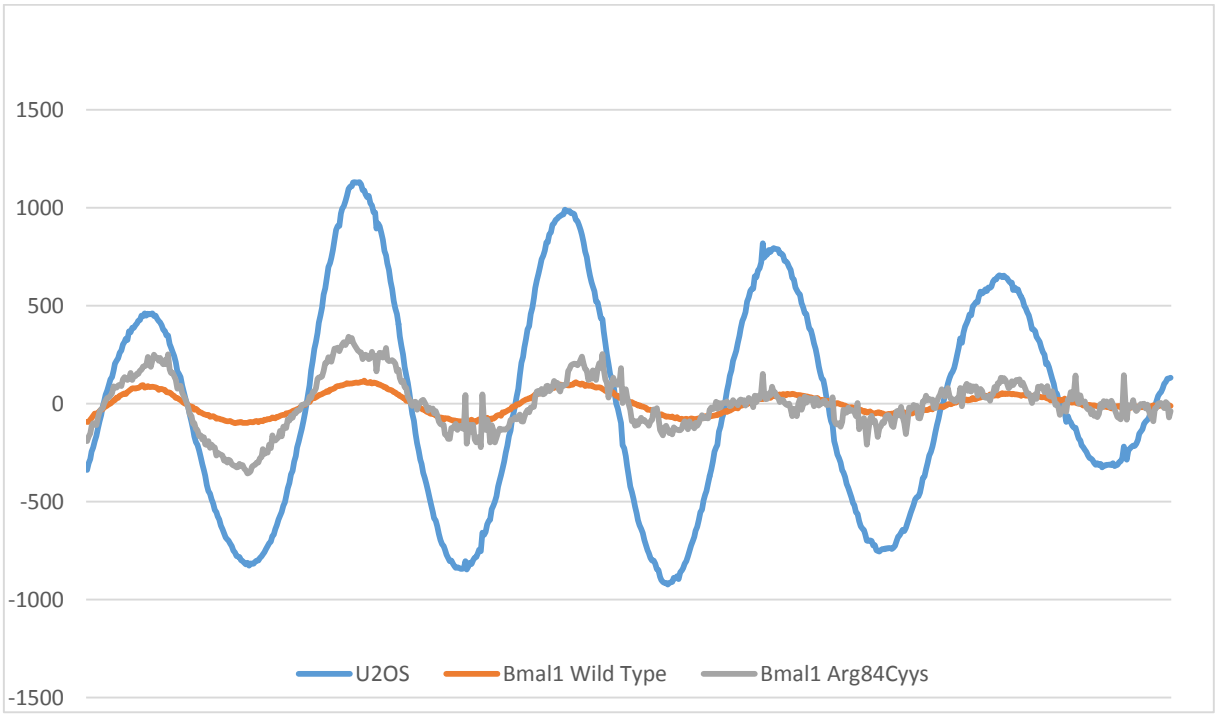
Figure 4. 17.Expression control for (A) BMAL1, (B) CRY1 and (C) CLOCK variants. All the variants are expressed in the HEK293T cell line and visualized on the gel. Total protein isolated from HEK293T cells are used as negative control.

4.6 The Effects of BMAL1, CLOCK and CRY1 Variants on Circadian Rhythm

In order to analyze if the variants of BMAL1, CLOCK and CRY1 proteins had a significant effect on the rhythm itself we aimed to analyze these variants by kinetic luminescence recording. For this purpose we produced lentivirus containing both the wild types of the genes

and their promising variants: Arg84Cys for Bmal1, Phe122Ser for Clock, Gly144Val, Arg293His and Arg348Cys for Cry1. Using the prepared lentivirus we transduced a U2OS cell line containing a luciferase gene attached to a Bmal1 promoter to observe the oscillation of luciferase gene expression and see if the variants caused a different rhythm than the wild types when overexpressed. Under normal conditions the cell line expresses luciferase in a circadian rhythmic manner. We also induced cells with a lentivirus containing GFP as a negative control where it is not expected to effect the rhythm even when overexpressed (Data not shown). The same lentivirus containing GFP was also used to see the transduction efficiency. mRNA levels of overexpressed proteins in our transduced cells were checked using RT-PCR. All cells were prepared for kinetic luminescence assay using the protocol mentioned in section 3.10.

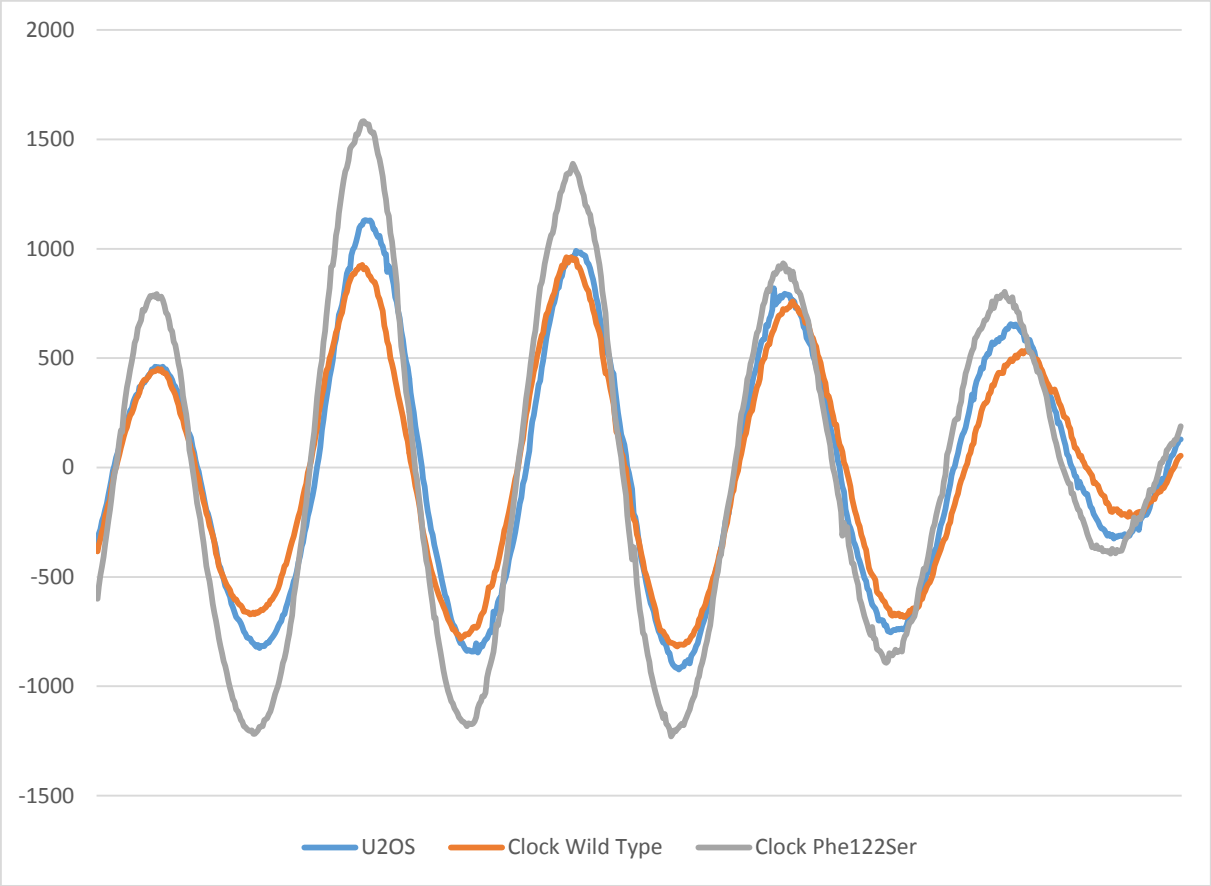
Luminescence readings were taken from each plate every 10 minutes to generate the graphs below. Represented data are fitted to a 4^{th} degree polynomial in order to calculate their respective period lengths.



	Period (hours)	Amplitude
U2OS	22.29451	889.2349
Bmal1 Wild Type	22.94779	80.31715
Bmal1 Arg84Cys	22.13412	110.5744

Figure 4.18. U2OS cells containing luciferase reporter gene regulated by Bmal1 promoter is infected with Bmal1 wild type and Arg84Cys variant using lentivirus. Luciferase readings were taken for 6 days. The data is baseline subtracted.

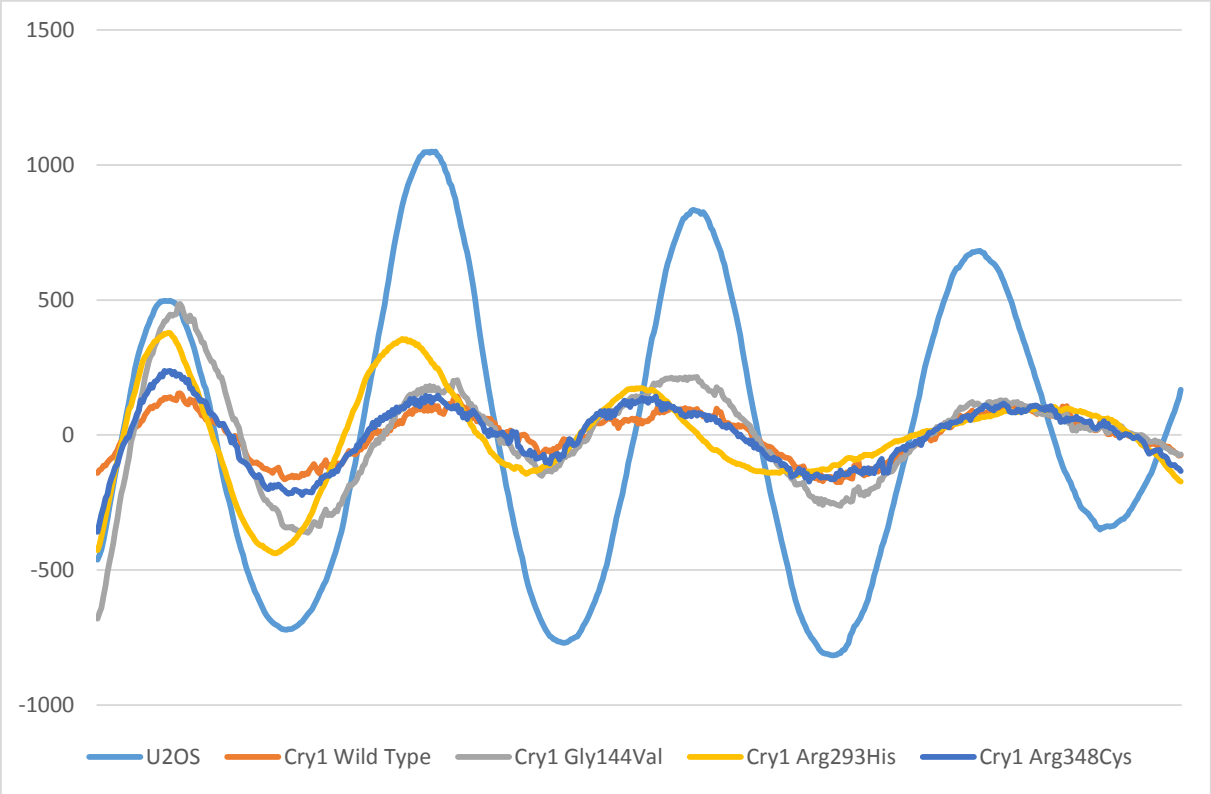
When overexpressed Bmal1 Arg84Cys variant loses its rhythmic oscillation in U2OS cell line. It is possible that overexpressed BMAL1 Arg84Cys proteins bind to endogenous CLOCK more often than the endogenous BMAL1, however the decreased transactivational capacity of the variant decreases the expression PERs and CRYs decreasing the suppression on Bmal1 promoters. This gives rise to arrhythmic production of luciferase protein. On the other hand when wild type BMAL1 is overexpressed in the cell, due to its transactivational capacity being the same as endogenous BMAL1 protein, it decreases the overall expressions regulated by Bmal1 promoters (Figure 4.6.1).



	Period	Amplitude
U2OS	22.29451	890.6728
Clock Wild Type	22.94779	804.1675
Clock Phe122Ser	23.45028	997.6219

Figure 4. 19.U2OS cells containing luciferase reporter gene regulated by Bmal1 promoter is infected with Clock wild type and Phe122Ser variant using lentivirus. Luciferase readings were taken for 6 days. The data is baseline subtracted.

Clock variant Phe122Ser showed stronger transactivational capacity during the luciferase assays. Phe122Ser also shows a slightly longer period when overexpressed, compared with the wild type overexpressed and U2OS cell lines (Figure 4.6.2).



	Period (hours)	Amplitude
U2OS	22.61879	876.1854
Cry1 Wild Type	22.61879	95.44559
Cry1 Gly144Val	21.504	170.3822
Cry1 Arg293His	20.15105	150.0797
Cry1 Arg348Cys	21.81678	103.5317

Figure 4.20. U2OS cells containing luciferase reporter gene regulated by Bmal1 promoter is infected with Cry1 wild type and Gly144Val, Arg293His and Arg348Cys variants using lentivirus. Luciferase readings were taken for 6 days. The data is baseline subtracted.

CRY1 overexpression in U2OS cell line causes the cells to lose their rhythmicity as expected. CRY1 functions as a repressor and its repression on BMAL1:CLOCK complex is extremely high compared with the endogenous CRY1 levels. Due to their overabundance in the cells CRY1 can continuously repress endogenous BMAL1:CLOCK complex. However in the variant Arg293His it is possible to observe the rhythmicity until it diminishes after 3 days. This

corresponds with the luciferase assays, especially mammalian two hybrid assays where the variant shows almost no interaction with BMAL1 wild type. The oscillation is very similar to that of U2OS cell line control, suggesting that Arg293His variant is not able to repress the complex at all even when overexpressed (Figure 4.6.3).

CHAPTER 5

DISCUSSION

The connection between circadian clock dysfunction and NCDs (non-communicable diseases) has been a hot topic in the recent years. With more and more diseases being associated with circadian rhythm dysfunctions it has been critical to assess the causes of circadian rhythm dysfunctions and produce solutions accordingly [31, 119]. Most of the studies have used forward genetics to understand the underlying problem of diseases. SNPs in the clock genes are associated with many physiological disorders like sleep disorders, metabolic disorders, and infertility [126, 149]. However, the effects of SNPs on biological clock and the related disorders are not well understood.

In this thesis we aimed to find SNPs in core clock genes that may have an effect on circadian rhythm dysfunctions. For this purpose we used 1000 genome project database to find a total of 18 SNPs in core clock genes, Bmal1, Clock and Cry1, which may play a significant role in circadian rhythm dysfunctions. We selected a number of SNPs that may cause a loss-of-function and or gain-of-function using the SIFT and PolyPhen prediction tools. We then produced the selected SNPs in vitro through site-directed mutagenesis. BMAL1 and CLOCK are the main transactivators of molecular clock mechanism in mammals. To find if any of the SNPs we selected caused a change in the transactivational ability of these proteins we used each variant in luciferase assays with the wild type of other protein. Bmal1 Arg84Cys variant showed a 35% decrease in the transactivational activity compared with the wild type. Clock variant Phe122Ser also showed a 35% increase in its tranactivational capacity during the screenings. Afterwards we wanted to see if Cry1 variants had an effect on its function as a suppressor to BMAL1:CLOCK complex. We screened all the variants with the same luciferase assay using Cry1 variants with wild types of Bmal1 and Clock. Three of the selected variants, namely Gly144Val, Arg293His and Arg348Cys, showed decreased repressional activites. Gly144Val and Arg348Cys variants both showed a 25% decrease in their repression capacity whereas Arg293His variant showed almost no effect on the BMAL1:CLOCK transactivation rate.

We also wanted to see if any of the Bmal1 or Clock variants showed a significant change in the Cry1 repressional activity. By using Bmal1 variants with wild type Clock and Cry1 we found

an increase in the repressional capacity of CRY1 on Arg84Cys and Leu196Gln. We also screened Clock variants to see their effect on Cry1 repression. Luciferase assay results showed however there was no significant effect of the selected Clock variants on CRY1 repression.

For the circadian molecular mechanism to function properly BMAL, CLOCK and CRY1 should interact with each other. To screen the variants for their interactions with each other we used mammalian two hybrid screening, a powerful method to detect protein-protein interaction. We first wanted to see the interaction between BMAL1 and CRY1 proteins. Gly144Val, Arg293His and Arg348Cys variants of CRY1 showed decreased interaction with BMAL1 compared to that of wild type. Gly144Val and Arg348Cys showed a decrease of almost 75% and 60% respectively. Arg293His variant on the other hand showed no interaction at all with BMAL1. Looking at the drastic decrease in interactions of Cry1 variants we conducted another mammalian two hybrid screening of CRY1 variants with mPER2. The assay gave a similar result where the same three variants decreased interaction between CRY1 and mPER2. Gly144Val showed almost 60% of decrease, Arg293His showed a 75% decrease and Arg348His showed a 40% decrease in their interactions with mPER2 compared with the wild type CRY1. We also screened the effect of CLOCK variants on the interaction of BMAL1 and CRY1. For this screen we used BMAL1 and CRY1 as the bait and prey proteins in the presence of different CLOCK variants. However there was no statistically significant difference in any of the screened variants. The interaction between the Bmal1 variants and Clock on the other hand yielded a notable result where Arg84Cys variant of Bmal1 showed an increased interaction with wild type CLOCK, almost by a factor of 3.2.

After the luciferase assays we chose the most promising variants of each protein – Arg84Cys of BMAL1, Phe122Ser of CLOCK and Gly144Val, Arg293His and Arg348Cys of CRY1. We overexpressed these variants together with their wild types in a reporter U2OS cell line using lentiviral transduction, in order to see their effect on biological rhythm. BMAL1 Arg84Cys variant caused the loss of rhythmicity as opposed to wild type. CLOCK variant Phe122Ser showed a very similar pattern to the wild type with a slight increase in its period. When overexpressed CRY1 variant on the other hand showed a completely different phenotype than the other two variants and the wild type. Gly144Val, Arg293His and wild type CRY1 completely destroyed the circadian rhythm when overexpressed, on the other hand Arg293His showed a similar rhythmicity to the untreated U2OS cell line with a slightly shorter period for 3 days.

The cumulative data gathered on BMAL1 variant Arg84Cys suggests a significant decrease in its ability to activate the expression of clock controlled genes. This effect may be explained in different ways. First of all, looking at the 3D structure of BMAL1, arginine in position 84 of bHLH domain is critical for BMAL1 binding to DNA (Figure 4.1.1, A) [66, 178]. A change at this position may cause the direct effect on the decreased levels of BMAL1 activation. Looking at the mammalian two hybrid data of BMAL1 and CLOCK proteins the same variant shows a significant increase in the protein-protein interaction. As BMAL1 and CLOCK are transactivators normally an increase in the interactions between the two proteins would suggest an increase in their activational rate as opposed to the decrease showed through luciferase assays. It is possible that arginine changing to cysteine in position 84 creates a much stronger interaction with CLOCK through the bHLH domain, so that the 3D structure of the combined bHLH domain is altered decreasing its ability to bind to the E-box elements. Kinetic luminescence assay also supports this idea showing that, when overexpressed, Bmal1 Arg84Cys variant shows an arrhythmic oscillation as opposed to the wild type. It is possible that overexpressed BMAL1 Arg84Cys proteins in U2OS bind to endogenous CLOCK more often than the endogenous BMAL1, however the decreased transactivational capacity of the variant decrease the expression PERs and CRYs. Since CRY1 wild type is functioning normally as the endogenous BMAL1 the competition does not change the transactivational efficiency. To understand the molecular mechanism of this variant however more experiments should be conducted. EMSA (Electron mobility shift assay) can be performed to see if there really is a direct effect in BMAL1 binding to the E-box elements. We also suspect a possible disulfide bridge formation due to arginine to cysteine change, thus experimenting with EMSA might shed a light on this issue. The significant increase of protein-protein interaction of BMAL1 Arg84Cys with CLOCK also makes it possible for a disulfide bridge formation in between the BMAL1 bHLH domain and CLOCK protein, however the 3D crystal structure reveals no nearby cysteine residues on CLOCK that are in close proximity to BMAL1 position Arg84. Another cysteine residue is available on BMAL1 bHLH domain position 102, which may make it possible for an intramolecular disulfide bridge causing a structural change in the bHLH domain of BMAL1 [66, 178].

CRY1 variants also show a significant variance from the wild type in luciferase assays. The significant decrease of interaction to BMAL1 is sufficient enough to explain the decrease in repressional capacity. However the variants also show a similar fashion in PER2 interaction suggesting a structural change rather than a change in binding efficiency. Crystal structure of

mCRY1 with mPER2 shows a heterodimer interface between mCRY1 α12 and mPER2 α1-2 [82]. Arginine in position 293 of CRY1 is a part of this α11' helix and it is possible that arginine changing to histidine may cause a structural change in this alpha helix resulting in disposition of α12 causing decreased interaction between mCRY1 and mPER2. Also trypsin in position 292 of mCRY1 was shown to be a part of FAD binding domain [82]. A structural change in the helical structure may cause the side chains to rotate, interfering with mPER2 and FAD binding. Close residues, Tyr287/Gly288 in α11, were also shown to be critical for transcriptional repressions [179]. Glycine in the position 144 of mCRY1 is found in between α5 and α6. Other residues in the same region, Leu148 and Thr149 were shown to have direct interactions with mPER2 α4 [82]. Being very close to these two residues any structural change caused by Gly144Val variant may have caused the decrease of interaction between mCRY1 and mPER2. mCRY1 Arg348Cys variant is positioned on α13 which is close to α12, any changes at this region may cause a structural change at the helical structure, causing the difference in mPER2 interaction observed in the luciferase assays [82]. Crystal structure of BMAL1:CRY1 interaction has not been proposed yet and thus it is not possible to talk about the difference in mammalian two hybrid assays of these two proteins. However all of the variants are in the PHR domain, close to mPER2 binding region, and in critical positions. BMAL1 interaction may be caused by a structural change due to these SNPs directly. Looking at the luciferase data, mammalian two hybrid assay data and the kinetic luminescence data it is possible to see CRY1 Arg293His is not able to function properly, probably due to a significant structural change. It has also shown a very different phenotype from wild type and other variants in kinetic luminescence assay, with a rhythm almost similar to U2OS cell line, supporting this idea where Arg293His is not able to effect the circadian rhythm as a repressor. Even though Gly144Val and Arg348Cys variants showed very low repressional activity and BMAL1 binding affinity in the luciferase assays, they give arrhythmic circadian oscillation when overexpressed even though they may actually have a change in their functions. A possible way around this is to use Cry1 knockout strains and induce them with these variants regulated by a Cry1 promoter instead of overexpressing them, and use them for kinetic luciferase assay. Since Arg293His seems to show a complete loss-of-function it is possible to see this effect in cells overexpressing the variant, however a variant with a decrease in the repressional function will cause the rhythm to completely disappear when overexpressed.

Clock variant Phe122Ser shows an increased activation coupled with wild type Bmal1 however it does not show a significant change in the proteins binding affinity towards BMAL1. It is

possible the residual change in this position alters the binding of BMAL1:CLOCK transactivator complex to E-box elements however it should be further researched using an assay such as EMSA to see whether it has a direct effect on DNA binding. Even though the variant does not have a significant effect on the circadian rhythm it is possible to see a slight increase in the period length compared to overexpressed wild type. However similar to the CRY1 variants this longer period should be tested using Clock knockout cells expressing the variant regulated by a Clock promoter to imitate the actual molecular system.

1000 genome project is based on the data gathered from healthy individuals. However, studies have put forth some of the SNPs in the database to be associated with chronic diseases [180]. Some of the variants we showed in this study does seem to have a significant effect on the circadian rhythm at a molecular level. It is possible that these variants are found as heterodimers, not showing the full effect in clock mechanism. Another possibility that is not negligible is the probability of the circadian rhythm dysfunction to show phenotypic disorders after a certain time point such as the onset of alzheimer's disease, which usually effects people after a certain age [126]. To relate these variants with a disease different gene pools from different diseased individuals should be sequenced.

To sum it up, as to our knowledge this has been the first study investigating the effects of SNPs resulting in amino acid changes in clock proteins at molecular level. We have been able to discover 5 SNPs in core clock proteins, BMAL1, CLOCK and CRY1, which cause functional alterations. These 5 variants may, in the future, be associated with circadian rhythm dysfunction and related diseases.

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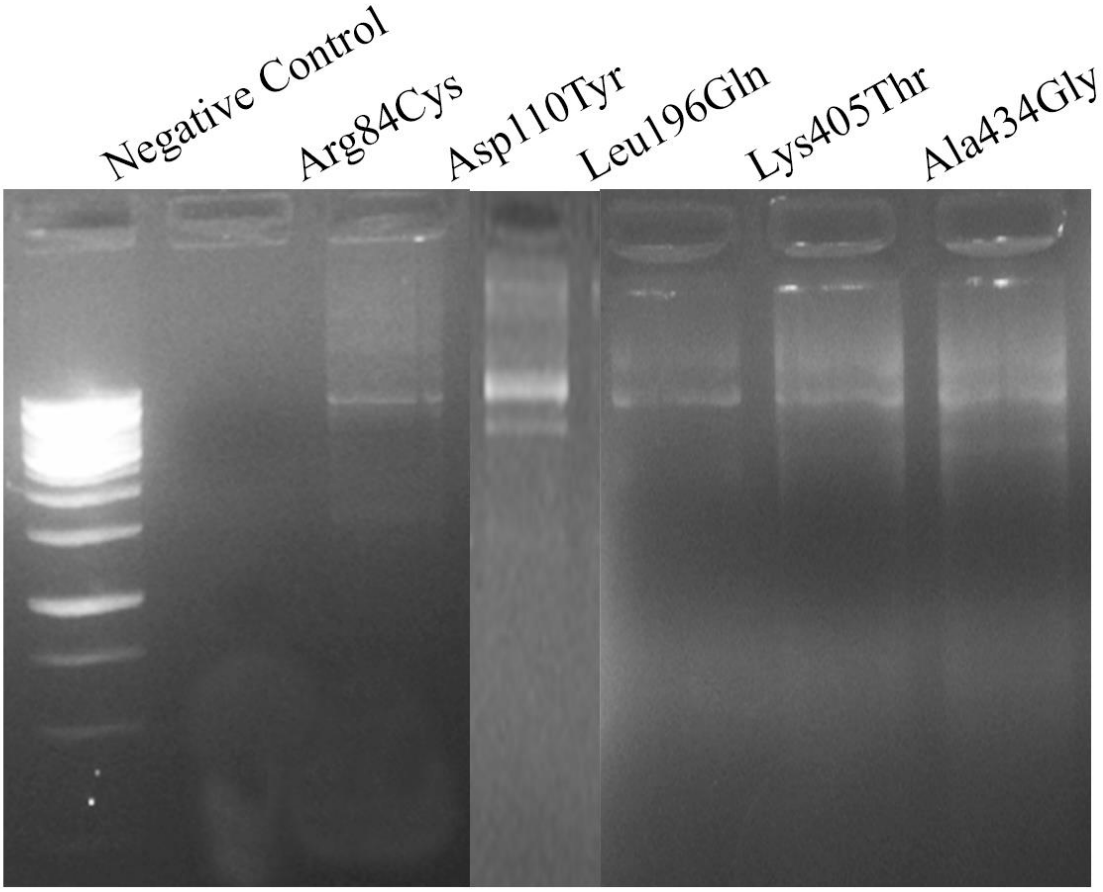
VITA

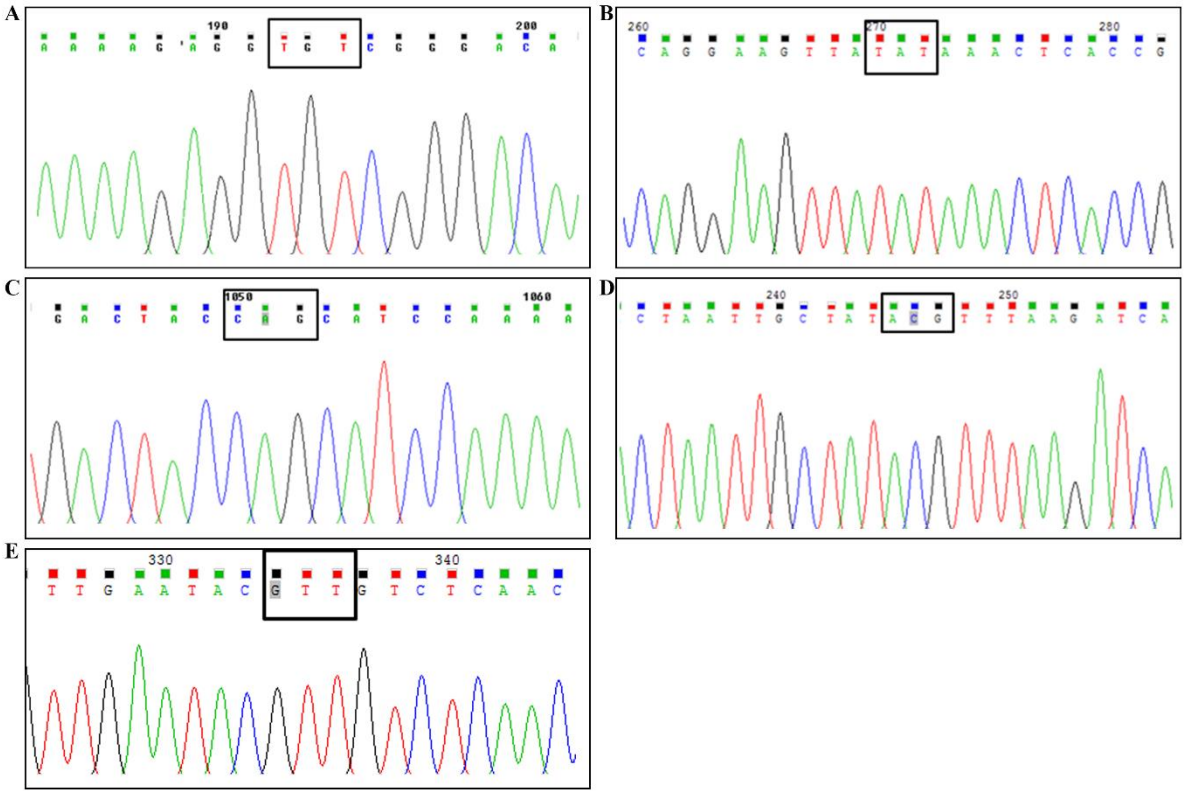
Berke Gürkan was born in Ankara, Turkey, on February 15, 1989. He received her B.Sc. Degree in Molecular Biology and Genetics and Chemical Engineering from Middle East Technical University, Ankara, in 2012. From September 2012 to September 2015 he worked as teaching and research assistant at Koç University, Istanbul, Turkey as a member of Molecular Biology and Biochemistry Laboratory. He has worked on “Molecular Analyses of the Effects of Clock Gene SNPs on the Biological Clock” during his M.Sc. study.

APPENDIX A: SITE-DIRECTED MUTAGENESIS RESULTS

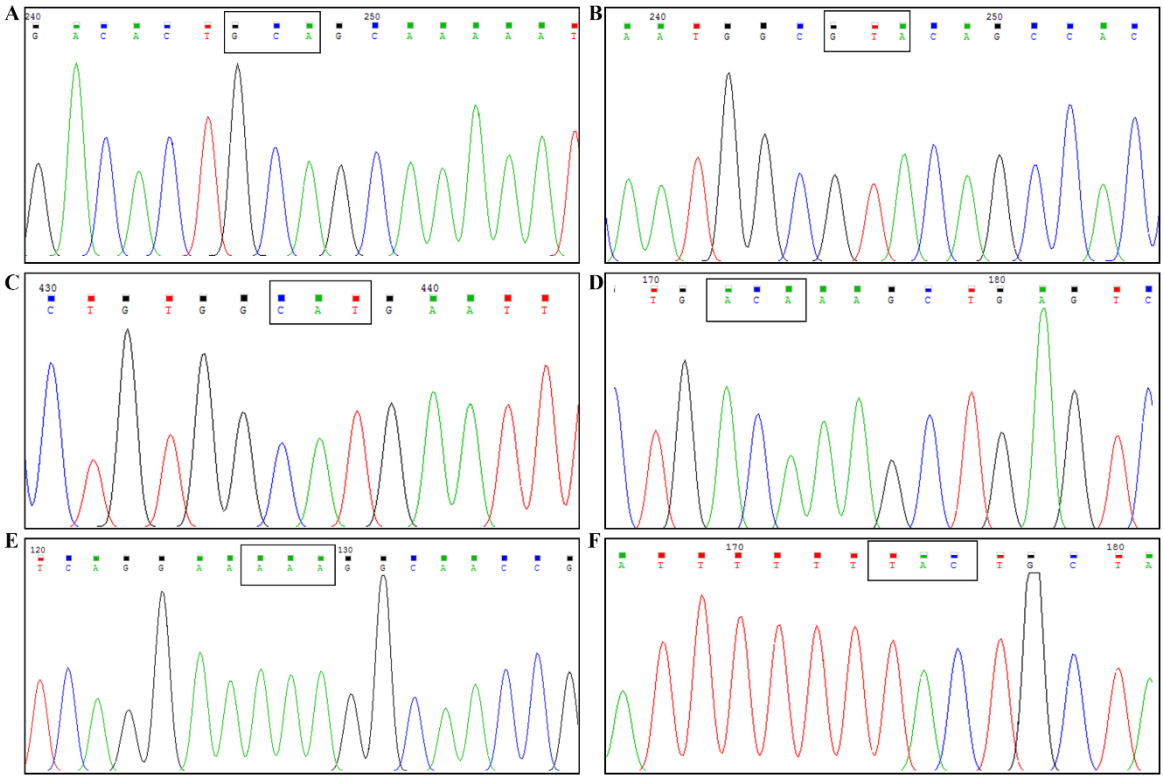


Site directed mutagenesis PCR amplicons of Bmal1 variants after DpnI digestion.

APPENDIX B: SEQUENCING RESULTS



Sequencing results for Bmal1 mutants. (A) Arg84Cys, (B) Asp110Tyr, (C) Leu196Gln, (D) Lys405Thr, (E) Ile434Val



Sequencing results for Cry1 mutants. (A) Leu56Arg, (B) Gly144Val, (C) Arg293His, (D) Arg348Cys, (E) Cys363Phe, (F) His411Tyr

APPENDIX C: PRIMER SEQUENCES

mBmal1-R84C-F: GATTGAAAAGAGGTGTCGGGACAAAATG
mBmal1-R84C-R: CATTTTGTCCCGACACCTCTTTTCAATC
mBmal1-D110Y-F: GTCCAGGAAGTTATATAAACTCACCGTG
mBmal1-D110Y-R: CACGGTGAGTTTATATAACTTCCTGGAC
mBmal1-L196Q-F: GAGCTTGTTTGACTACCAGCATCCAAAAGATATTG
mBmal1-L196Q-R: CAATATCTTTTGGATGCTGGTAGTCAAACAAGCTC
mBmal1-L405T-F: CACAACTAATTGCTATACGTTTAAGATCAAAGATG
mBmal1-L405T-R: CATCTTTGATCTTAAACGTATAGCAATTAGTTGTG
mBmal1-I434V-F: GGAAGTTGAATACGTTGTCTCAACCAAC
mBmal1-I434V-R: GTTGGTTGAGACAACGTATTCAACTTCC
Clock-R82Q-F: GAAAAGCATTGATTTTTTACAAAAACATAAAGAAATCACTGC
Clock-R82Q-R: GCAGTGATTTCTTTATGTTTTTGTAAAAAATCAATGCTTTTC
Clock-F122S-F: GGCTCTTGATGGTTTTTCTTTAGCAATCATGACAGA
Clock-F122S-R: TCTGTCATGATTGCTAAAGAAAAACCATCAAGAGCC
Clock-D128G-F: GCAATCATGACAGGTGGAAGCATAA
Clock-D128G-R: TTATGCTTCCACCTGTCATGATTGC
Clock-S208C-F: CCCAAAGGAGCCATGTACCTATGAATATGT
Clock-S208C-R: ACATATTCATAGGTACATGGCTCCTTTGGG
Clock-G235E-F: CACAATGGTTTTGAAGAAACTATACAACGCAC
Clock-G235E-R: GTGCGTTGTATAGTTTCTTCAAAACCATTGTG
Clock-L395I-F: GGCATTGAAGAGCTTATTCCTGAGACAGCTG
Clock-L395I-R: CAGCTGTCTCAGGAATAAGCTCTTCAATGCC
Clock-H542L-F: GATAGAAGCAAATATTCTTCGGCAACAAGAAGAAC
Clock-H542L-R: CTGAGCCTGAGATGGATGCTGAACTGAAGTG
Clock-Q772H-F: CACTTCAGTTCAGCATCCATCTCAGGCTCAG
Clock-Q772H-R: CTGAGCCTGAGATGGATGCTGAACTGAAGTG
mCry1-L56R-F: CAGGTGGCGATTTTTGCTGCAGTGTCTTGAGGATCTTG
mCry1-L56R-R: CAAGATCCTCAAGACACTGCAGCAAAAATCGCCACCTG
mCry1-G144V-F: CATAGAACTCAATGGCGTACAGCCACCTCTAAC
mCry1-G144V-R: GTTAGAGGTGGCTGTACGCCATTGAGTTCTATG
mCry1-R293H-F: GGGCAACTCCTGTGGCATGAATTTTTTTATACAG

mCry1-R293H-R: CTGTATAAAAAAATTCATGCCACAGGAGTTGCCC
mCry1-R348C-F: CATCATGACTCAGCTTTGTCAGGAGGGCTGGATC
mCry1-R348C-R: GATCCAGCCCTCCTGACAAAGCTGAGTCATGATG
mCry1-C363F-F: CCAGACACGCGGTTGCCTTTTTCCTGACTCGTGGTGAC
mCry1-C363F-R: GTCACCACGAGTCAGGAAAAAGGCAACCGCGTGTCTGG
mCry1-H411Y-F: CTTTTTTCAGCAATTTTTTTACTGCTACTGCCCTGTGGG
mCry1-H411Y-R: CCCACAGGGCAGTAGCAGTAAAAAAATTGCTGAAAAAAG
mBmal1-pAct-F: GCGTTGATATCAGCAGACCAGAGAATGG
mBmal1-pAct-R: GTCAAGGCGGCCGCTTACAGCGGCCATGGC
Clock-pBind-F: GCGTTGATATCATTGTTTACCGTAAGCTG
Clock-pBind-R: GTACCATGCGGCCGCCTACTGTGGTTGAACC
mBmal1-pENTR1A-F: ATGCAGTCGACATGGCGGACCAGAGAA
mBmal1-pENTR1A-R: GTAGCGATATCTTACAGCGGCCATGGCAAGTCACTA
Clock-pENTR1A-F: CTGCGGTCGACATGTTGTTTACCGTAAGCTG
Clock-pENTR1A-R: GTAGCGATATCCTACTGTGGTTGAACCTTGGA
mCry1-pENTR1A-F: ATGCAGTCGACATGGGGGTGAACGCCGT
mCry1-pENTR1A-R: GTAGCGATATCTTACTGCTCTGCCGCTGGA
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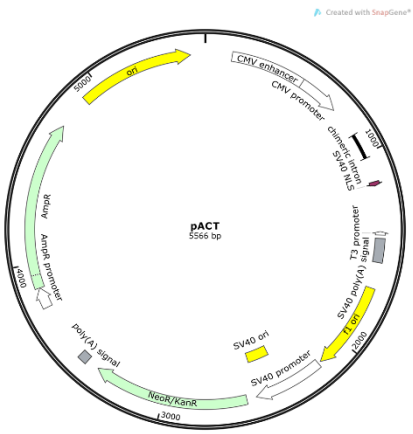
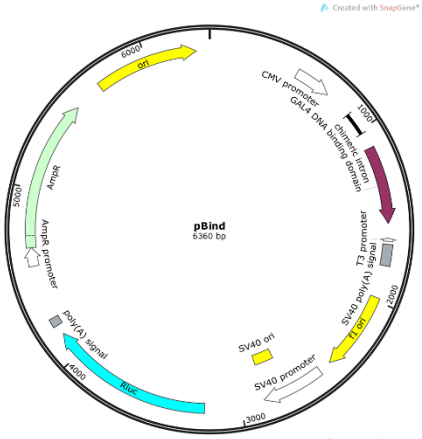
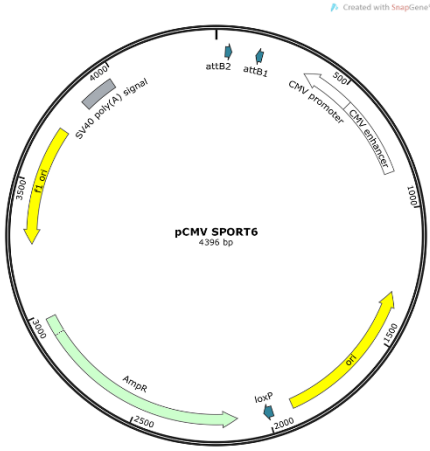
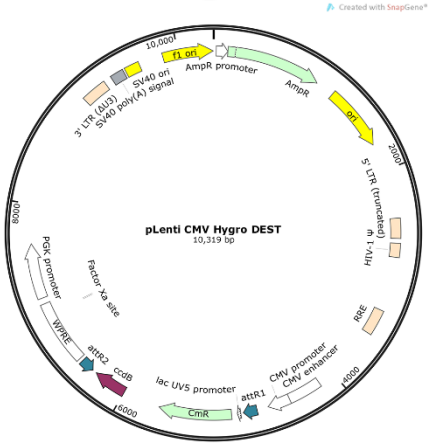
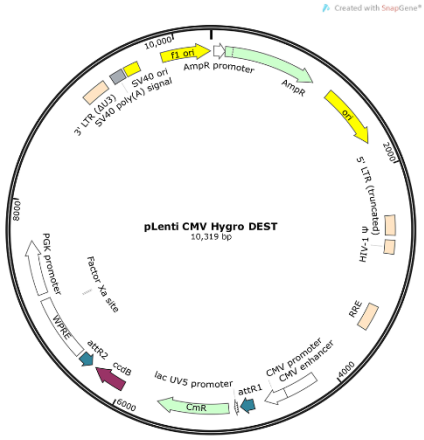
APPENDIX D: VECTOR MAPS

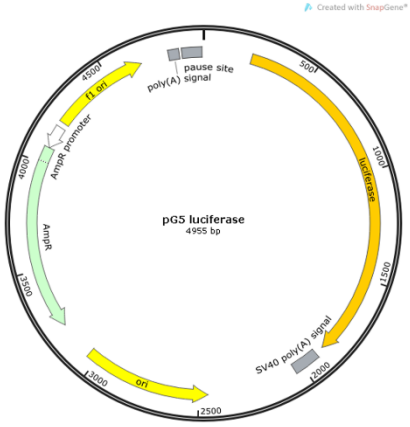






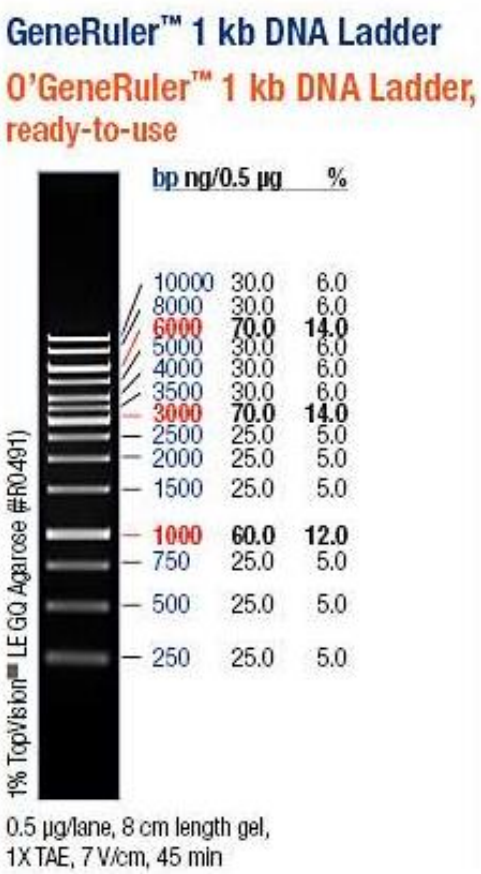




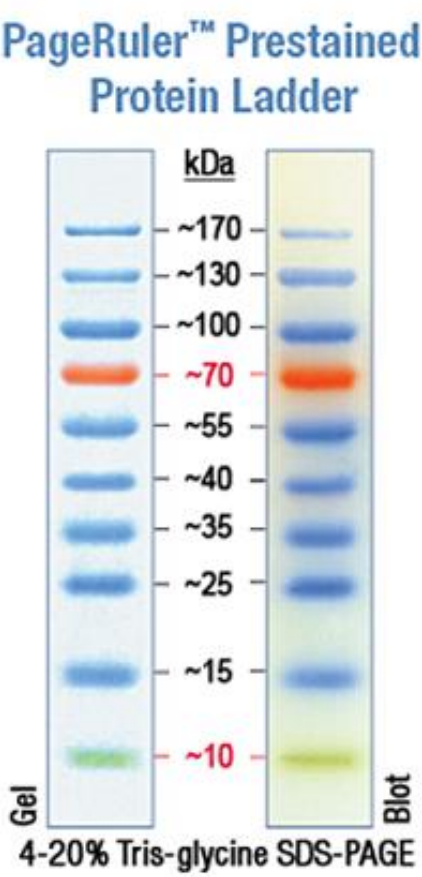


APPENDIX E: DNA AND PROTEIN MARKERS

DNA Molecular Weight Markers



Protein Molecular Weight Markers



APPENDIX F: LABORATORY EQUIPMENT

Autoclaves	**:** CL-40S/SDP (60L) ALP autoclave
Centrifuges	**:** 4K15, Sigma Laboratory
	: Microfuge 14-15, Sigma Laboratory
Deep freezes and refrigerators	**:** Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.
	: 2021 D deep freezer, Arcelik.
	: 1061 M refrigerator, Arcelik.
Gel documentation system	**:** UVIpro GAS7000, UVItec Limited.
Ice Machine	**:** AF 10, Scotsman.
Shaker	**:** Innova 4300 incubator shaker
Magnetic stirrer	**:** Heidolph MR 3001
Pipettes	**:** Pipetteman P10, P 100, P1000, Eppendorf
pH meter	**:** Inolab pH level 1, order# 1A10-1113,
Power supply	**:** PowerPac Basic (300V,400mA,75W) Biorad
Pure water systems	**:** DV25 PureLab Option ELGA
Spectrophotometer	**:** W-1700 PharmaSpec, Shimadzu Corporation.
Vortexing machine	**:** Reax Top, Heidolph2.2.