Characterization of the Single Nucleotide Polymorphisms that effect repression activity and stability of Cryptochrome2

by

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Koç University

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Bilge Bahar Çamur

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Date:

To my beloved family…

ABSTRACT

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Bilge Bahar Çamur
Master of Science in Molecular Biology and Genetics
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The daily rhythm of sleep/wake cycles, body temperature, metabolism, hormone secretion and many other physiological activities are in control of the circadian mechanism which is conserved from simple cyanobacteria to complex humans. In mammals, the circadian clock is endogenous and highly regulated with complex transcriptional and translational feedback loops (TTFL). Entrainment of the rhythm through the whole body is maintained by suprachiasmatic nuclei (SCN) located in the anterior hypothalamus with the cues from the environment. Many physiological processes are linked to the circadian clock, and it is not surprising that disruption of the clock can lead to certain disorders as demonstrated in various studies.

At the molecular circadian mechanism of mammals, Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like-1 (BMAL1) heterodimerizes and transactivates the expression of clock controlled genes including *Period* (*Per*) and *Cryptochrome* (*Cry*) by binding to E-box(CACGTG). PER and CRY accumulate in the cytoplasm and translocate into the nucleus, where they repress BMAL1/CLOCK-driven transactivation. The inhibition of BMAL1/CLOCK is relieved by the breakdown of CRY and PER proteins, resetting the cycle.

In this study, the effect of the rare CRY2 variants in the central clock mechanism is aimed to be investigated. To this end, the fifteen rare *Cry2* variants are identified from the database of 1000 Genomes Project and Ensembl. The initial assessment of these fifteen variants is predicated to be pathogenic. Further experimental and structural studies lead us to study p.Pro123Leu CRY2, p.Ser210Ile CRY2, and p.Asp406His CRY2 variants, which are located at a functionally important domain of the CRY2 called the secondary pocket, plays a role in CLOCK binding. CRY2 variants were unable to repress BMAL1/CLOCK driven transcription. Further biochemical studies indicated these

variants were less stable than the wild type CRY2. Additionally, compared to wild type CRY2 the p.Pro123Leu CRY2, the p.Asp406His CRY2 and p.Ser410Ile had reduced affinity to CLOCK assessed by co-immunoprecipitation. Finally, it has been observed that p.Ser210Ile CRY2 and p.Asp406His CRY2 are not capable of rescuing the circadian rhythm in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double knockout mouse embryonic fibroblast cell line by complementation test. Collectively our data suggest that the secondary pocket of CRY2 plays a significant role not only for the CLOCK binding but also for the stability and the proper circadian rhythm at the cellular level.

ÖZETÇE

Kriptokrom2 Proteininin Stabilite ve Baskılama Aktivitesini Etkileyen Tek Nükleotit Polimorfizmlerinin Karakterizasyonu

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Uyku düzeni, vücut sıcaklığı, metabolik aktiviteler gibi bir çok fizyolojik ve davranışsal olayın 24 saatlik periyotlar halinde düzenlenmesine sirkadyen ritim denir ve siyanobakteriden insanlara bir çok organizmada geçmişten günümüze korunmuştur. Bu ritim, dış faktörlere bağlı olmaksızın oluşmakta ve döngüsüne devam etmektedir, fakat dış etmenlerden gelen bilgiler içsel saatin dış ortam ile senkronize olmasını sağlar. Memelilerde dıştan alınan bilgilerle vücuttaki tüm dokuların hassas ayarını yapan ana saat süprakiazmatik çekirdekte (SCN) bulunur. Ana saat tüm dokuları, hormanlar ve sinir yolu iletimi ile senkronize etmektedir.

Sirkadyen ritmin moleküler mekanizmasına bakıldığında karşımıza iç içe geçmiş transkripsiyonel-translasyonel geri bildirim döngüleri çıkmaktadır. Bu döngülerin pozitif kolunda CLOCK (Circadian locomotor output cycles kaput) ve BMAL1 (Brain and Muscle ARNT-Like-1) proteinleri kompleks oluşturup E-box (CACGTG) üstünden gerçekleşen gen ifadesini aktifleştirici rol oynarlar. Gen ifadesi aktifleştirilen genler arasında *Per* (*Period*) ve *Cry* (*Cryptochrome*) genleri de bulunur ve bu iki protein döngülerin negatif kolunu oluşturmaktadır. Sitoplazmada biriken PER ve CRY kompleks halinde hücre çekirdeğine giderek BMAL1/CLOCK ile etkileşime girer. Bu etkileşim zamana bağlı olarak gen ifadesi aktifleştirilmiş genlerin ifadelerinin baskılanmasına neden olur. Bu şekilde PER ve CRY kendi gen ifadelerini kendileri düzenlemiş olur, bu durum döngünün tekrar başlamasına olanak sağlamaktadır.

Bu çalışmada 1000 Genom Projesi ve Ensembl Veri Bankasından seçilen nadir *CRY2* varyantlarının sirkadiyen mekanizması üzerine olan etkilerinin çalışılması amaçlanmıştır. Bu amaç için öncelikle varyantların baskılayıcı aktiviteleri ölçülmüş, ardından, fare CRY2 proteinin 3 boyutlu yapısı kullanılarak varyantarın yerleri belirlenmiştir. Bu iki data seti verilerinin değerlendirilmesi sonunda CRY2'nin ikincil

cep bölgesinin girişinde bulunan p.Pro123Leu CRY2, p.Ser210Ile CRY2 ve p.Asp406His CRY2 varyantları ile çalışılmaya karar verilmiştir. Doza bağlı baskılayıcı aktivite ölçümü deneyleri sonucunda PER2 proteinin bu üç CRY2 varyantının baskılayıcı görevlerini yerine getirmesi için elzem olduğu, hatta varlığında hala daha varyantların tam olarak yabanıl tipe kıyasla baskılayıcı etki sergileyemedikleri gösterilmiştir. Ardından yapılan kararlılık çalışmaları yabanıl tip CRY2 proteinin varyantlara göre çok daha kararlı olduğunu ve PER2 varlığında yabanıl tip ve varyantlar olmak üzere tüm CRY2 proteinlerinin yarılanma ömrünün yaklaşık iki katına çıktığı belirlenmiştir. Bunlara ek olarak, protein-protein etkileşim çalışmaları p.Pro123Leu CRY2, p.Asp406His CRY2 ve p.Ser410Ile varyantlarının yabanıl tipe göre CLOCK proteinine olan afinitelerinin azaltığı gözlemlenmiştir. Son olarak, bu çalışma p.Ser210Ile CRY2 ve p.Asp406His CRY2 varyantlarının $Cry1^{-/-}Cry2^{-/-}$ fare embiriyonik fibroblast hücrelerinde ritimi geri getirebilecek kadar iyi bir baskılayıcı olmadığını göstermiştir. Bir bütün olarak bu çalışma ikincil cebin CRY2 proteinin kararlılık ve hücre seviyesinde düzgün çalışan sirkadiyen ritim için büyük rol oynadığını önermektedir.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

AMPK	AMP-activated protein kinase
bHLH	Basic helix-loop-helix domain
BMAL1/ ARNTL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
CKIε/Iδ	Casein kinase 1 epsilon/1 sigma
CLOCK	Circadian locomotor output cycles kaput
CR	Circadian rhythm
CRE	cAMP response element
CREB	(cAMP)-response-element-binding protein
CRY	Cryptochrome
DBP	D site of albumin promoter (albumin D-box) binding protein
DSPD	Delayed sleep phase disorder
E-box	Enhancer box
FASP	Familial advanced sleep phase syndrome
FBXL3/21	F-box/LRR-repeat protein 3/21
HEK 293T	Human embryonic kidney cells
INL	Inner nuclear layer
ipRGC	Intrinsically photosensitive retinal ganglion cells
MEF	Mouse embryonic fibroblast cells
NFIL3	Nuclear factor, interleukin 3 regulated
NPAS2	Neuronal PAS domain protein 2
PAS	Per-arnt-sim domain

PER	Period
PRC	Photoreceptor cells
RBC	Retinal bipolar cells
REV-ERB	Nuclear receptor subfamily 1, group D
RGC	Retinal ganglion cells
RHT	Retinohypothalamic tract
ROR	RAR-related orphan receptor
RORE	ROR/REV-ERB-response element
SCN	Suprachiasmatic nucleus
TTFL	Transcriptional-translational feedback loops
β-TrCP1	β-transducin repeat-containing protein 1

CHAPTER 1

INTRODUCTION

The circadian clock is an endogenous timing system that controls physiological, metabolic, and behavioral processes in 24 hours period (Lowrey & Takahashi, 2004). This rhythmicity is conserved in all live beings from simple cyanobacteria to complex humans and is entrained by environmental cues like light and temperature (Cohen & Golden, 2015; Panda, Hogenesch, & Kay, 2002). The central pacemaker of the mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) and is responsible for the regulation and synchronization of peripheral clocks via neuronal signals and hormones (Fu & Lee, 2003).

The mammalian circadian clock is composed of interlocked transcriptional-translational feedback loops (TTFL) at the molecular level. Transcriptional factors Brain and Muscle ARNT-Like-1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) initiate the transcription of clock-controlled genes including the *Period* (*Per*) and *Cryptochrome* (*Cry*) by forming heterodimer and binding to E-box elements so that they serve as the positive arm of the TTFL (Gekakis et al., 1998; Rudic et al., 2004). When phosphorylated by casein kinase IΔ, PER and CRY heterodimer translocates into the nucleus and suppresses BMAL1/CLOCK-mediated transcription. PER and CRY, in the end, function as negative regulators of their own transcription and forms the negative arm of the loop (Shearman et al., 2000; Vitaterna et al., 1999). The repression on BMAL1/CLOCK is relieved when CRY and PER proteins are degraded. This process resets the cycle (J. S. Takahashi, 2016). The ubiquitination of CRY and PER by F-box and leucine-rich repeat protein 3 (FBXL3) and β-transducin repeat-containing protein 1 (β-TrCP1), respectively, initiates proteasomal degradation of these proteins (Reischl et al., 2007; Siepka, Yoo, Park, Song, et al., 2007).

Disruption of the circadian clock could cause numerous behavioral and physiological diseases (Sahar & Sassone-Corsi, 2009). Several genetic studies indicated that there is a relationship between CRY mutations and a variety of disorders such as advanced sleep phase, type 2 diabetes, and depression (Hirano et al., 2016; Lavebratt et

al., 2010; Liu et al., 2011). However, it is not well understood the contribution of the CRY2 mutations on disease progressions through the circadian rhythm mechanism at the molecular level. The purpose of this study is to establish a pipeline to study the effect of CRY2 mutations at the molecular level using molecular and cellular methods.

To this end, *Cry2* variants are identified from the database of 1000 Genomes Project were used to understand the effect of the mutants on circadian rhythm. Using the *Per1*-d*Luc* assay, the repressor capacity of each CRY2 variant is evaluated on BMAL1/CLOCK-driven transcription. After the identification of CRY2 variants with altered repressor activity, we mapped amino acid residues on the 3D structure to identify the variants that reside at the functionally important domains of CRY2. Among variants, p.Pro123Leu, p.Ser210Ile, and p.Asp406His CRY are located at the gate of the secondary pocket, which is shown to be important for the CLOCK binding. The repression activity of CRY2 variants on BMAL1/CLOCK-driven transcription located at the gate of the secondary pocket is examined in a dose-dependent manner in both the presence and absence of PER2. After that, the stabilities of p.Pro123Leu CRY2, p.Ser210Ile CRY2, and p.Asp406His CRY2 are determined with/without PER2 by using cycloheximide chase assay. Additionally, Co-immunoprecipitation is used to examine the physical interaction between CRY2 variants and the BMAL1/CLOCK complex in the presence and absence of PER2. Finally, under endogenous conditions, the circadian rhythm rescue analysis of CRY2 variants is examined.

Chapter 2 covers a comprehensive literature review about the structure and molecular mechanism of the mammalian circadian clock. In addition, CRY structure and important regions for protein-protein interactions that affect CRY activity and stability were reviewed in detail.

Details of the materials and methods used in this research are given in Chapter 3.

Chapter 4 provides all the experimental results of the study as well as detailed explanations of the findings.

Chapter 5 includes the discussion of the results about CRY2 variants that are investigated in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Rhythms and Characteristics

Periodic changes in nature like daily temperature, humidity and light fluctuations, tides, seasons, and changes in the length of day and night over the year are caused by the Earth's geometry, rotation, and position with respect to the sun and moon. In order to adapt to these cyclic changes in their environment, nearly all organisms have their own rhythms which are greatly studied by the field of chronobiology (Kuhlman, Craig, & Duffy, 2018). These rhythms are classified into three main categories: ultradian, circadian, and infradian rhythms (Figure 2.1). Ultradian rhythms, such as the rapid eye movement (REM)-nonREM (NREM) sleep cycle, cell division, breathing, feeding, and urination cycles last less than 24 hours and can occur several times per day (Weber, 2017). The circadian rhythm controls many physiological variables in mammals such as, the sleep-wake cycle, the concentration of numerous circulating hormones, and oscillations of body temperature in mammals and occurs approximately every 24 hours (Giebultowicz, 2004). Infradian rhythms, on the other hand, have periods that are longer than 24 hours, ranging from days to weeks, months, or even years. The human menstrual cycle, hibernation, and migration can be given as examples (Laje, Agostino, & Golombek, 2018) (Figure 2.1). The term “circadian” derives from the Latin words *circa*, which means “about,” and *diem*, which means “a day”. So, cycles that take 24-hour named circadian rhythms (CR). Circadian rhythms have been the most interesting among all others for humans. Because ultradian rhythms are too short while infradian rhythms are too long to attract attention. However daily rhythms are the most conspicuous ones because it is in the perfect alignment with the human’s way of life. With this great attention, it has become one of the most studied disciplines of molecular biology and physiology.

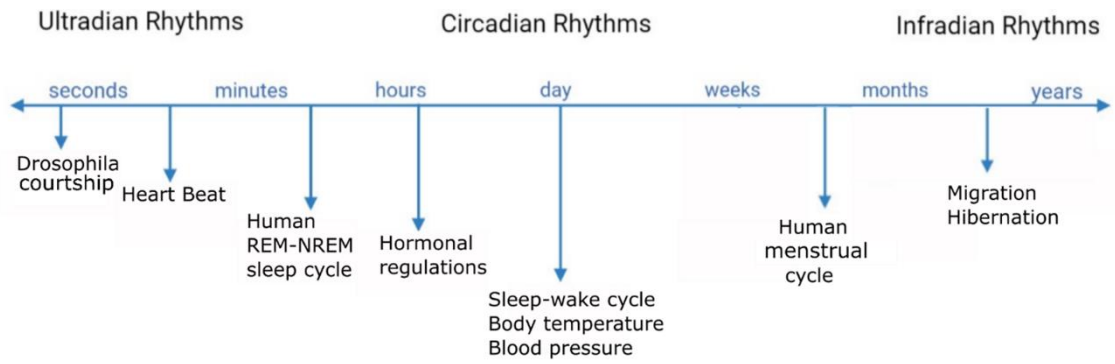


Figure 2.1 Schematic visualization of the rhythms
Ultradian, circadian, and infradian rhythms, as well as biological examples, are depicted schematically.

For a rhythm to be considered as circadian rhythm, it should display three distinct characteristics. Firstly, when external stimuli are removed, CRs should maintain their endogenous oscillations for 24 hours, a behavior is known as free-running (Wever, 1986). Although, because each organism has its own set of physiological regulations, the circadian clock might vary between 23 and 25 hours (Ripperger, Jud, & Albrecht, 2011). Secondly, CRs exhibit temperature compensation, which means that the duration of the endogenous period remains constant within the physiological temperature range (Dunlap, 1999). Finally, extrinsic stimuli such as light, eating behavior, emotional mood, social engagement, and temperature are entrained CRs, while light emerging as the most powerful zeitgeber (in German, zeitgeber means time-giver) (Aschoff, 1984; Damiola et al., 2000; Mohawk, Green, & Takahashi, 2012). As a whole, the circadian rhythms are composed of endogenous behavioral, physiological, and biochemical events which are cycling around 24 hours which are entrained by environmental cues such as light and temperature and form the circadian clock (Aschoff, 1984). From cyanobacteria to humans, the circadian clock that allows them to make the most use of their resources is conserved (Dunlap, 1999) which is not that surprising as the whole process of survival depends on it. In this thesis, the circadian clock mechanism of mammals was studied, therefore it will be covered in depth in the flowing text.

2.1.1 Features of Circadian Rhythm: Period, Amplitude and Phase

The circadian rhythms can be represented by plotting a specific oscillator like body temperature or locomotor activity over time. This representation reveals a sine wave which is a continuous periodic oscillation. As it is seen in Figure 2.2, amplitude and period are the primary elements of a wave. The distance between the highest (peak) and lowest (trough) values is known as amplitude. The time between one peak and the next, or from any position to the next matching point, is called a period. The circadian period of mammals is around 24 hours (a day) (Reppert & Weaver, 2002). For circadian rhythmicity, phase is another important parameter. It can be described as the difference between a reference point on the curve with the same reference point of other curves. Phase shifts according to a stimulus are depicted by the Phase Response Curve (PRC). The stimulus can cause a phase advance or a phase delay in the curve (Boulos & Rusak, 1982; Daan & Pittendrigh, 1976; C. Johnson, 1990)

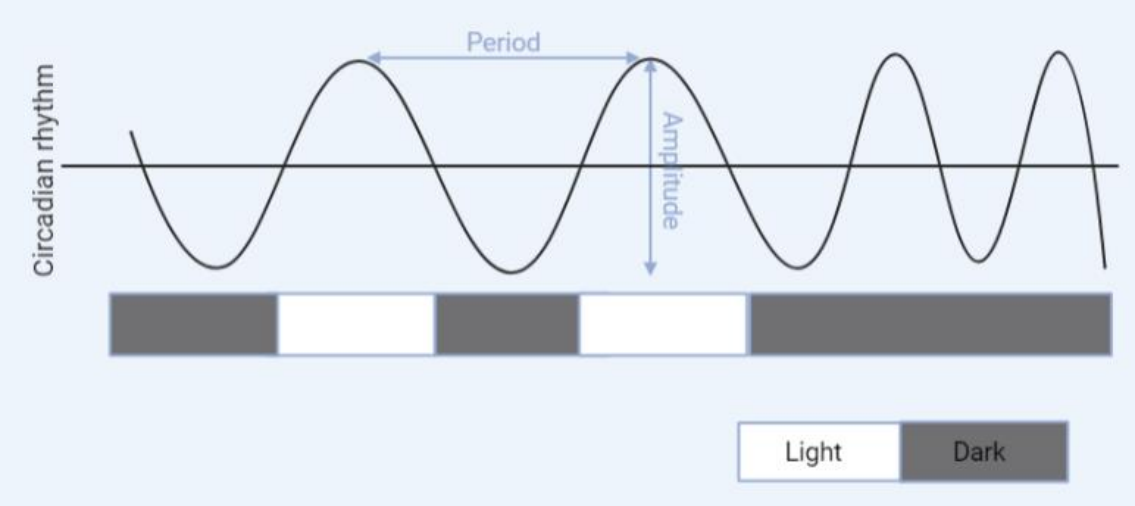


Figure 2.2 Schematic representation of rhythm parameters in the entrained system followed by the free-running system
The period and amplitude of the circadian rhythm are illustrated in the presence of the light/dark cycle (entrained system) followed by the free-running system (constant dark condition). The figure is adapted from (Nydegger, Escobar, Risch, Risch, & Stanga, 2014).

2.1.2 Historical Overview of Circadian Clock

The history of the circadian clock goes back to 2.5 billion years. It is believed that organisms' circadian clocks evolved to drive the detoxification of reactive oxygen species during the Great Oxidation Event (Loudon, 2012). However, the first known experiment about the circadian clock was held by the astronomer, Jean Jacques de Marian in 1729. He noticed that the leaves of *Mimosa pudica* opened and closed according to the time of day and night. He conducted a controlled experiment in which he established that the

movement of the leaves was retained even in the presence of constant darkness which led him to the realization that the leaf movement must be an endogenous characteristic of plants rather than a response to environmental cues (De Mairan, 1729). Augustin Pyramus de Candolle, a Swiss scientist, also confirmed de Marian's discovery and added that the rhythm is shorter than 24 hours by reasoning it with dual control of both endogenous clock and environmental cues of the biological clock (De Candolle, 1832).

Nearly two centuries later, the endogenous free-running locomotor activity of mice was measured by Maynard S. Johnson which was the first attempt at determining the period of a mammalian organism (M. S. Johnson, 1939). Then, a German scientist Jürgen Aschoff, one of the founders of chronobiology, published the findings of his experiment where he monitored the sleep-wake cycle, body temperature, and urinary excretion of over 150 human subjects under the constant-dark conditions, and he stated that the average period of the subjects measured as 25 hours (Aschoff, 1965). He also defined the term “zeitgeber” and identified the organisms’ endogenous rhythm.

Investigations of core clock components were started with *Period (Per)* gene in 1971 by Konopka and Benzer. By using forward genetic methods on *Drosophila melanogaster,* they found three different phenotypes; longer (28 h) shorter (19 h), and altered periods. These three mutations were located at the X chromosome and found as the alleles of the same gene later named as *Period*. After that, another forward genetic experiment on *Mus musculus* revealed *Circadian Locomotor Output Cycles Kaput* (*CLOCK)* from a period-lengthening mutation which is responsible for sustaining the rhythm and period length (Antoch et al., 1997; Vitaterna et al., 1994). In 1997, John B. Hogenesch discovered the Brain and Muscle Arntl like-1 protein (BMAL1). On the other hand, when Sancar and Ikenaga discovered CRYs, they were classified as photolyase orthologs and because of that for a time CRYs were thought to have a role in light sensation or DNA repair (Hsu et al., 1996; Todo et al., 1996). However, mCRY1 and mCRY2 exhibit distinct subcellular localization and DNA binding affinities than photolyases, according to a study (Kobayashi et al., 1998). Then, CRYs were hypothesized to be circadian photoreceptors for mammals; nonetheless, entrainment of circadian clock was proven to be possible with just ganglion cells in the inner retina (I. C. Van Gelder et al., 2002). Finally, studies that are conducted in 1999 were revealed the function of CRYs as circadian clock regulators (Horst et al., 1999; Vitaterna et al., 1999).

2.2 The Central and Peripheral Mammalian Circadian Clocks

The mammalian circadian clock mechanism on large scale is made up of output signals generated by the master clock in response to input signals. Input signals derived from environmental cues (Zeitgebers) such as light and temperature are assessed by the central pacemaker which is the suprachiasmatic nucleus (SCN) in mammals. The SCN is located at the anterior hypothalamus, where it orchestrates and synchronizes the peripheral clocks by producing neuronal and hormonal output signals (Mohawk et al., 2012).

SCN is composed of approximately 20,000 neurons each of which is cell-autonomous circadian oscillators (R. N. Van Gelder, 2003). Collectively they produce a robust, coherent rhythm. Because the most predominant Zeitgeber is light, there is a very specialized pathway where SCN receives the light signals. Light is sensed by intrinsically photosensitive retinal ganglion cells (ipRGC) which are the third members of photoreceptors after rods and cones (Wong, Dunn, & Berson, 2005). These are neuronal cells that are located at the inner retina and melanopsin is the key protein responsible for ipRGC's intrinsic photosensitivity (Do & Yau, 2010). The inner nuclear layer (INL) and ganglion cells transmit light signals to the SCN through the retinohypothalamic tract (RHT) (Figure 2.3) where neurotransmitters and peptide co-transmitters are released (Berson, Dunn, & Takao, 2002; Gooley, Lu, Chou, Scammell, & Saper, 2001). The release of the neurotransmitter glutamate increases the Ca^{+} levels which provokes the phosphorylation of the cyclic adenosine monophosphate (cAMP)-response-element-binding protein (CREB) (Meijer & Schwartz, 2003). The activated CREB via phosphorylation induces the transcription of *Per1* and *Per2* by binding to cAMP response elements (CREs) which are located on the *Per* promoters (Travnickova-Bendova, Cermakian, Reppert, & Sassone-Corsi, 2002). SCN neurons form a web where they evaluate signals to build a unified clock across the body as a result of this sensitive signaling system.

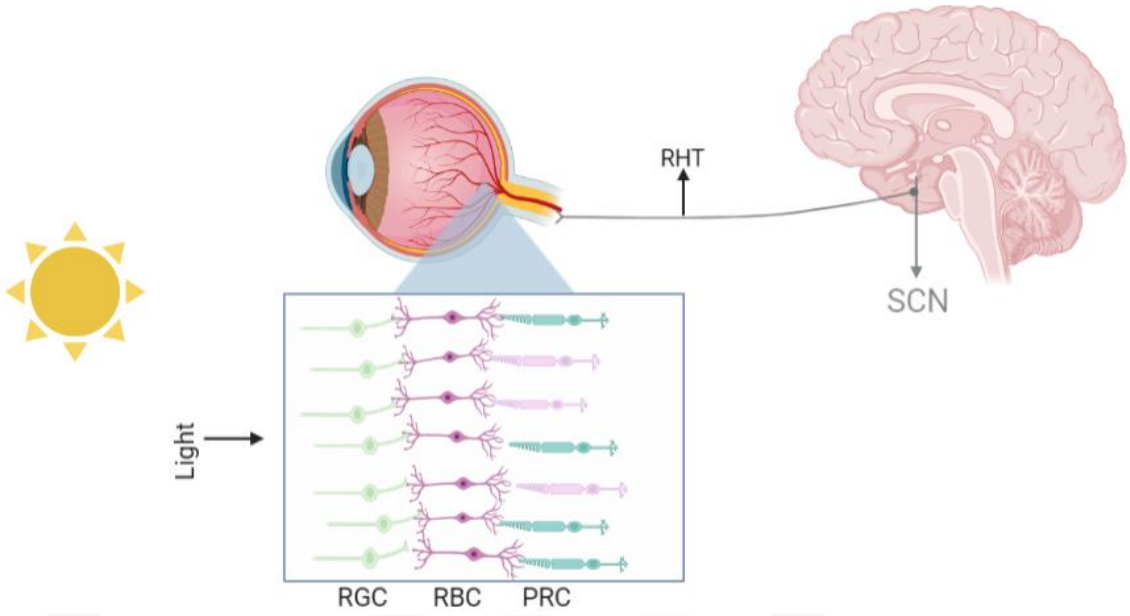


Figure 2.3 Illustration of light signal transmission to SCN
The suprachiasmatic nucleus (SCN) is in the hypothalamus and light signals are transmitted to SCN through the retinohypothalamic tract (RHT). Light is mainly sensed by retinal ganglion cells (RGC) and by rodes and cones which are photoreceptor cells (PRC) and retinal bipolar cells (RBC). The figure is adapted from Kavakli and Sancar (Kavakli and Sancar 2002).

Besides the central clock, each tissue has its own clock which is name as the peripheral clock. Despite the fact that the clock's molecular mechanism is shared by all tissues, peripheral clocks have a vital and distinct role in each tissue by regulating the circadian expression of specific genes involved in a wide range of physiological tasks (Yamazaki et al., 2000; S.-H. Yoo et al., 2004). The previous study reported that the circadian clock regulates the expression of 43% of the protein-coding genes, at least in one organ, in mammals. (Ray Zhang, Nicholas F. Lahens, Heather I. Ballance, Michael E. Hughes, & John B. Hogenesch, 2014). Various studies have also revealed that these expressions are tissue specific due to the specialized function, physiology, and biochemistry of each tissue (Panda, Antoch, et al., 2002; Storch et al., 2002; R. Zhang, N. F. Lahens, H. I. Ballance, M. E. Hughes, & J. B. Hogenesch, 2014). The detoxification process in liver, kidney, small intestine (Gachon, Olela, Schaad, Descombes, & Schibler, 2006); cardiovascular parameters like heart rate and blood pressure (Gachon, Nagoshi, Brown, Ripperger, & Schibler, 2004); carbohydrate and lipid metabolism in liver, muscle and adipose tissues can be given as examples for peripheral clock control and for tissue specific physiological activities (Lamia, Storch, & Weitz, 2008).

SCN synchronizes peripheral clocks and produce a robust rhythm by regulating neuronal and hormonal processes such as melatonin, prolactin, cortisol, and growth

hormone release; bowel movement, core body temperature, and immune system functioning (A. J. Brown, Pendergast, & Yamazaki, 2019; Dibner, Schibler, & Albrecht, 2010; Gamble, Berry, Frank, & Young, 2014). Also, there are other synchronization mechanisms for peripheral clocks like food intake and temperature fluctuations (Figure 2.4) (S. A. Brown, Zumbrunn, Fleury-Olela, Preitner, & Schibler, 2002; Le Minh, Damiola, Tronche, Schütz, & Schibler, 2001).

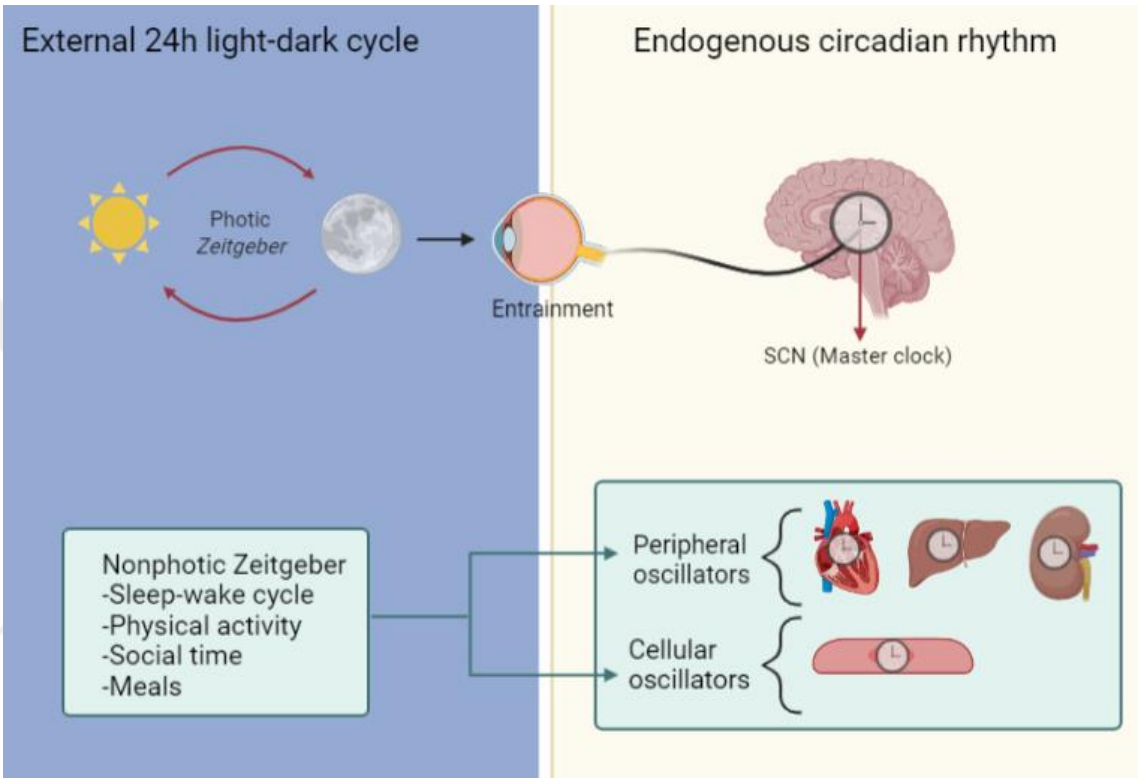


Figure 2.4 Representation of master and peripheral clocks which entrained by environmental factors
The figure is adapted from (Buttgereit, Smolen, Coogan, & Cajochen, 2015).

2.3 Molecular Components of the Mammalian Circadian Rhythm

2.3.1 Molecular Architecture of Circadian Clock Mechanism

The mammalian circadian clock mechanism is composed of interlocked transcriptional and translational feedback loops (Dunlap, 1999). In the main loop, the positive arm is composed of BMAL1 and CLOCK. NPAS2 is the paralog of CLOCK and has similar functions in clock mechanism (Hogenesch et al., 1997; Reick, Garcia, Dudley, & McKnight, 2001). They heterodimerize through bHLH-PAS (Period-Arnt-Single) domains and bind to E-box (enhancer box) elements of clock-controlled genes (Gekakis et al., 1998; Hogenesch et al., 1997). Expression of clock-controlled genes including *Per*s (*mPer1*, *mPer2*, *mPer3*) and *Cry*s (*Cry1*, *Cry2*) which are built the negative arm of the

main TTFL, are transactivated in the afternoon with this binding (Kume et al., 1999; Partch, Green, & Takahashi, 2014). In the late afternoon, PERs and CRYs accumulate in the cytoplasm and are heterodimerized. Along with the serine/threonine kinases; casein kinase 1δ (CK1δ) and casein kinase 1ε (CK1ε), PER and CRY translocate into the nucleus to repress the BMAL1/CLOCK transactivation at night and autoregulate their own transcription (Cao, Yang, Selby, Liu, & Sancar, 2021; Gekakis et al., 1998; Kume et al., 1999). For resetting the cycle CRY and PER should be degraded. SKP1–CUL1–F-box protein complex (SCF^{FBXL3}) is served as E3 ubiquitin ligase and controls the degradation of CRY (Siepka, Yoo, Park, Song, et al., 2007; S. H. Yoo et al., 2013). When CRY is phosphorylated by AMP-activated protein kinase (AMPK) the interaction of CRY with FBXL3 is enhanced which leads to the polyubiquitination and proteasomal degradation of CRY where, FBXL21, which has an amino acid sequence that is 84 percent identical to FBXL3, is described as an antagonist of FBXL3 in CRY degradation (Lamia et al., 2009; Xing et al., 2013). PER is degraded through CK1δ and CK1ε phosphorylation which leads to ubiquitination of PER by β-TrCP. The ubiquitination drives PER to proteasomal degradation (Ko & Edery, 2005).

The secondary (auxiliary) TTFL is composed of retinoid-related orphan receptors (RORα, β, and γ) and REV-ERBα, REV-ERBβ (*Nr1d1*, *Nr1d2*) which are under the control of BMAL1/CLOCK transactivation (Preitner et al., 2002). RORs bind to ROR enhancer element (RORE) and initiate the transcription of *Nfil3 and Bmal1* which cause a delay in *Cry1* expression (Akashi & Takumi, 2005; Sato et al., 2004; Ukai-Tadenuma et al., 2011). Whereas REV-ERBs counteract and repress the *Bmal1* transcription. This secondary feedback loop is crucial for proper clock timing. There is also another feedback loop that regulates the genes expressions that are under the control of D-box elements where DBP and NFIL3 activated the expression of *Rorα/Rorβ/Rorγ* and *Nr1d1*/*Nr1d2* (Ueda et al., 2005). As a result of these three feedback loops, ~24-hour periodicity is highly regulated (Figure 2.5).

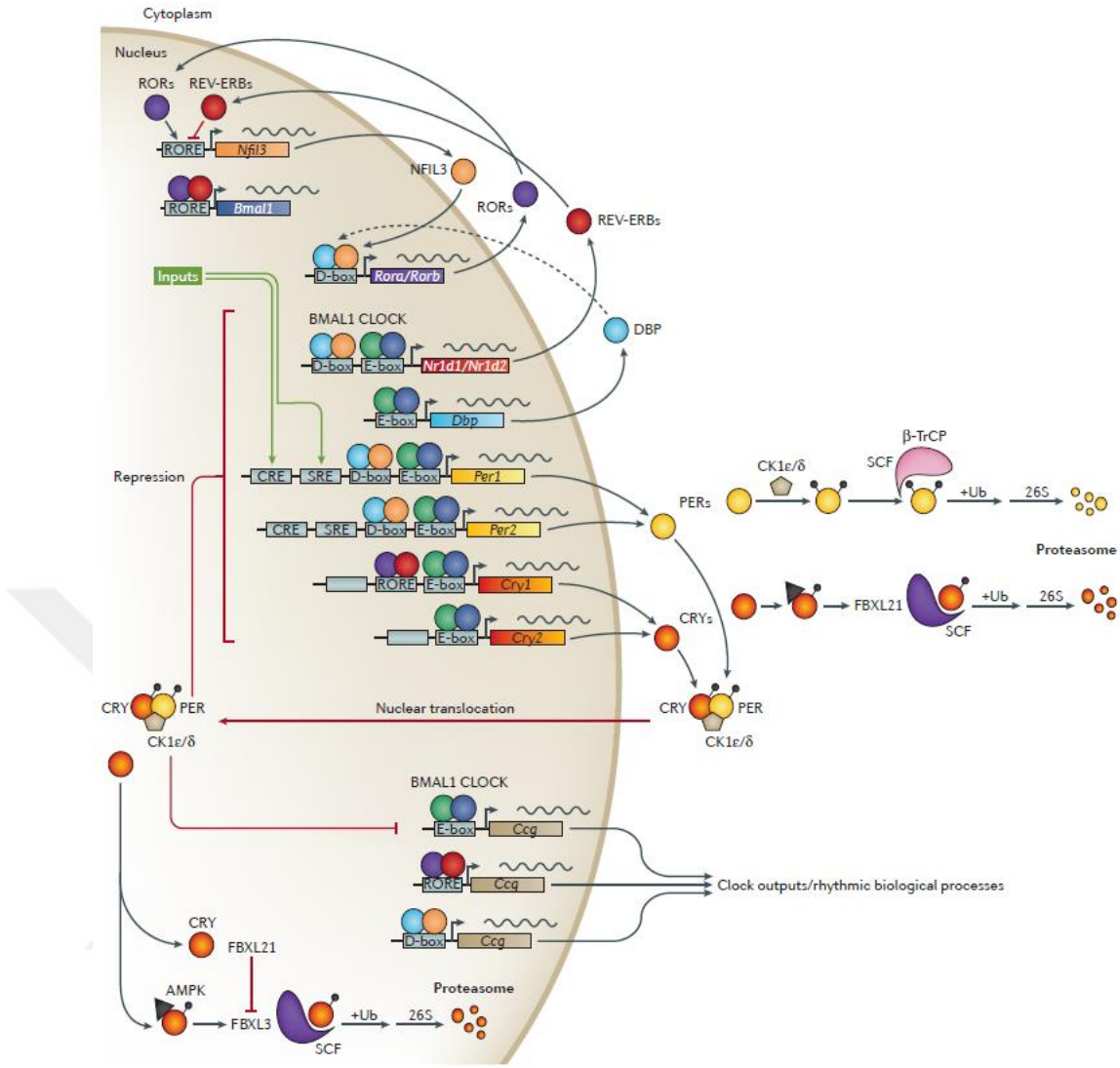


Figure 2.5 The gene network of the circadian clock mechanism in mammals is depicted schematically
Three interlocked transcriptional and translational feedback loops (TFFL) are demonstrated in detail. The figure is retrieved from (J. S. Takahashi, 2017). Permission is attached in the appendix.

2.3.2 Structure of CRY

Cryptochromes are classified in the cryptochrome/photolyase family (CPF) (Kavakli et al., 2017). Photolyases harvest blue light to repair DNA damage induced by UV irradiation (Sancar et al., 2000). Cryptochromes are proteins found in bacteria, plants, and animals and show high similarity to photolyase but do not have any repair function (Sancar, 2004). It is demonstrated that CRYs in mammals can also bind to FAD-like photolyases, but they have no DNA repair activity (Özgür & Sancar, 2003). Photolyase homology region (PHR) which is located at the N-terminus and highly variable C-terminal tail are the two distinct domains in the CRY structure. PHR domain is composed

of α-helices and β-sheets with a spherical tertiary structure that form the primary (FAD-binding) and the secondary pockets. On the other hand, C-terminal α-helix named as coiled-coil (CC) helix referring to structural similarity to coiled coils, is disordered and varies along the species (Czarna et al., 2013; Kavakli et al., 2017; Öztürk et al., 2008). A flexible interdomain linker attaches these two domains to each other (Figure 2.6).

The PHR domain is essential for maintaining the rhythmicity (Khan et al., 2012) because it has been recently discovered that the secondary pocket of CRY mediates interaction with CLOCK's PAS-B domain (Michael et al., 2017; Nangle et al., 2014). Additionally, C-terminal region of CRY is demonstrated to interact with the transactivation domain (TAD) of BMAL1 (Chaves et al., 2006; Czarna et al., 2011; Xu et al., 2015). Also, PER2 and FBXL3 interact with CRY through the coiled-coil helix in the C-terminal (Nangle et al., 2014; Ozber et al., 2010) which makes the C-terminal region is critical for the amplitude of the circadian rhythm (Gao et al., 2013).

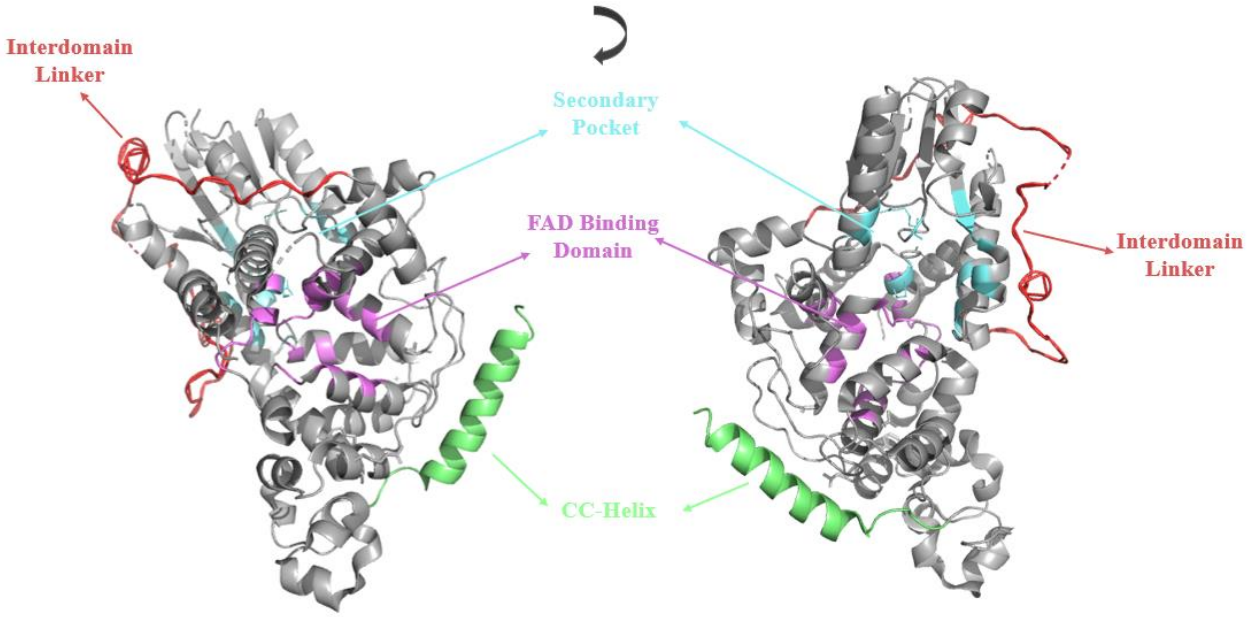


Figure 2.6 Visualization of and important functional domains on 3D Structure of mCRY2 (PDB ID: 4I6E) using PyMOL2
The mCRY2 structure is shown as a cartoon diagram. Residues forming the secondary pocket are colored as cyan, the FAD-binding domain is colored as purple, the CC-helix is colored as green and the interdomain linker is colored as red.

2.3.3 Differences Between CRY1 and CRY2

In mammals, there are two *Cryptochrome* genes *Cry1* and *Cry2*. They belong to the photolyase/cryptochrome family and serve as transcription repressors (Kavakli et al., 2017; Kume et al., 1999). There are numerous studies about the roles of CRYs in the

circadian clock and they suggest that CRY1 and CRY2 may have different roles. Behavioral studies indicate that under constant dark, *Cry1*$^{-/-}$ mice have a shorter free-running circadian rhythm, while *Cry2*$^{-/-}$ mice exhibit a longer free-running circadian rhythm and unlike all, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice display arrhythmicity (Horst et al., 1999; Thresher et al., 1998; Vitaterna et al., 1999) as shown in Figure 2.7. Besides different behavioral effects of CRYs, as transcription factors, CRY1 and CRY2 are located on DNA at different times of the day as transcription factors (Koike et al., 2012). Also, CRY1 shows a stronger repression activity than CRY2 on BMAL1/CLOCK transactivation (Khan et al., 2012; Rosensweig & Green, 2020).

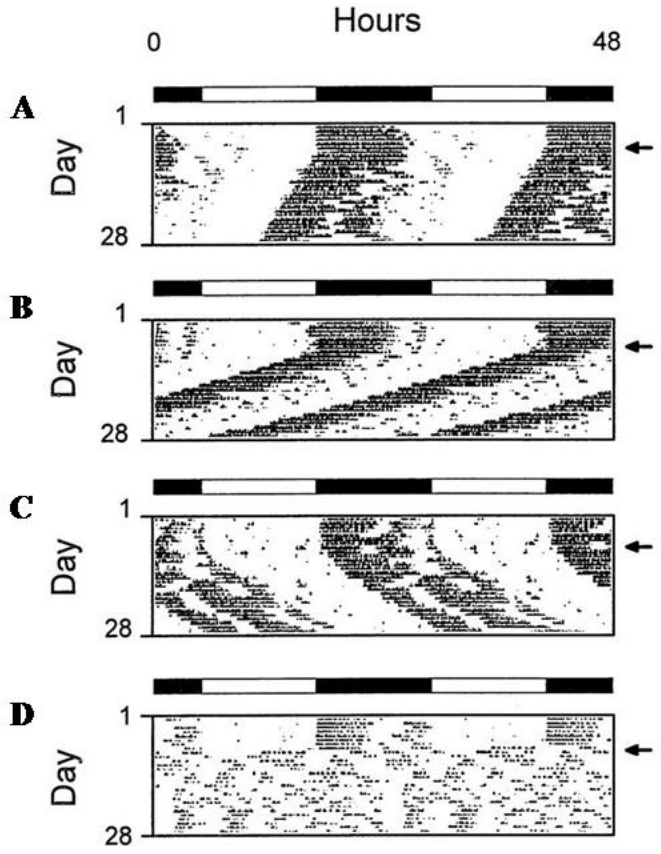


Figure 2.7 Phenotypic differences of Circadian rhythms in *Cry* knockout mice
The Actogram of the individual mouse is plotted. Black dots represent the times of activity. The bar above shows 12h light and 12h dark cycles. Arrow indicates the beginning of the constant darkness. (A) The activity of (A) wild type mouse, (B) *Cry1*$^{-/-}$ mouse, (C) *Cry2*$^{-/-}$ mouse, (D) *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse. The figure is retrieved from (Vitaterna et al., 1999). Permission is attached in the appendix.

2.4 Effects of Core Clock Gene Mutations on Human Health Physiology

As it is mentioned in the earlier sections circadian clock is involved in many different physiological processes like hormone secretion, neuronal functioning, sleep-

wake cycles and feeding behavior. As a result, any disruption in the clock is very likely to affect disease-causing pathways (Mohawk et al., 2012; Joseph S. Takahashi, Hong, Ko, & McDearmon, 2008). From past to present, malfunctioning of the clock has been correlated with sleep deprivation, impaired cognitive capabilities, diabetes, obesity, weakened immune system, accelerated aging, cardiovascular diseases and various cancer types (Krishnaiah et al., 2017; Ma et al., 2009; Marcheva et al., 2010; Zhang et al., 2010). These are only the ones we are aware of, and they are most likely only the tip of the iceberg.

Several different types of diseases are associated with mutations in the core clock genes; *PERs, CRYs, CLOCK and BMAL1*. Familial advanced sleep phase disorder (FASPD) causes early-onset and offset of sleep in those who are affected, and it occurs because of a heterozygous mutation on PER2 (Toh et al., 2001). Furthermore, mutants of CRY1 and CRY2 have been linked to FASPD and delayed sleep phase disorder (DSPD) (Hirano et al., 2016). Recent research has linked a mutation in the *CRY1* gene to family delayed sleep phase and attention deficit hyperactivity disorder (Onat et al., 2020; Patke et al., 2017). Mutations in *CLOCK* cause metabolic syndrome with obesity, higher blood glucose levels and insulin resistance (Marcheva et al., 2010; Turek et al., 2005). Type 2 diabetes and impaired fasting glucose are also linked with a *CRY2* variant (Liu et al., 2011). DEC2 mutations, which are linked to the core clock regulator (Honma et al., 2002), have a role in sleep disorders (He et al., 2009; Pellegrino et al., 2014). In addition to schizophrenia (E. W. Lamont, Coutu, Cermakian, & Boivin, 2010; Elaine Waddington Lamont, Legault-Coutu, Cermakian, & Boivin, 2007; Mansour et al., 2006; Moons et al., 2011), unipolar major depression (Soria et al., 2010) and bipolar disorder (Benedetti et al., 2008; McCarthy & Welsh, 2012) are also associated with circadian clock genes.

Cancer is another significant disease that correlates with circadian clock defects. Large-scale genomic screening of breast cancer cases by Sjöblom is revealed that the vast majority of them have *PER1* or *PER2* mutations (Sjöblom et al., 2006). To the best of my knowledge, lung, endometrial, glioma, hepatocellular carcinoma, ovarian, and pancreatic cancers have been linked to certain mutations which led to dysfunctioning of circadian clock proteins (Davis & Mirick, 2006; Viswanathan & Schernhammer, 2009; Zhu, Brown, Zhang, Stevens, & Zheng, 2005). In addition to all these physiological effects, clock dysfunction can cause psychology-related diseases. For instance, a *CRY2* mutant is associated with depression (Lavebratt et al., 2010). All these suggest that clock

disruption by SNPs on core clock genes may reveal new disease relations. In this thesis characterization of three CRY2 variants is explained.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell lines and Regular Cell Culture Procedures

In this study, human embryonic kidney 293 cells (HEK293T) and mouse embryonic fibroblast double knockout *Cry1*$^{-/-}$*/Cry2*$^{-/-}$ (DKO-MEF) cell lines were used. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. No. 11573397) and 100 μg/ml penicillin and streptomycin (P/S; Gibco, Cat. No. 15140122). The cells were grown in 37°C, humidified cell incubator supplemented with 5% CO_2.

As standard subculturing procedure, 80-90% confluent cells were washed with phosphate-buffered saline solution (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and detached with trypsin-EDTA (Wisent Multicell, Cat. No. 325-043-CL). To neutralized trypsin, 10% FBS-containing medium was used for resuspension and resuspended cells were split into new cultures with a fresh medium. To store cell for long period of time, cells were centrifuged at 400xg for 5 minutes and resuspended with an ice-cold freezing medium (50% FBS, 40% DMEM, 10% DMSO). The cells were placed in cryotubes and stored in liquid nitrogen. To harvest the cells, trypsin-treated cells were collected with PBS and centrifuged at 400xg for 5 minutes. PBS was vacuumed and the cell pellet was kept at -20°C for further studies.

3.2 Plasmid Constructs and Site-Directed Mutagenesis

The following plasmids backbone were used throughout studies: pCMV-Sport6, pFLAG-CMV, pcDNA3.1(+)/Myc-His A, pGL3, and pMU2-MCS.

Site-Directed Mutagenesis (SDM) is used for single nucleotide change which is also known as quick-change mutagenesis as described in (Ozber et al., 2010). SDM was performed on pcDNA-*Cry2*-Myc-His for 15 different *Cry2* variants and on pMU2-P(*CRY1*)-(intron 336)-*Cry2* for 3 variants using the primers in Table 3.1. The presence of

the mutation for each variant was confirmed by Sanger sequencing (Macrogen, Netherland)

Table 3.1 The primer sequences for site-directed mutagenesis

mCRY2_R28H-F	GGTGCACTGGTTCCACAAAGGACTACGG
mCRY2_28H-R	CCGTAGTCCTTTGTGGAACCAGTGCACC
mCRY2_P122L-F	GAATATGACTCTGAACTCTTTGGGAAAGAACGGG
mCRY2_P122L-R	CCCGTTCTTTCCCAAAGAGTTCAGAGTCATATTC
mCRY2_G161W-F	CAGAATCATCGAACTGAATTGGCAGAAACCACCCCTTACC
mCRY2_G161W-R	GGTAAGGGGTGGTTTCTGCCAATTCAGTTCGATGATTCTG
mCRY2_R274C-F	CTCAGCCCCTACCTGTGCTTTGGATGCCTC
mCRY2_R274C-R	GAGGCATCCAAAGCACAGGTAGGGGCTGAG
mCRY2_C277Y-F	CGCTTTGGATACCTCTCCTGCC
mCRY2_C277Y-R	GGCAGGAGAGGTATCCAAAGCG
mCRY2_S279T-F	CTTTGGATGCCTCACCTGCCGCCTCTTC
mCRY2_S279T-R	GAAGAGGCGGCAGGTGAGGCATCCAAAG
mCRY2_S279C-F	GCTTTGGATGCCTCTGCTGCCGCCTCTTCTAC
mCRY2_S279C-R	GTAGAAGAGGCGGCAGCAGAGGCATCCAAAGC
mCRY2_L282P-F	CTCTCCTGCCGCCCCTTCTACTACCG
mCRY2_L282P-R	CGGTAGTAGAAGGGGCGGCAGGAGAG
mCRY2_W357C-F	CAGGCTTCCCTTGCATTGACGCCATC
mCRY2_W357C-R	GATGGCGTCAATGCAAGGGAAGCCTG
mCRY2_G369V-F	CTGAGGCAGGAGGCCTGGATCCACCACC
mCRY2_G369V-R	GGTGGTGGATCCAGGCCTCCTGCCTCAG
mCRY2_V379M-F	CTGGCCCGGCACGCTATGGCCTGCTTCCTC
mCRY2_V379M-R	GAGGAAGCAGGCCATAGCGTGCCGGGCCAG
mCRY2_V379G-F	CCGGCACGCTGGGGCCTGCTTC
mCRY2_V379G-R	GAAGCAGGCCCCAGCGTGCCGG
mCRY2_D405H-F	GAGCTGCTCCTGCATGCCGATTTCAG
mCRY2_D405H-R	CTGAAATCGGCATGCAGGAGCAGCTC
mCRY2_S409I-F	GGATGCCGATTTCATTGTGAATGCAGGCAG
mCRY2_S409I-R	CTGCCTGCATTCACAATGAAATCGGCATCC
mCRY2_S419F-F	CTGGATGTGGCTGTTCTGCAGTGCTTTC
mCRY2_S419F-R	GAAAGCACTGCAGAACAGCCACATCCAG
mCRY2_R439H-F	GGCTTCGGCCGACATACAGACCCCAGTG
mCRY2_R439H-R	CACTGGGGTCTGTATGTCGGCCGAAGCC

Reaction for each SDM consists of 1X HF Buffer, 200 μM dNTP mix, 0.3 μM forward and reverse primers, 10 U Phusion Hot Start Flex DNA Polymerase (NEB, Cat. No. M0535S) and 50 ng template DNA. Details of the PCR cycling protocol is described in Table 3.2. After PCR amplification, the presence of the products were confirmed by 0.8% agarose gel. Then, to eliminate the parental plasmid used as template 1 U of FastDigest DpnI (Thermo Scientific, Cat. No. FD1703) was added directly to the PCR

product and reactions were incubated at 37°C for 1 hour. Then 7 µl of the sample was transformed to TOP10 *E. coli* by heat shock. Cells were plated onto LB agar containing appropriate antibiotics and incubated at 37°C for 16 hours. Then colonies were picked and transferred in 2 mL of LB with proper antibiotics and incubated at 37°C for 16 hours. Next, the plasmid was purified with Nucleospin Plasmid kit (Macherey Nagel, Cat. No. 740588.50) using manufacturer instruction.

Table 3.2 Detailed PCR cycling protocol for site-directed mutagenesis

Step			
1	Initial Denaturation	98°C	4 minutes
2	Denaturation	98°C	30 seconds
3	Annealing	62°C	30 seconds
4	Elongation	72°C	10 minutes
5	GO TO STEP2	29X(cycle)	
6	Final Elongation	72°C	5 minutes

3.3 Per1-Luc Reporter Gene Assay

The repressor activity of the CRY2 variants was measure as described in (Cal-Kayitmazbatir et al., 2021). In this assay pGL3-*mPer1*:d*Luc* marker plasmid was used, where m*Per1* promoter was fused with destabilized firefly luciferase. HEK 293T cells ($4x10^4$) were transfected with PEI (Polysciences, Cat. No. 23966-1) transfection reagent with the following amount of plasmids in a 96-well by reverse transfection: 25 ng of pCMV-Sport6-*CLOCK,* 50 ng of pCMV-Sport6-*Bmal1*, 50 ng of *mPer1*:d*Luc*, 20 ng of pFLAG-CMV-*PER2,* and dose dependent manner of wild type and *Cry2* variants plasmids. Also, pcDNA-empty was used for the equalized the transfected plasmid concentration. The plasmid mixture in DMEM was incubated with PEI for 20 minutes and mixed with the appropriate number of cells. Then, cells were plated to a 96-well opaque white plate. After 24-hour incubation in humidified cell incubator supplemented with 5% CO_2, cells were lysed with Firefly luciferase buffer (Table 3.3.). Firefly luciferase activity was measured with Fluoroskan Ascent FL microplate reader. Measurements are normalized with Renilla luciferase expression with the same protocol but using Renilla Buffer (Table 3.3.).

Table 3.3 Compositions of Firefly and Renilla Luciferase Buffer

Firefly Luciferase Buffer			
Triton Lysis Buffer			
Tris-HCl	0.1082 M	DTT	15 mM
Tris-base	0.0419 M	Coenzyme A	0.6 mM
NaCl	75 mM	ATP	0.45 mM
$MgCl_2$	3 mM	Luciferin	4.2 mg/ml
Triton X-100	0.25%	*prepared in Triton Lysis Buffer*	
prepared in ddH_2O			
Renilla Luciferase Buffer			
Renilla Salts Buffer			
Na_2EDTA	45 mM	PTC124 (in DMSO)	0.06 mM
Na4P2O7	30 mM	h-CTZ (in ethanol)	0.01 mM
NaCl	1.425 M	*prepared in Renilla Salts buffer*	
prepared in ddH_2O			

3.4 Protein Extraction and Western Blotting

3.4.1 Extraction of Proteins from Cells

For protein extraction, cells were harvested by centrifugation and resuspended in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Triton-X, 1% protease inhibitor cocktail (PIC; Cat. No. 78430)). After 15 minutes of incubation on ice, samples were vortexed for 5 seconds and left on ice for another 15 minutes. Lysed cells were centrifugated at 15.000g for 20 minutes at +4°C and supernatants were transferred to a new Eppendorf tube. To measure total protein concentration, Pierce assay (Thermo Fisher Scientific, Cat. No. 22660) was used according to the manufacturer manual. Measurements were taken by Epoch microplate reader at 660 nm. Afterward, 4X Laemmli Buffer (250 mM Tris-HCl at pH:6.8, 40% Glycerol, 4% SDS, 0.02% Bromophenol Blue and 10% β-mercaptoethanol, which is freshly added) was used as sample buffer for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After adding 4X Laemmli buffer, samples were boiled at 95°C for 7 minutes. Samples were used both directly and were stored at -20°C for further use.

3.4.2 Western Blotting

Total protein samples were subjected to 10% SDS-PAGE gel consists of stacking and resolving gels. For stacking, 5% gel was used, while for separating 10% gel was used (Table 3.4.). Proteins were separated in PAGE at 90V for 20 minutes, then the voltage was increased to 120V. When loading the dye run at the end of the gel, proteins were

transferred to polyvinylidene fluoride membrane (PVDF, Millipore, Cat. No. IEVH00005) by wet Western blot transfer. A sandwich was prepared in the order of sponge, Whatman paper, gel, PDVF, Whatman paper, and sponge. The sandwich located in the tank, where the gel was facing to the negative electrode, was filled with Western Blot transfer buffer (3.04 g Tris base, 14.4 g Glycine, 200 ml Methanol, ddH_2O up to 1 L). The transfer was performed for 1 hour at 100 V.

Table 3.4 SDS-PAGE Stacking and Separating gel recipe

Percentage	**Solution Compositions**	
10% Separating gel (for 5 ml)	dH_2O	1.9ml
	30 % acrylamide mix	1.7ml
	1.5 M Tris (pH=8.8)	1.3ml
	10% SDS	0.05ml
	10% ammonium persulfate	0.05ml
	TEMED	0.002ml
5% Stacking gel (for 1 ml)	dH_2O	0.68ml
	30 % acrylamide mix	0.17ml
	1.5 M Tris (pH=6.8)	0.13ml
	10% SDS	0.01ml
	10% ammonium persulfate	0.01ml
	TEMED	0.001ml

After the completion of the transfer protein to membrane, it was washed with TBS-T (Tris-buffered saline with 0.1% Tween-20 (Thermo Fischer Scientific, Cat. No. 003005) for 1 minute and was blocked with 5% non-fat milk solution prepared in TBS-T for 1 hour. After the blocking, the PDVF membrane was incubated overnight at +4°C or for 2 hours at room temperature on a rotator shaker with the appropriate primary antibodies; Anti-FLAG (Sigma Aldrich, Cat. No. SAB4200071), Anti-Myc (Abcam, Cat. No. ab18185), Anti-β-actin (Cell Signalling, Cat. No.4970). All the antibodies were prepared in a 5% non-fat milk solution with proper dilution as suggested by manufacture. Membranes were washed with TBS-T solution 3 times for 5 minutes with continuous shaking at room temperature. The washed membrane was incubated in secondary antibody conjugated with Horseradish Peroxidase (HRP) (Anti-Mouse: Santa Cruz Biotechnology, Cat. No. 516102; Anti-Rabbit: Cell Signalling, Cat. No. 7074) for 1 hour at room temperature. Before imaging, the membrane was again washed with TBS-T, 3 times for 5 minutes. For the imaging process, the homemade ECL buffer (Table 3.5) was

prepared freshly, and the membrane was incubated in it for 3-4 minutes. Since the homemade ECL buffer is sensitive to light, preparation and incubation were performed in dark. Bioluminescence imaging was performed with BioRad ChemiDoc Touch.

Table 3.5 Homemade ECL recipe

Solution Compositions	
250 mM Luminol	50 µl
90 mM p-Coumaric acid	25 µl
1.5 M Tris (pH=8.8)	700 µl
30 % H_2O_2	3 µl
ddH_2O	up to 10 ml

3.5 Protein Complex Immunoprecipitation (Co-IP)

A 6-well plate was seeded with $3.2x10^5$ HEK293T cells per well 24 hours before the transfection. Plasmid mixtures were prepared in DMEM with pcDNA4/myc-His-A-*Cry2* (wild type or variants, 500 ng), pFLAG-CMV-*Per2* (350 ng), pFLAG-CMV-*CLOCK* (150 ng), and pFLAG-CMV-*Bmal1* (10000 ng). Plasmids contain *Bmal1*, *CLOCK* and *Per2* cDNAs were transfected with empty pCMV-Sport6 instead as a negative control. 6 µl PEI was used as the transfection reagent for 2000 ng total plasmid. PEI and plasmid mixtures were incubated for 20 minutes and were transfected to cells. Transfected cells were incubated in humidified cell incubator supplemented with 5% CO_2 for 24 hours. The next day, cells are harvested as it was explained in section 3.4.1.

After harvesting the cells with centrifugation, cell pellets were resuspended in 300 µl passive lysis buffer (PLB; 15 mM HEPES, 5 mM NaF, 300 mM NaCl, 1% NP-40, 1% Protease inhibitor cocktail) and were incubated on ice for 15 minutes. After that, the samples were vortexed for 5 seconds and incubated on ice for another 15 minutes. The lysates were centrifuged for 20 minutes at +4°C at 15.000 x g, and the supernatants were transferred to a fresh Eppendorf tube. EZview Red Anti-c-Myc Affinity Gel beads (Cat. No. E6654) were equilibrated while the lysates were centrifuged.

15 µl resin per sample was added to the Eppendorf tube for equilibration. 225 µl PLB was added to the beads, which were then spun vertically at +4°C for 30-45 seconds before being centrifuged for 30 seconds at 8.000 x g at +4°C. The supernatants were

carefully removed, and the process was repeated. Supernatants from the cell lysates were collected after equilibration.

Samples were centrifugated for 30 seconds at 8.200 x g. Supernatants were removed and 50 µl of it was kept as flowthrough. Beads were washed by adding 225 µl of PLB without PIC by rotating at +4°C for 5 min. The washing steps were repeated two more times. After removing the final wash supernatant, 30 µl Laemmli buffer was added to the samples and was boiled for 10 minutes at 95°C. Finally, samples were subjected to SDS-PAGE followed by Western blotting (see section 3.4.2).

3.6 Protein Degradation Assay

A day before the transfection, $2.25x10^5$ HEK293T cells/well were seeded to a 12-well tissue culture plate. The pcDNA4/myc-His-A-*Cry2* (wild type (WT) or variants, 500 ng) and pFLAG-CMV-*PER2* (500 ng) plasmids were used for the transfection. For each *Cry2*-WT and CRY2 variants, 4 wells were seeded and were transfected to collect in time dependent manner. For 1000 ng of plasmid DNA, 3 µl of PEI was used. DMEM, plasmids, and PEI were mixed and were incubated for 20 minutes. The mixture was added to the attached cells drop by drop after the incubation.

One day later, cells were treated with cycloheximide (CHX; Sigma, Cat. No. 66819) with a final concentration of 20 µg/ml. CHX is a chemical that ceases protein expression by interfering with translational elongation in eukaryotes. Cells were harvested at 4-hour intervals up to the 12^{th} hour. Cells that were collected right after CHX treatment were marked as t=0 h. Protein amounts were detected by western blotting using anti-myc and anti-β-actin. The detailed protocol was explained in section 3.4.

3.7 Cell-based Circadian Assay

The mouse embryonic fibroblast *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ (DKO-MEF) cell lines (kindly provided by Prof. Ueda, RIKEN, Japan) were used for real-time circadian rhythm monitoring to assess the effect of the CRY2 variants on circadian rhythm. To this end, $3.2x10^5$ cells were seeded into 35 mm culture dishes a day before the transfection. pGL3-*Per2*-d*Luc* (luciferase reporter, 4000 ng) and pMU2-P(*CRY1*)-(intron 336)-*Cry2* (WT or variants, 150 ng) were transfected by using FuGENE6 transfection reagent (Promega, Cat. No. E2691). 12.45 µl FuGENE6 was used for 4.15 µg plasmid DNA. First DMEM and FuGENE6 were mixed and were incubated for 5 minutes. Then, the plasmid DNA

was added to the mixture and was incubated for 25 minutes. DMEM:FuGENE6:DNA mixture was added dropwise onto the cells and then incubated in CO_2 incubator at 37 ºC for 72 hours.

Then, the medium of cells was replaced with DMEM containing dexamethasone (DXM; Sigma Aldrich, Cat. No. D4902) with a final concentration of 0.1 μM and incubated for 2 hours for the synchronization of the circadian clock of cells. After that, the medium was vacuumed and was replaced with lumicycle medium (Table 3.6). The plates were sealed with silicon grease and were placed into LumiCycle 32 Luminometer (Actimetrics). This device provides the required environment for cell growth. Bioluminescence recordings were taken with a photomultiplier for 70 seconds every 10 minutes for 7 days.

Table 3.6 Lumicycle medium composition

Medium Composition	
DMEM powder	10g
$NaHCO_3$	0.35g
D-(+)-glucose powder	3.5g
1M HEPES buffer	10ml
100 μg/ml Pen/strep	2.5ml
5% FBS	50ml
Non-essential aminoacids	1% final
ddH_2O	Up to 1L

CHAPTER 4

RESULTS

The database of 1000 Genomes Project (Genomes Project et al., 2015) together with the Ensembl database (Zerbino et al., 2018) are used to identify *CRY2* variants (Table 4.1). According to the American College of Medical Genetics and Genomics (ACMG), variants are classified as "variant of uncertain significance-VUS" (Richards et al., 2015). However, in silico analysis indicated that these variants may have structural hence functional effects (PM2, PP3) on CRY2. Heterogeneities of SNPs are also indicated based on the gnomAD database in the table and only two of the variants detected at gnomAD. None of the variants are reported in the ClinVar database. Although the selected variants are not related to any disease, all variants are interpreted as "disease-causing" according to Mutation Taster (Schwarz, Cooper, Schuelke, & Seelow, 2014). These findings indicate that they exist a potential for disease development due to structural thus functional changes in the protein. The variants are selected for further experimental studies to investigate their role in the circadian clock mechanism **(Table 4.1)**.

Table 4.1 The clinical features of CRY2 SNPs

Position in CDS(NM_021117.4)	Position in protein(NM_021117.4)	ACMG	gnomAD	ClinVar
c.86G>A	p.Arg29His	VUS (PM2, PP3)	not detected	—
c.368C>T	p.Pro123Leu	VUS (PM2, PP3)	not detected	—
c.484G>T	p.Gly162Trp	VUS (PM2, PP3)	not detected	—
c.823C>T	p.Arg275Cys	VUS (PM2, PP3)	not detected	—
c.833G>A	p.Cys278Tyr	VUS (PM2, PP3)	not detected	—
c.838T>A	p.Ser280Thr	VUS (PM2, PP3)	not detected	—
c.839C>G	p.Ser280Cys	VUS (PM2, PP3)	not detected	—
c.848T>C	p.Leu283Pro	VUS (PM2, PP3)	not detected	—
c.1074G>C	p.Trp358Cys	VUS (PM2, PP3)	not detected	—
c.1138G>A	p.Val380Met	VUS (PM2, PP3)	f = 0.000003991	—
c.1139T>G	p.Val380Gly	VUS (PM2, PP3)	Not detected	—
c.1216G>C	p.Asp406His	VUS (PM2, PP3)	not detected	—
c.1229G>T	p.Ser410Ile	VUS (PM2, PP3)	not detected	—
c.1259C>T	p.Ser420Phe	VUS (PM2, PP3)	not detected	—
c.1319G>A	p.Arg440His	VUS (PM2, PP3)	f = 0.000003991	—

4.1 The structure and functional analyses of the CRY2 variants

After generating the CRY2 variants by site directed mutagenesis, the repressor capacity of each CRY2 variants was assessed on BMAL1/CLOCK-driven transcription using *Per1*-d*Luc* assay. The raw luminometric data are given in Table F1. Three variants (p.Gly162Trp, p.R275Cys, and p.Arg440His) had comparable repressor to wild type (WT) CRY2 while the following CRY2 variants had reduced activity: p.Arg29His, Cys278Tyr, p.Ser280Thr, p.Ser280Cys, p.Leu283Pro, p.Trp358Cys, p.Val380Met, p.Val380Gly, p.Asp406His, p.Ser410Ile, p.Ser420Phe, p.Pro123Leu (**Figure 4.1**).

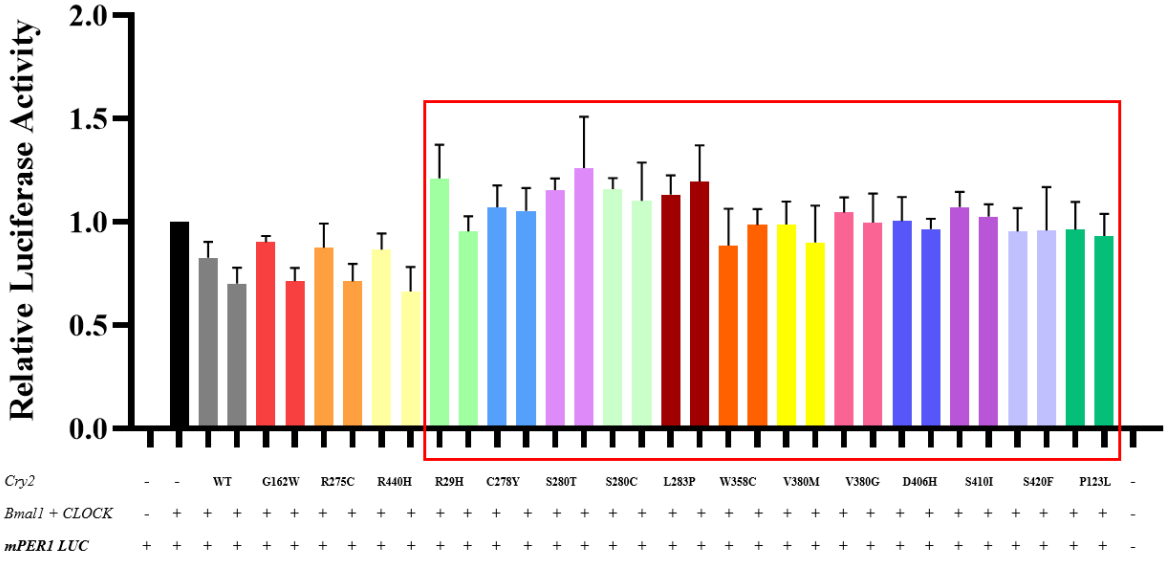


Figure 4.1 The repressor activity of wild type CRY2 and fifteen CRY2 variants
Variants; p.Arg29His, Cys278Tyr, p.Ser280Thr, p.Ser280Cys, p.Leu283Pro, p.Trp358Cys, p.Val380Met, p.Val380Gly, p.Asp406His, p.Ser410Ile, p.Ser420Phe and p.Pro123Leu could not repress the BMAL1:CLOCK mediated transcription while p.Gly162Trp, p.R275Cys, and p.Arg440His were comparable repressor compared to wild type (WT) CRY2. HEK293T cells were transfected with indicated plasmids. 0.25 ng and 0.5 ng of plasmids were used sequentially for each *Cry2*. (Data represent the mean ± SEM, n≥3 biological replicates).

The locations of these variants on the 3D structure of CRY2 were mapped using PyMOL2 to see whether they are located on the functionally important regions of the CRY2. CRY2 has two important domains called PHR domain consisting of primary (FAD binding) and secondary pockets and extended C-terminal region (Kavakli et al., 2017). Several studies indicate that these domains are essential for circadian rhythm generation. For example, FAD binding domain is required for the interaction with a ubiquitin E3 ligase while the secondary pocket is required for the CLOCK binding

(**Figure 4.2A**) (Rosensweig et al., 2018). Additionally, the C-terminal domain is required for the interactions with BMAL1 and PER2 (Czarna et al., 2011; Ozber et al., 2010).

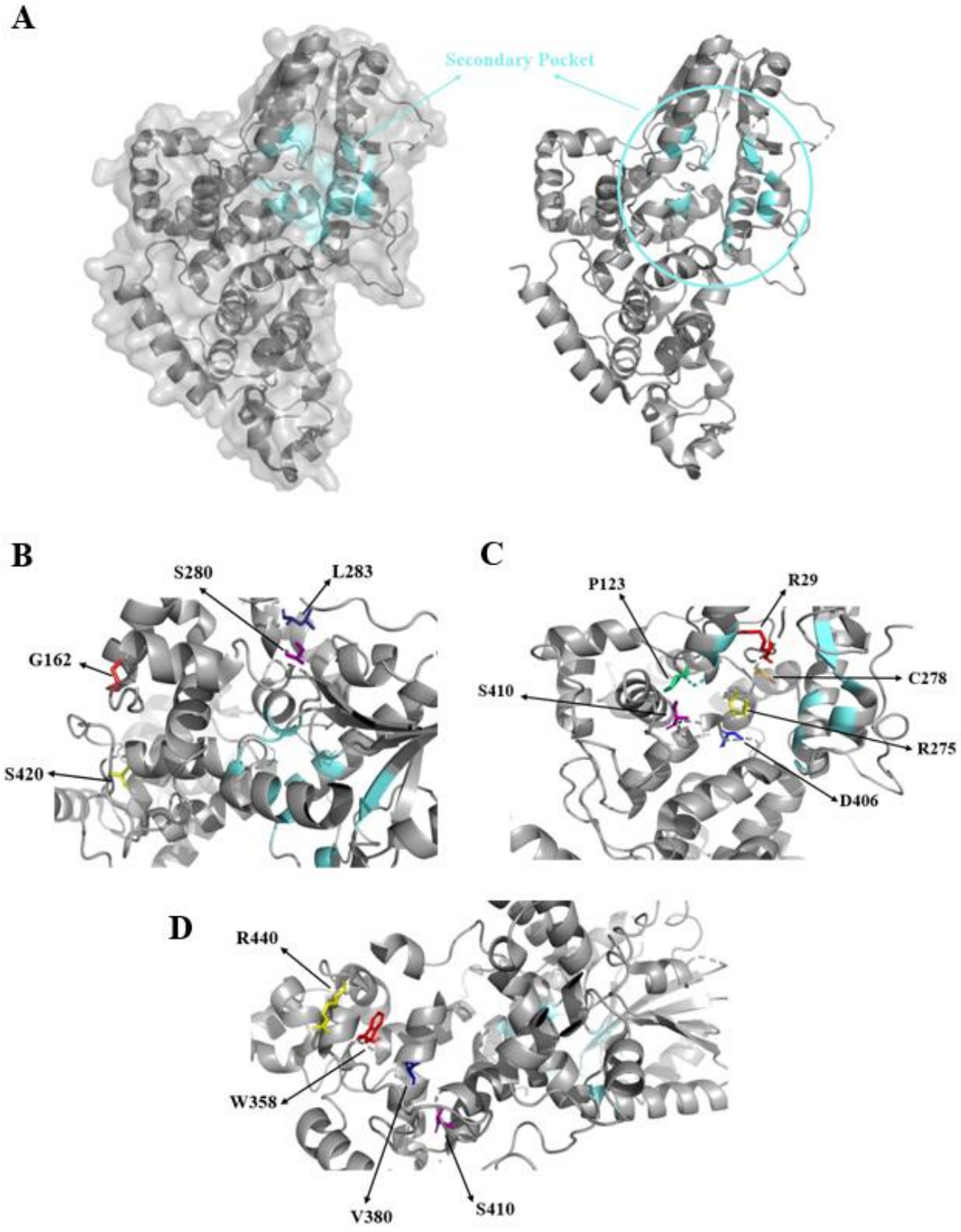


Figure 4.2 Visualization of SNP positions on the mCRY2 (PDB ID: 4I6E) structure using PyMOL2
(A) The mCRY2 structure is shown as ribbon diagram(right) or surface(left). Surface(left) and ribbon(right) representation of CRY2 residues in secondary pocket are colored as cyan. The mutant residues are shown as stick, **(B)** G162(red), S280(purple), L283(blue), S420(yellow); **(C)** R29(red), P123(green), R275(yellow), C278(orange), D406(blue), S410(purple); **(D)** W358(red), V380(blue) and R440(yellow).

As shown in **Figure 4.2**, residues Pro-123, Asp-406, and Ser-410 are located at the gate of the secondary pocket; Arg-29 and Arg-275 are inside the secondary pocket; Cys-278, Ser-280, and Leu-283 are at the back of the secondary pocket; and Trp-358 and Val-380 are close to the C-terminal lid. The residue Ser-420 extends towards the C-terminal coiled-coil-like helix (CC Helix) while the residue Arg-440 resides near the FBLX3 interface, and it is solvent-exposed. The exact locations of these residues are as follows; α4 (Arg-29), α11 (Arg-275), between α11 and α12 (Cys-278), α12 (Ser-280 and Leu-283), α15 (Trp-358), α16 (Val-380), between α17 and α18 (Asp-406), α18 (Ser-410 and Ser-420), and α19 (Arg-440) (Xing et al., 2013).

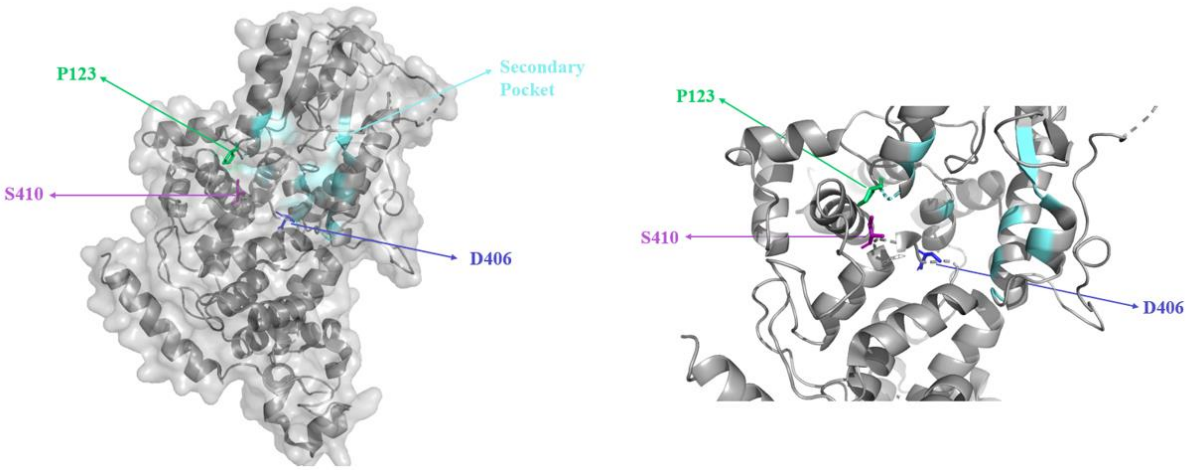


Figure 4.3 Visualization of SNP positions on the mCRY2 (PDB ID: 4I6E) structure using PyMOL2
The mCRY2 structure is shown as ribbon diagram(right) or surface(left), the mutant residues are shown as a stick; P123(green), D406(blue), S410(purple). Surface(left) and ribbon(right) representations of CRY2 residues that generate the secondary pocket are colored cyan.

Among these, p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants (**Figure 4.3**) located on the gate of the secondary pocket of the CRY2. To assess the degree of conservation of these three residues, the multiple sequence alignment was performed using the primary amino acid sequences of CRY2 from different organisms. As shown in **Figure 4.4**, Pro-123, Asp-406, and Ser-410 are conserved across the

species. Such high conservation suggests a potential role in the protein function. Therefore, three *CRY2* variants were selected for further characterization in this thesis to investigate their roles in CLOCK binding and circadian periodicity.

Figure 4.4 Multiple sequence alignment of selected Cryptochrome2 proteins
Human CRY2 is highlighted in gray and residues Pro-123(green), Asp-406(blue), and Ser-410(purple). The protein accession number of organisms are as follows; *Cyprinus carpio* (XP_018938637.1), *Danio rerio* (NP_571861.2), *Drosophila melanogaster* (AAF55649.1), *Ephemera danica* (KAF4523712.1), *Homo sapiens* (AAH41814.1), *Mus musculus* (AAD46561.1), *Rattus norvegicus* (NP_596896.2), *Xenopus laevis* (NP_001082139.1).

4.2 The repression activity of CRY2 variants with/without PER2 overexpression

The repression activity of the *CRY2* variants (located at the gate of the secondary pocket) on BMAL1/CLOCK-driven transcription was investigated in a dose-dependent manner using the *Per1*-d*Luc* assay. The raw experimental results are given in Table F2 and are shown in **Figure 4.5**. The analysis of the results indicates that none of the CRY2 variants are able to repress BMAL1/CLOCK transactivation with exception p.Pro123Leu CRY2 at dose of 2 ng, 20 ng and 50 ng compared to wild type CRY2, where it repressor activity is highly reduced. To eliminate the expression problem, the protein extracts obtained from cells that express these variants were subjected to the Western blot analysis. Results revealed that three variants had comparable expression levels compared to wild type CRY2 **(Figure 4.6)**. These results suggest that p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants may have a loss of function characteristics.

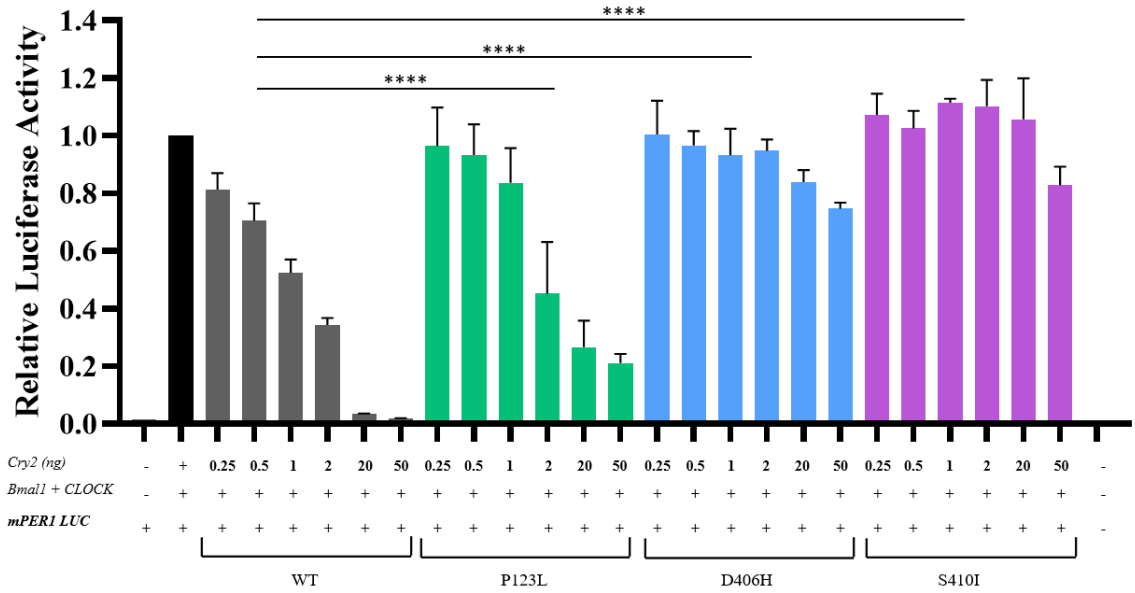


Figure 4.5 Dose-dependent repressor activity of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the absence of PER2
Wild type (WT) CRY2 was used as a positive control. HEK293T cells were transfected with indicated plasmids. 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng, and 50 ng of plasmids were used sequentially for each *Cry2*. (Data represent the mean ± SD, n≥3 biological replicates with triplicate samples). Significance analysis was performed using two-way ANOVA (****, $p<0.0001$). Comparisons were made between wild type CRY2 and each mutant separately.

On the other hand, as it is known, the transcription-translation negative feedback loop of the mammalian circadian clock is composed of two distinct protein groups, CRYs and PERs. PER has two functions on CRY. First, it localizes CRY into the nucleus (Schmutz, Ripperger, Baeriswyl-Aebischer, & Albrecht, 2010) and by competing with FBXL3, it increases the stability of CRY (S. H. Yoo et al., 2013). Considering these effects, it was decided to use PER2 for repression assay. The experimental results with PER2 overexpression are listed in Table F3 and are shown in **Figure 4.7**.

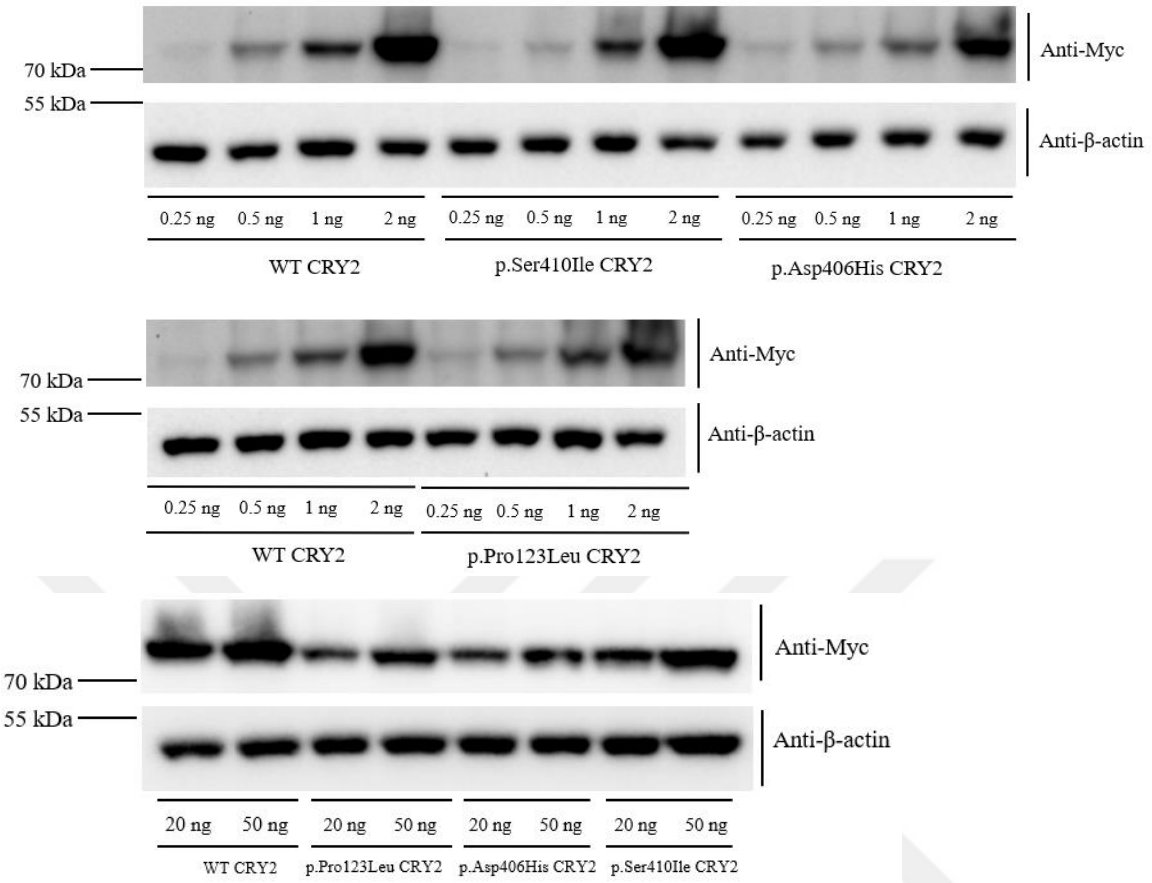


Figure 4.6 Representative Western blot images of dose-dependent expression of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the absence of PER2

HEK293T cells were transfected with 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng, and 50 ng of wild type (WT) *Cry2* and variants. The wild type CRY2 and variants were detected with anti-Myc. β-actin was used as a loading control. Since 20 ng and 50 ng of *Cry2* expressions mask lower doses, they were represented in separate membranes.

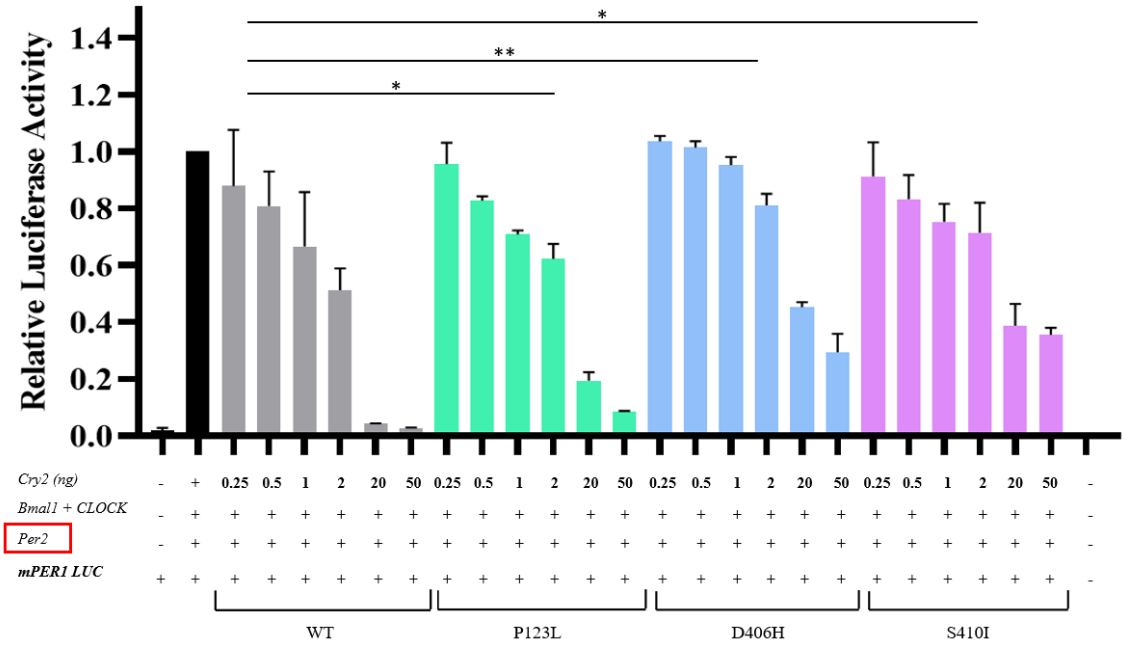


Figure 4.7 Dose-dependent repressor activity of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the presence of PER2
Wild type (WT) CRY2 was used as a positive control. HEK293T cells were transfected with indicated plasmids. 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng, and 50 ng of plasmids were used sequentially for each *Cry2*. (Data represent the mean ± SD, n≥3 biological replicates with triplicate samples). Significance analysis was performed using two-way ANOVA (*, $p<0.05$). **, $p<0.005$). Comparisons were made between wild type CRY2 and each mutant separately.

The results show that the CRY2 variants in the presence of PER2 exhibit some repression activity on the BMAL1/CLOCK transactivation. Although the rescued activity by PER2 is to a certain degree, the CRY2 variants had severely altered repressor activity on BMAL1/CLOCK. In detail, the average of dose differences between the wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants indicate about 8%, 27%, and 17% less repression, respectively. Note that the p.Pro123Leu CRY2 is more effective than the others. In order to eliminate the expression problem of the CRY2 variants, Western blot was performed on samples and the results indicate that protein levels of CRY2 variants and wild type were comparable **(Figure 4.8).** These results suggest that PER2 might increase stability of the CRY2 variants, therefore, result

in partial rescue of their repressor activities. Their stability measurements are presented in the following section.

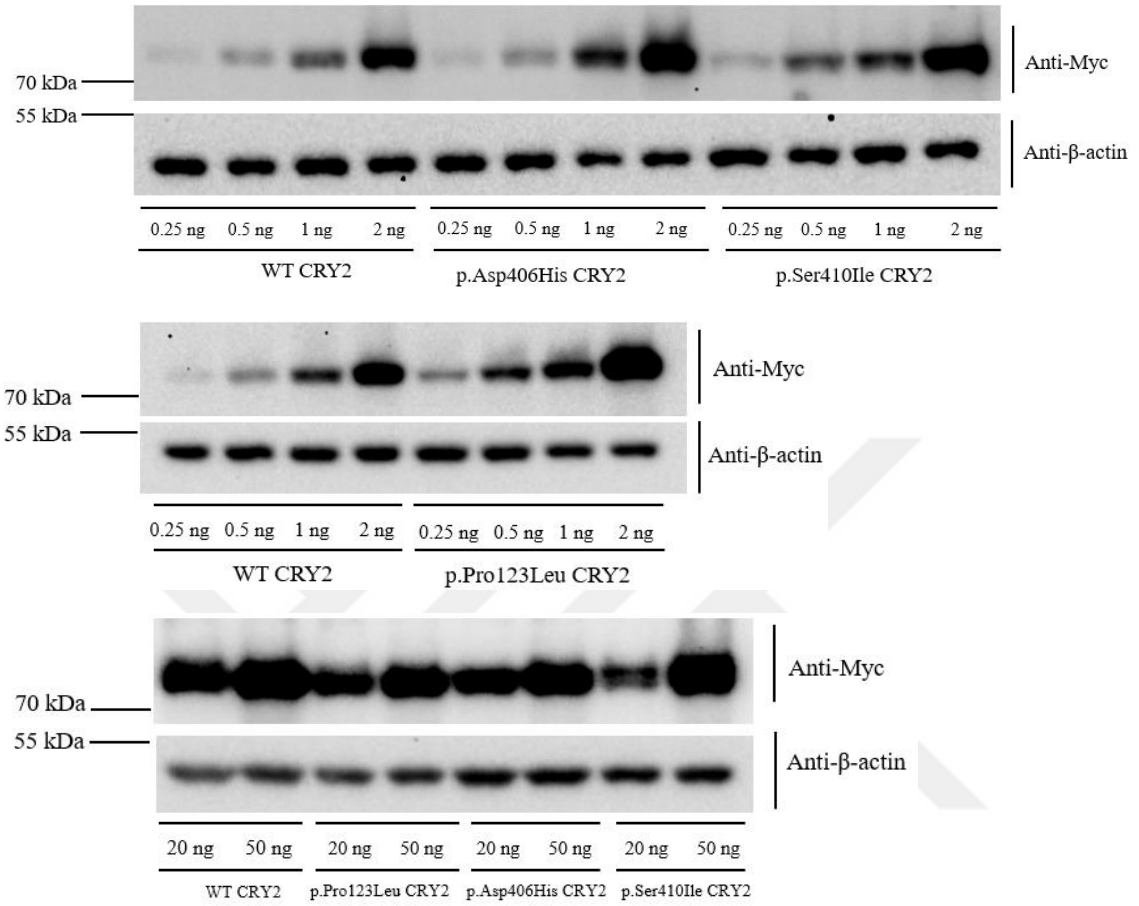


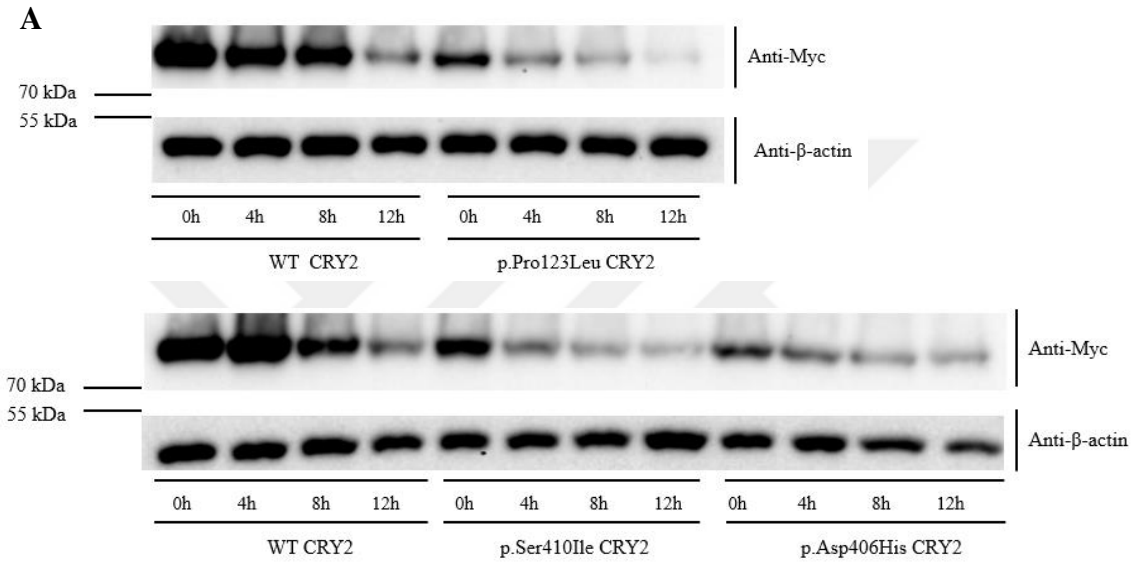
Figure 4.8 Representative Western blot images of dose-dependent expression of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the presence of PER2

HEK293T cells were transfected with 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng, and 50 ng of wild type (WT) *Cry2* and variants and 20 ng of *Per2*. The wild type CRY2 and variants were detected with anti-Myc. β-actin was used as a loading control. Since 20 ng and 50 ng of *Cry2* expressions mask lower doses, they were represented in separate membranes.

4.3 The stability of CRY2 variants with/without PER2 overexpression

The stability of the CRY2 variations was investigated in a system with and without PER2 overexpression. The cycloheximide chase test was used for this investigation because it is a well-known and widely used assay for measuring protein degradation in the chronobiology field (Eide et al., 2005; Kao et al., 2015; Siepka, Yoo, Park, Lee, & Takahashi, 2007). Details of the experiment were explained in the methodology of the experimental procedure Section 3.6.

The raw data of CRY2 variants degradation assay are given in Table F4. The representative images from the experiments are shown in **Figure 4.9A**. The p.Pro123Leu CRY2, p.Ser210Ile CRY2, and p.Asp406His CRY2 had reduced half-lives in comparison to wild type CRY2 (**Figure 4.9B**). The $t_{1/2}$ of wild type CRY2 was 7.14 hours, while the t1/2 of p.Pro123Leu CRY2, p.Ser410Ile CRY2, and p.Asp406His were 3.83 hours, 1.95 hours, and 2.13 hours, respectively. The one phase exponential decay function from Figure E1 in the appendices was used in the calculations.



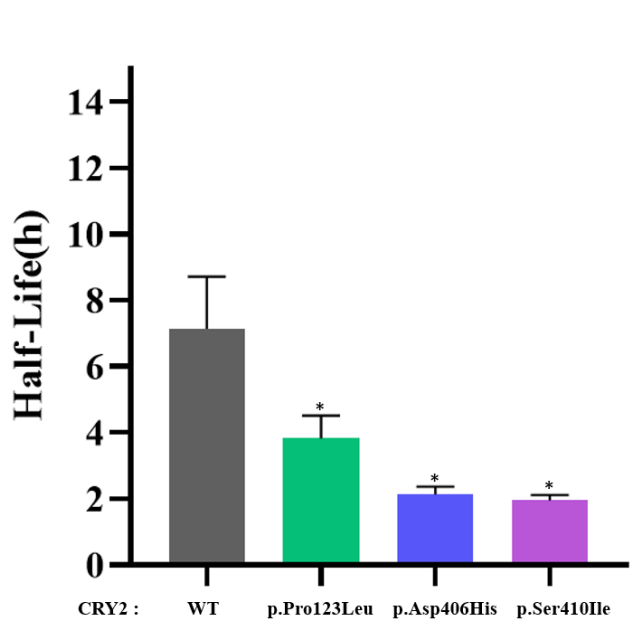
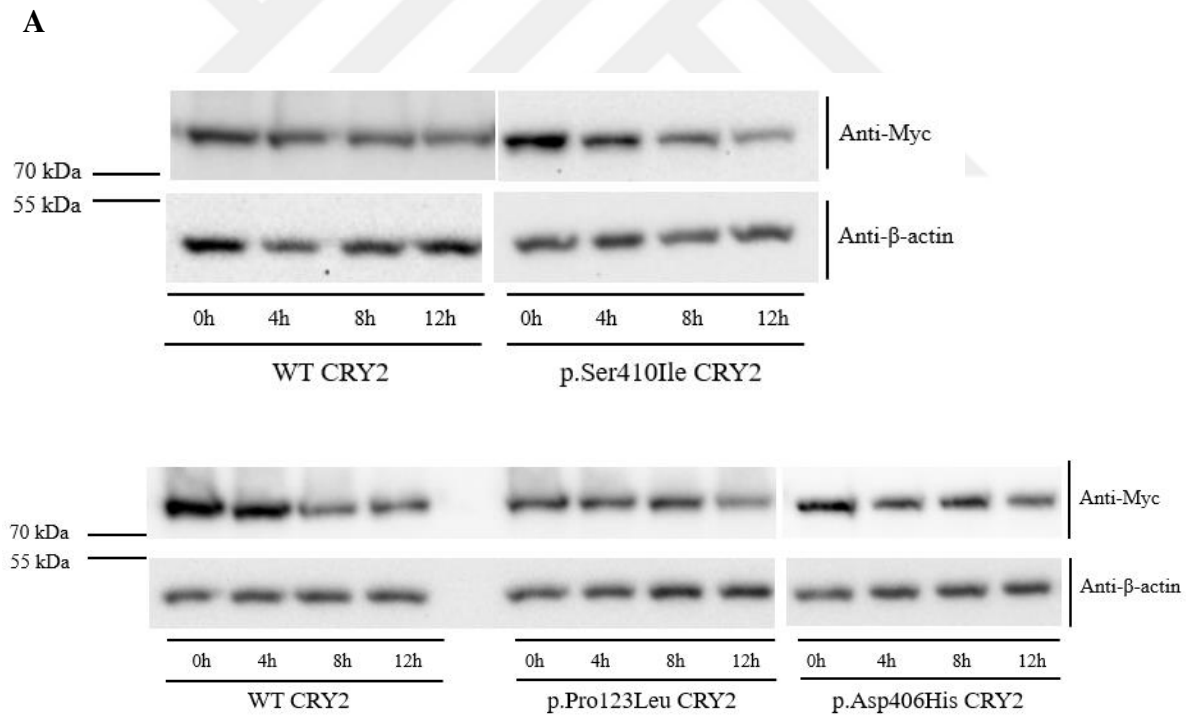


Figure 4.9 Representative Western blot images of protein degradation assay and calculated half-lives of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the absence of PER2
HEK293T cells were transfected with 500 ng wild type (WT) *Cry2* and variants, 20 µg/ml CHX was used to stop protein expression. **(A)** Representative Western blot images at the different time points after CHX treatment. The WT CRY2 and variants were detected with anti-Myc. β-actin was used as loading control. **(B)** Half-lives of WT CRY2 and variants which were calculated using a one-phase exponential decay function. Average $t_{1/2}$ values ± SD from three independent experiments. (Data represent the mean ± SD, n≥3 biological replicates with triplicate samples). Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$). Comparisons were made between WT CRY2 and variants.

Since the increasing repressor capacity of the CRY2 variants in the presence of PER was observed, it is decided to determine half-lives of CRY2 variants with PER2 using CHX assay. The results of the CHX experiment with PER2 overexpression are listed in Table F5. The representative images from these experiments are shown in **Figure 4.10A**. Notably the half-lives of CRY2 variants are increased in the presence of PER2.



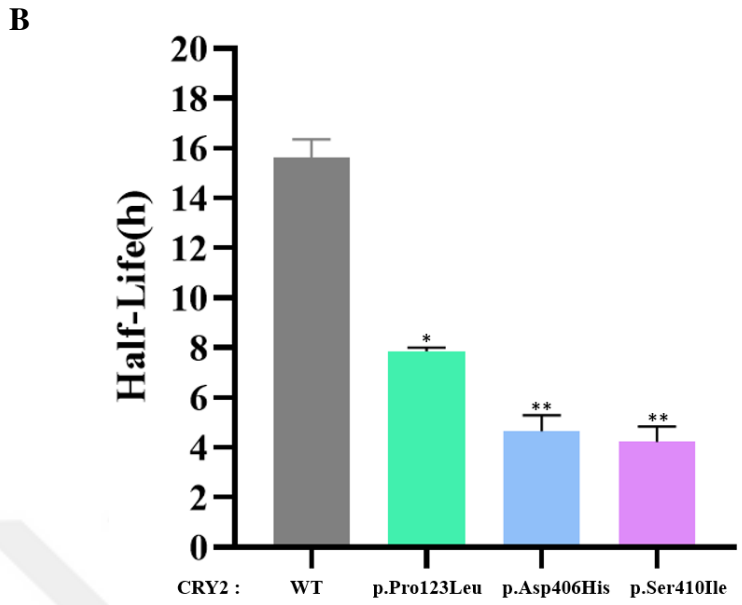


Figure 4.10 Representative Western blot images of protein degradation assay and calculated half-lives of wild type CRY2 and p.Pro123Leu CRY2, p.Asp406His CRY2, and p.Ser410Ile CRY2 variants in the absence of PER2

HEK293T cells were transfected with 500 ng wild type (WT) *Cry2* and variants, 20 µg/ml CHX was used to stop protein expression. **(A)** Representative Western blot image at the different time points after CHX treatment. The WT CRY2 and variants were detected with anti-Myc. β-actin was used as a loading control. **(B)** Half-lives of WT CRY2 and variants which were calculated using a one-phase exponential decay function. Average $t_{1/2}$ values ± SD from three independent experiments. Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$; **, $p<0.005$;). Comparisons were made between WT CRY2 and variants.

The half-lives of wild type CRY2, p.Pro123Leu CRY2, p.Asp406His CRY2 and p.Ser410Ile CRY2 are 15.65 h, 7.87 h, 4.67 h, and 4.22 h respectively **(Figure 4.10B).** Although there is no data point at 16 h, calculations performed by one-phase exponential decay by using non-linear fit (Figure E2). In comparison to the absence of PER2 overexpression, the half-lives of p.Pro123Leu CRY2, p.Asp406His CRY2 and p.Ser410Ile CRY2 with the overexpression of PER2 are 2.19, 2.05, 2.19, and 2.16 times higher, respectively.

Collectively, all these results indicate that CRY2 variants had less stability compared to wild type CRY2. In the stability increase of CRY2 variants, PER2 was not able to completely rescue the repressor activity. This observation led me to investigate the physical interaction between CRY2 variants and BMAL1/CLOCK complex in the presence and absence of the PER2 by co-immunoprecipitation.

4.4 Determination of protein-protein interaction between CRY2 variants and BMAL1/CLOCK complex in the absence and presence of PER2 overexpression

The protein complex immunoprecipitation experiments were carried out for understanding the affinity between the CRY2 variants and BMAL1/CLOCK complex and for gaining more insight to the protein-protein interaction control on the repression activity. The experiments were performed in the absence and presence of PER2 overexpressed cells by co-immunoprecipitation (Co-IP). The detailed experimental procedure was explained in the methodology Section 3.5. The experimental results with and without the PER2 overexpression are given in Table F6 and Table F7. The Co-IP results between the p.Pro123Leu CRY2 and BMAL1/CLOCK is shown in **Figure 4.11**. The amount of BMAL1 was comparable in the presence and absence of the PER2 to the results of co-immunoprecipitation of wild type CRY2 and p.Pro123Leu CRY2. Notably, p.Pro123Leu CRY2 had a higher affinity to CLOCK in the presence of PER2 while p.Pro123Leu CRY2 had a lower affinity to CLOCK when the is no PER2 compared to the control.

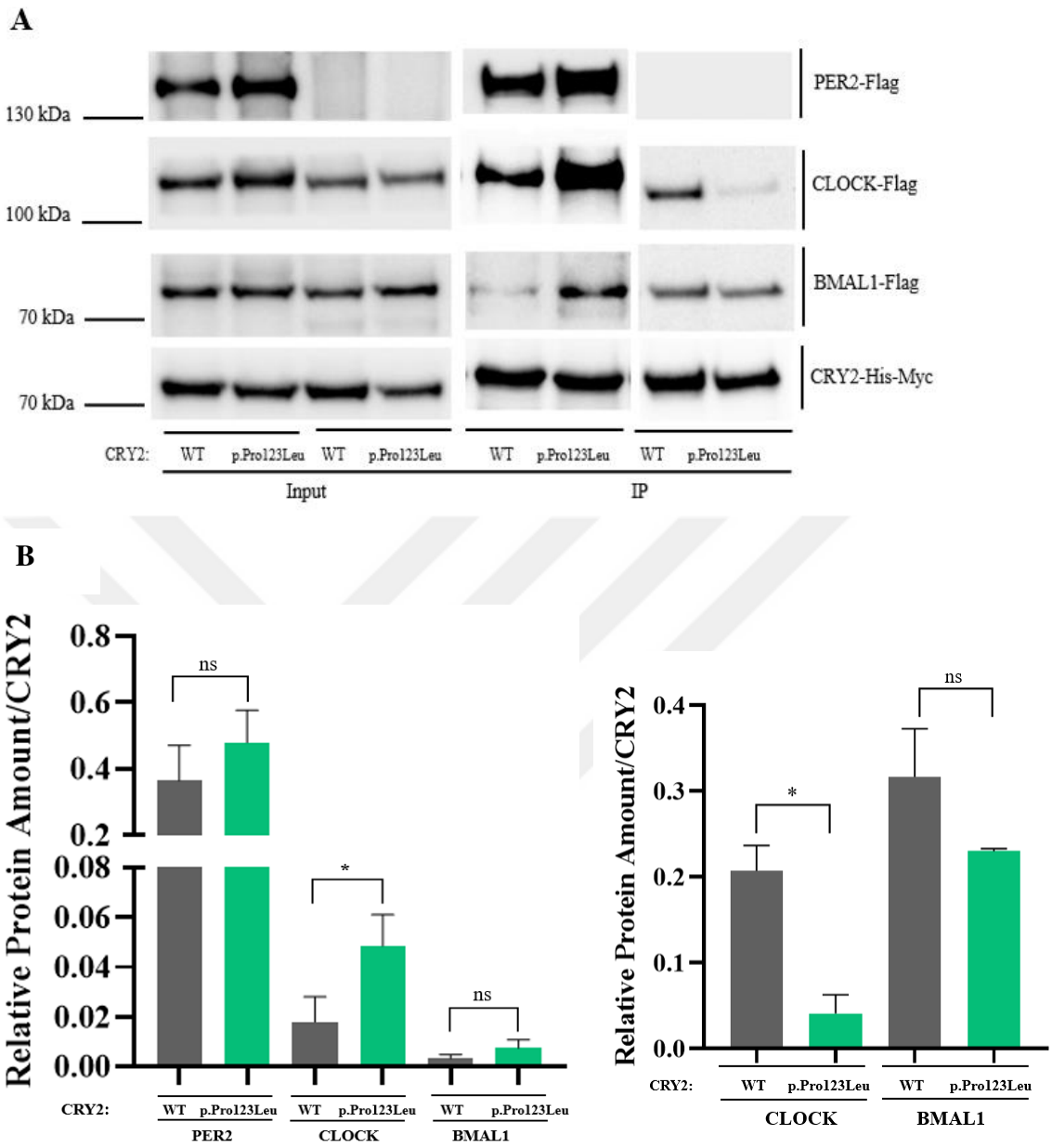


Figure 4.11 Complex immunoprecipitation of p.Pro123Leu CRY2 with CLOCK and BMAL1 in the presence and absence of PER2

Physical interaction between wild type (WT) CRY2 and p.Pro123Leu CRY2 variant with BMAL1/CLOCK and BMAL1/CLOCK/PER2. Proteins were pulled with anti-Myc resin and analyzed by Western blotting. Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$). **(A)** The blot shown is representative of three independent experiments. **(B)** Graph of relative pulled protein amounts in the absence and presence of PER2. (Data represent the mean ± SD, $n\geq3$ biological replicates with triplicate samples). Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$; ns=non-significant)).

The experimental results with and without the PER2 overexpression are given in Table F8 and Table F9. The results of Co-IP between wildtype/p.Asp406His variant CRY2 and BMAL1/CLOCK are given in **Figure 4.12**. The data indicate that the affinity

to CLOCK in the presence of PER2 is similar for wild type CRY2 and the mutant but the p.Asp406His variant CRY2 had 70% reduced affinity to BMAL1/CLOCK complex in the absence of PER2. The affinity of either wild type or variant CRY2 to BMAL1 was comparable irrespectively to the presence of PER2.

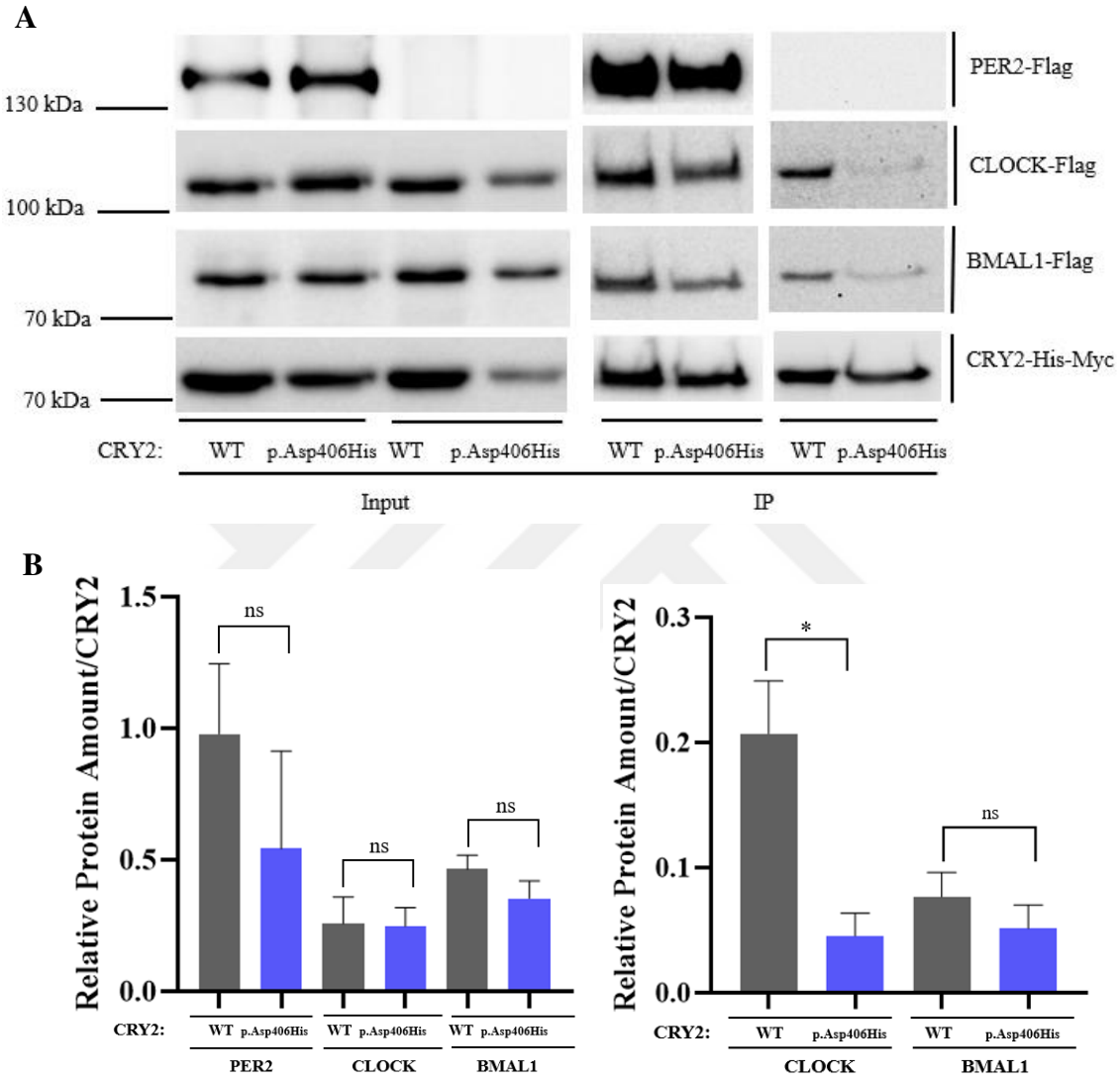


Figure 4.12 Complex immunoprecipitation of p.Asp406His CRY2 with CLOCK and BMAL1 in the presence and absence of PER2

Physical interaction between wild type (WT) CRY2 and p.Asp406His CRY2 variant with BMAL1/CLOCK and BMAL1/CLOCK/PER2. Proteins were pulled with anti-Myc resin and analyzed by Western blotting. Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$). **(A)** The blot shown is representative of three independent experiments. **(B)** Graph of relative pulled protein amounts in the absence and presence of PER2. (Data represent the mean ± SD, $n\geq3$ biological replicates with triplicate samples). Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$; ns=non-significant)).

The experimental results with and without the PER2 overexpression are given in Table F10 and Table F11. Like in the case of other two CRY2 variants the affinity of p.Ser410Ile CRY2 to BMAL1 were similar **(Figure 4.13)**. The interaction between p.Ser410Ile CRY2 and CLOCK was 40% reduced in the absence of the PER2 compared wild type CRY2/CLCOK interaction. However, the affinity between p.Ser410Ile CRY2 and CLOCK was 20% increased. In summary, the affinity between CRY2 variants and the CLOCK were differentially regulated by PER2. The circadian rhythm rescue analysis of CRY2 variants is investigated under endogenous conditions in the following section.

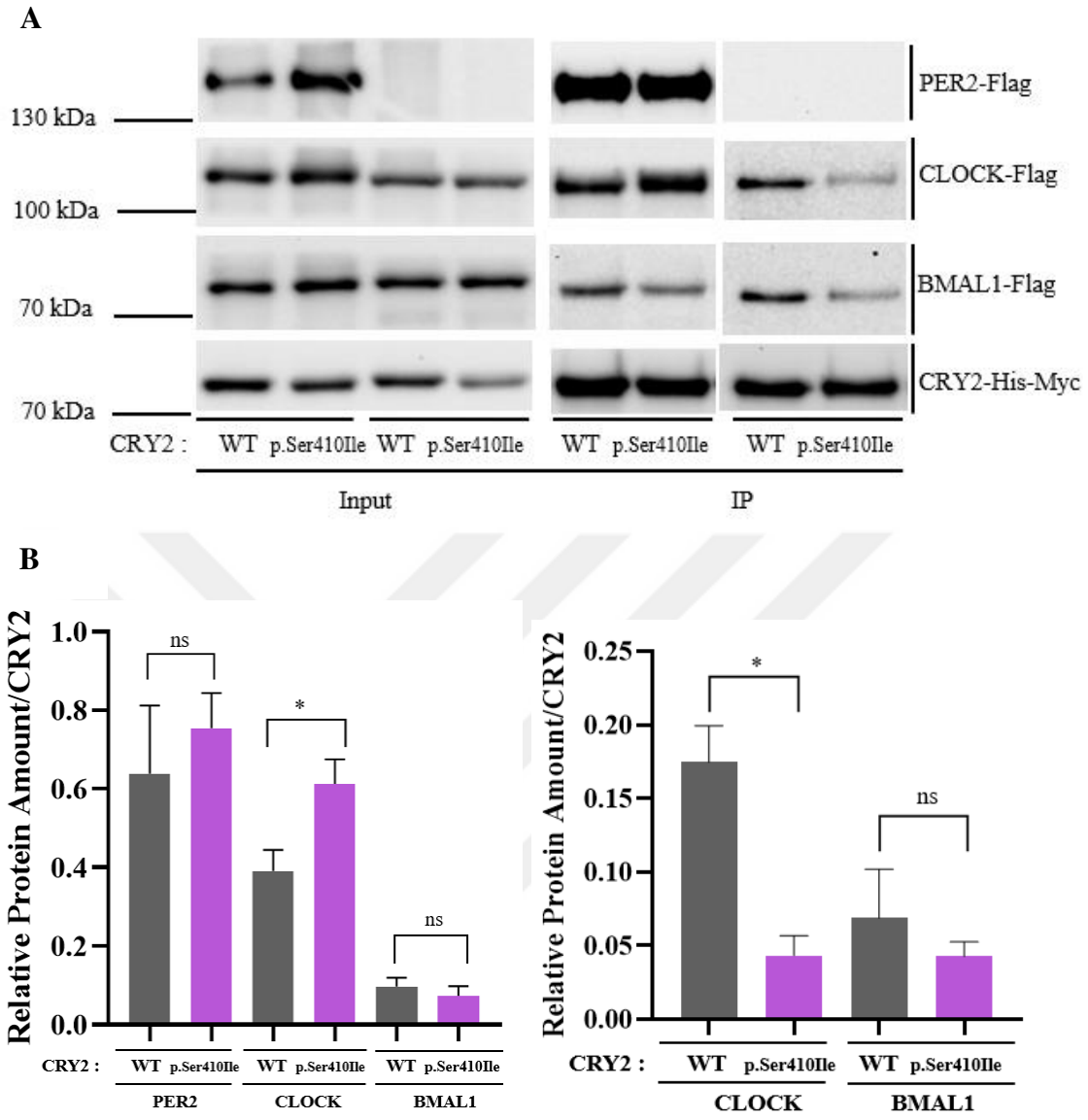


Figure 4.13 Complex immunoprecipitation of p.Ser410Ile CRY2 with CLOCK and BMAL1 in the presence and absence of PER2

Physical interaction between wild type (WT) CRY2 and p.Ser410Ile CRY2 variant with BMAL1/CLOCK and BMAL1/CLOCK/PER2. Proteins were pulled with anti-Myc resin and analyzed by Western blotting. Significance analysis was performed using unpaired t-test with Welch's correction (*, $p<0.05$). **(A)** The blot shown is representative of three independent experiments. **(B)** Graph of relative pulled protein amounts in the absence and presence of PER2. (Data represent the mean ± SD, n≥3 biological replicates with triplicate samples). Significance analysis was performed using an unpaired t-test with Welch's correction (*, $p<0.05$; ns=non-significant)).

4.5 The effect of CRY2 variants on circadian rhythm at cellular level

Here the interest is to investigate effects of the CRY2 variants on circadian rhythm at cellular level. A pervious study indicates that CRY2 can rescue circadian rhythm in

mouse embryonic fibroblast *Cry1*$^{-/-}$*Cry2*$^{-/-}$ cell line (DKO MEF) (Gul et al., 2020). Therefore, similar approach was undertaken to assess how CRY2 variants affect circadian rhythm in the cell line compare to wild type CRY2. The raw data of the circadian rhythm rescue experiment with real-time bioluminescence monitoring are listed in Table F9. The data of bioluminescence **(Figure 4.14 A)** calculated periods **(Figure 4.14 B)** are shown below. To note that, the periods of the variants cannot be calculated due to the absence of oscillations. Expectedly, wild type of *Cry1* and *Cry2* were able to rescue circadian phenotype compared control, where the period length is shorter in the presence of the *Cry2* while the period length was longer in the presences of *Cry1*. This observation is in agreement with previously published results (Gul et al., 2020; Khan et al., 2012; Onat et al., 2020). On the other hand, none of the CRY2 variants were able rescue circadian phenotype. To solve the stability problem, higher dose of the variants was used but again no rescue was observed (Figure E3).

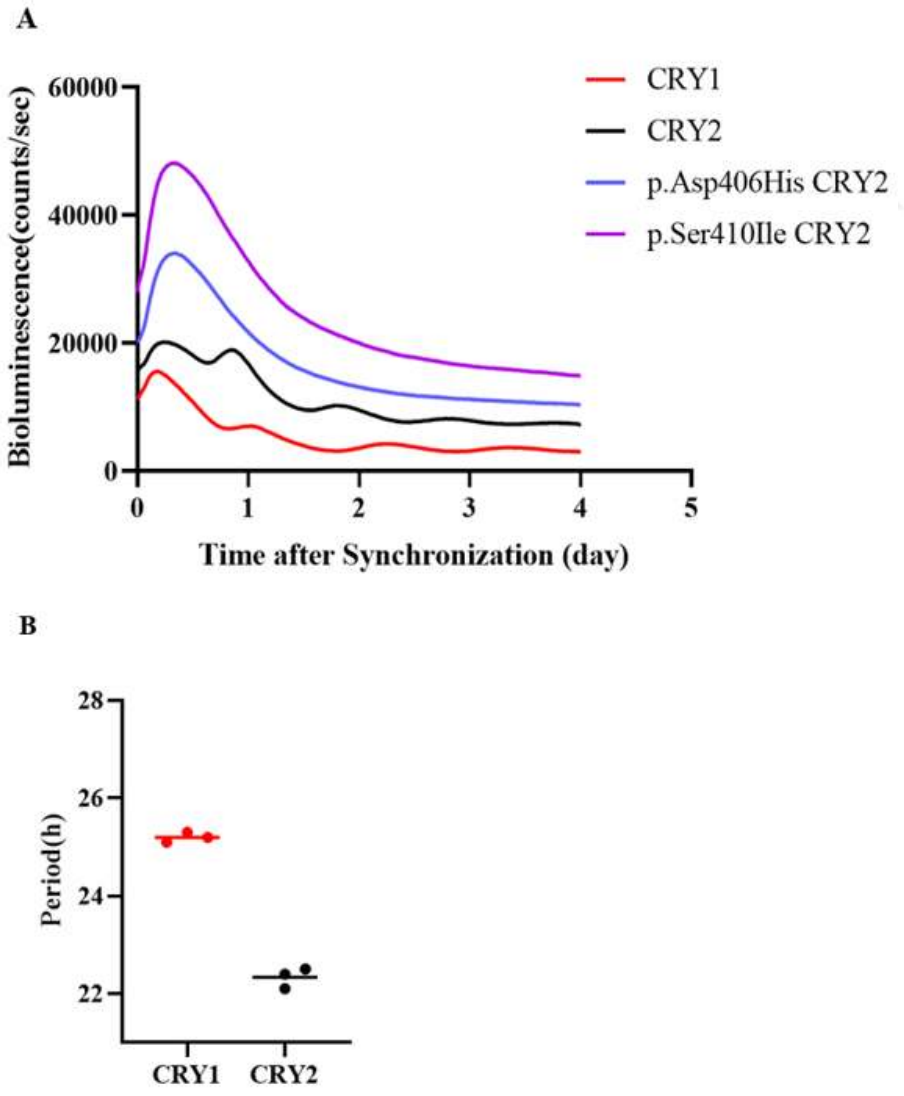


Figure 4.14 Bioluminescence rhythm of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MEF cells expressing wild type CRY2, CRY1, p.Asp406His CRY2 and p.Ser410Ile CRY2 variants endogenously.

DKO MEF cells transiently transfected with 2000 ng of pGL3-*Per2*-d*Luc* and 150 ng of wild type (WT) *Cry2, Cry1* and *Cry2* variants in rescue plasmids. After 72 h, cells were synchronized with dexamethasone and luminescence values were recorded for 4 days. **(A)** The representative bioluminescence of three independent experiments. **(B)** Calculated period lengths of *Cry1* and *Cry2*. Periods of p.Asp406His CRY2 and p.Ser410Ile CRY2 variants cannot be calculated due to the absence of the rhythms. Calculations were performed from 3 independent experiments. (Data represent the mean ± SD, n≥3 biological replicates with triplicate samples).

DISCUSSION

The circadian clock orchestrates the daily regulation of physiological, metabolic, and behavioral processes in mammals and nearly in all living organisms. The circadian clock in mammals is a highly conserved system of endogenous timers that generate 24-hour oscillation throughout the body with highly regulated interlocked transcriptional-translational feedback loops. CRYs are potent transcriptional proteins of the mammalian circadian clock and play role at the negative feedback loop by repressing the BMAL1/CLOCK transactivation (Kavakli et al., 2017; Kavakli & Sancar, 2002; Partch et al., 2014). The association between CRY mutations and various diseases has been shown in several genetic investigations. A CRY2 variant, for example, causes to advanced sleep phase in humans (Hirano et al., 2016). The mutation changes the conformation of CRY2 and makes it more accessible to FBXL3 (an E3 ubiquitin ligase) which leads to protein degradation. A study showed that type 2 diabetes and impaired fasting glucose are linked with another CRY2 variant (Liu et al., 2011). Yet, another study revealed that CRY2 is associated with depression (Lavebratt et al., 2010). All these different clinical studies suggest that SNPs on *Cry2* are associated with wide range of diseases. One reason for this diversity is that different cellular systems at tissue levels are controlled in circadian dependent manner (R. Zhang et al., 2014). Since ample evidence show the intact rhythm is important for mental, sleep, metabolic and physical health, Michael W. Young, Michael Rosbash, and Jeffrey C. Hall were awarded the 2017 Nobel Prize in Physiology or Medicine for their contributions to understanding of the molecular mechanisms controlling the circadian rhythm in Drosophila. In this study, rare genetic variants of *CRY2* which have not been associated with any diseases yet, are aimed to be identified from the 1000 Genomes Project and Ensembl database.

The initial functional characterization of CRY2 variants with *mPer1:*d*luc* repression assay showed that twelve variants out of fifteen have different repression activity on CLOCK/BMAL1 transactivation when compared with the wild type (Figure 4.1). These variants are mapped on 3D structure of the CRY2 to locate their positions on

functional domains. The coiled-coil helix is a highly conserved helix found at the C-terminus of mammalian CRYs that has a periodic spacing of nonpolar residues similar to coiled-coil (Rosensweig et al., 2018). This region has an affinity to both PER2 and FBXL3 which are competing for the region to bind (Ozber et al., 2010; Xing et al., 2013). These two proteins counteracting with each other and while FBXL3 drives CRYs to degradation, PER2 helps the stabilization of CRYs by binding to them (Chaves et al., 2006). In addition, the domain of CRY at the N-terminal (which is named as PHR domain) is composed of two distinct regions called primary and secondary pockets. The secondary pocket is required for the interaction with CLOCK PAS B domain and by binding to CLOCK, CRY keeps the BMAL1/CLOCK transcription factor in a suppressed state. Because the repression is the most important activity of CRY2 in the clock mechanism, the variants at the gate of the secondary pocket were subjected to further investigation (Figure 4.3).

The repressor activity of the three CRY2 variants, which are located at the gate of the secondary pocket, were severely impaired on BMAL1/CLOCK transactivation in the absence of PER2. However, addition of PER2 partially restores their repressor activities and this may be due to the protection of CRY2 variants by PER2. In fact, this is supported by the CHX chasing assay where the stabilities of the three variants are significantly lower when compared to the wild type, stabilities of both wild type CRY2 and variants are increased in the presence of PER2. It is known that there is a competition between PER2 and FBXL3 for binding to CRY2 (Siepka, Yoo, Park, Song, et al., 2007). It is possible that the increasing stoichiometry of PER2 during the assay may reduce affinity to FBXL3 and, in turn, increase the stabilities of the CRY2 variants. Despite having more stable CRY2 variants in the presence of PER2, they are unable to repress BMAL1/CLOCK-driven transcription, which suggests that there might be altered affinity between the CRY2 variants and BMAL1/CLOCK complex. Indeed, several studies show that PER2 acts as scaffold which brings core clock proteins together and physically helping the mutants to bind CLOCK and increases protein-protein interaction. Nonetheless, it should be kept in mind that PER2 does not completely rescue the repression activity of the variants.

In the absence of PER2, the interaction of the CRY2 variants with CLOCK is significantly lower than wild type CRY2, which is correlated with their reduced repressor

activities. Notably, p.Pro123Leu and p.Ser410Ile CRY2 variants exhibit a noticeably higher affinity to CLOCK than the wild type CRY2 in the presence of PER2 (Figure 4.11 and 4.13). The p.Asp406His CRY2 has an interaction to CLOCK similar degree to wild type CRY2 (Figure 4.12). These data indicate that residues at the position of 123 and 410 seem extremely important for CLOCK binding aided by PER2 although they are far away from the PER2 binding site (Schmalen et al., 2014). Since the region is dynamic, alterations at these residues could change the side-chain conformation hence change the interaction. On the other hand, as it is mentioned earlier, residue 406 is near the FBXL3 interaction site. Although the alteration of this residue possibly increases the affinity of CRY2 to FBXL3, in the presence of the excess amount of PER2, FBXL3 affinity is overcome by PER2 as a result the stability of p.Asp406His CRY2 is increased.

As the last point, p.Asp406His and p.Ser410Ile CRY2 variants cannot rescue the circadian rhythm of mouse embryonic fibroblast *Cry1*$^{-/-}$*Cry2*$^{-/-}$ cell line (Figure 4.14). This is direct evidence for phenotypic change in the function of CRY2. Although PER2 has some mild restoration effects on repressor activities of the variants when considered there is no rescue at endogenous conditions, the possibility of the variants to be disease causing is highly increased.

Overall, the results of this study indicate that Pro123Leu, Asp406His, and Ser410Ile mutations have functional effects on repressor activity of CRY2 and these effects cause CRY2 to become unable to repress BMAL1/CLOCK transactivation. This requires the evaluation of the pathogenicity of these variants. As it is mentioned earlier, there is no data in gnomAD about these three variants which shows that they are not seen in healthy people. Although, they are not yet related to any diseases, probably due to the fact that they are rare mutations, this study suggests these variants should also be taken into consideration especially in circadian-related diseases like sleep disorders, hormonal and neural defects, and depression. Moreover, this study provides molecular-level insights about how CRY2 mutants cause diseases and fills the related gap in the literature.

VITA

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APPENDICES

APPENDIX A Maps of Expression Vectors

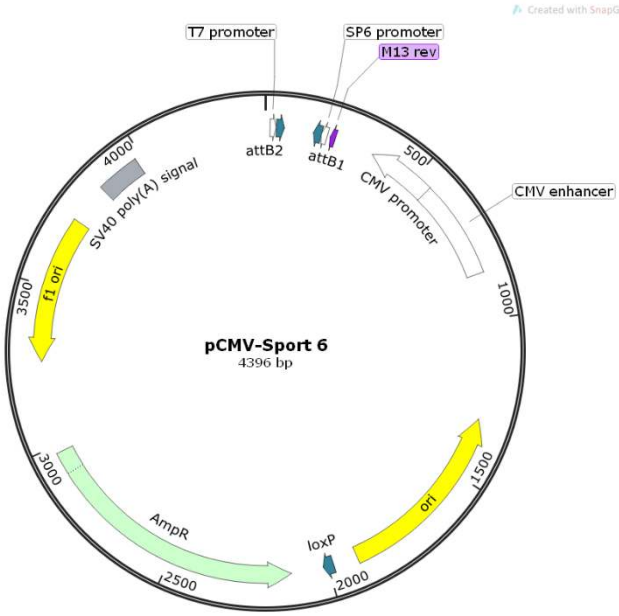

Figure A1. Plasmid map of pCMV-Sport 6.

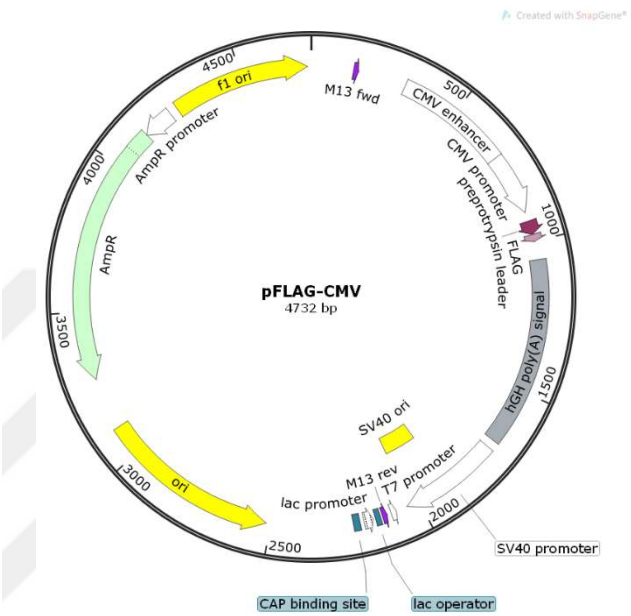

Figure A2. Plasmid map of pFLAG-CMV.

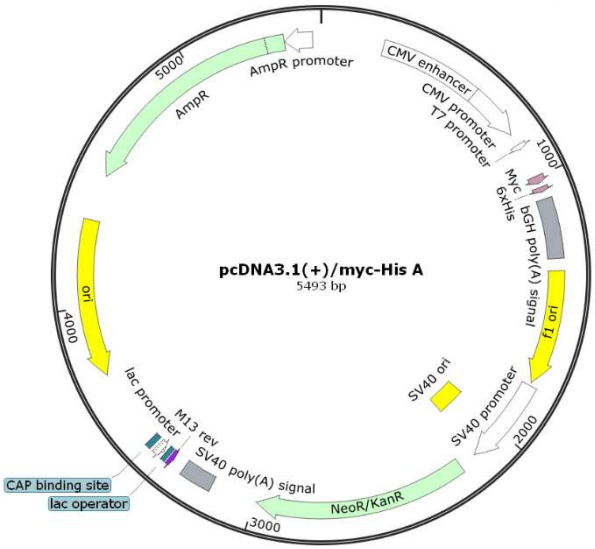

Figure A3. Plasmid map of pcDNA3.1(+) /myc-His A.

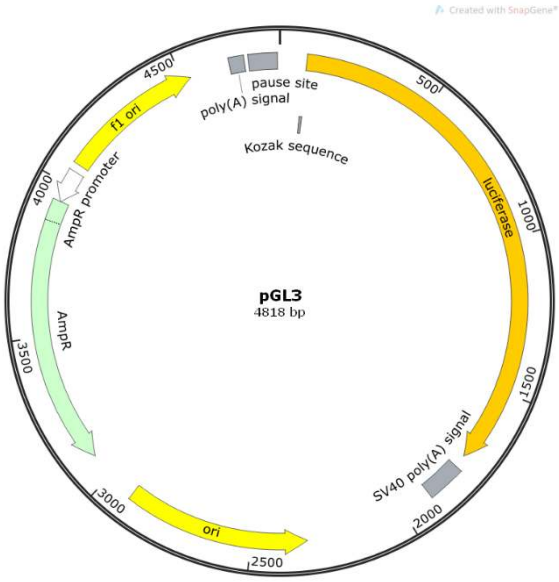

Figure A4. Plasmid map of pGL3.

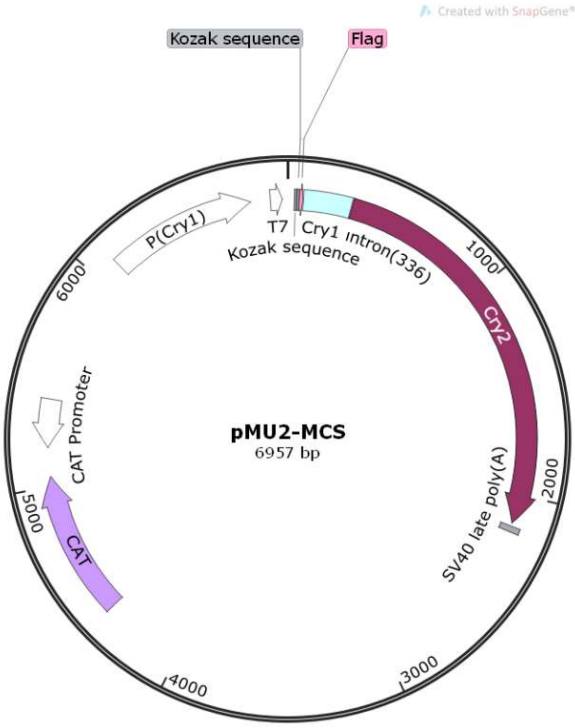

Figure A5. Plasmid map of pMU2-MCS.

APPENDIX B DNA and Protein Ladder

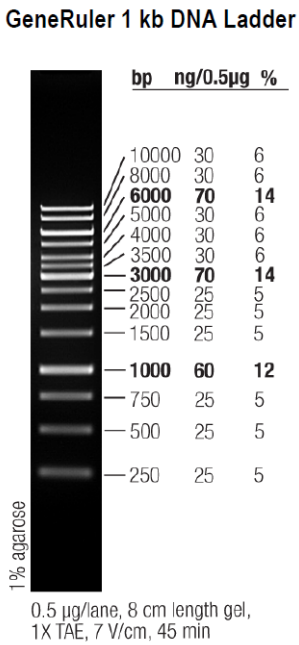

Figure B1. 1 kilobase pairs DNA Ladder for agarose gel electrophoresis.

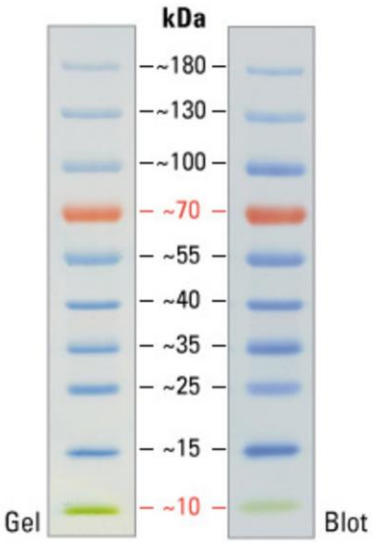

Figure B2. Protein ladder for SDS-PAGE.

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APPENDIX D Laboratory Equipment

Corning Lampda pipettes, Innova 4300 incubator shaker, AF 10 Scotsman ice machine, CL-40S/SDP (60L) ALP autoclave, Sigma Microfuge 1-14K and Beckman Coulter Allegra X-15R centrifuges, Mettler Toledo SevenCompact pH/Ion meter S220 pH meter, Thermo Scientific GenPure ultrapure water system, Heidolph Reax Top vortex, Arcelik 1061 M refrigerator and New Brunswick Scientific Premium U570 Ultra-Low Temperature Freezer were used for general lab equipment in this study.

Cell culture experiments were performed in Cell and Tissue facility. Esco Incucell incubator, ESCO Airstream® Class II Biological Safety Cabinets, Gen 3 (D-Series). And Motic AE31E microscope were used.

Epoch Microplate Reader and Thermo Scientific Fluoroskan™ Microplate Fluorometer were the microplate readers that were used in this study.

Last but not least, Bio-Rad Mini-Sub Cell GT, Bio-Rad Mini-PROTEIN 3 Cell and Single-Row AnyGel and BioRad PowerPac power supply were used to perform different gel electrophoresis'. Imaging of the electrophoses were performed with Bio-Rad ChemiDoc and Touch Imaging System.

APPENDIX E Supplementary Figures

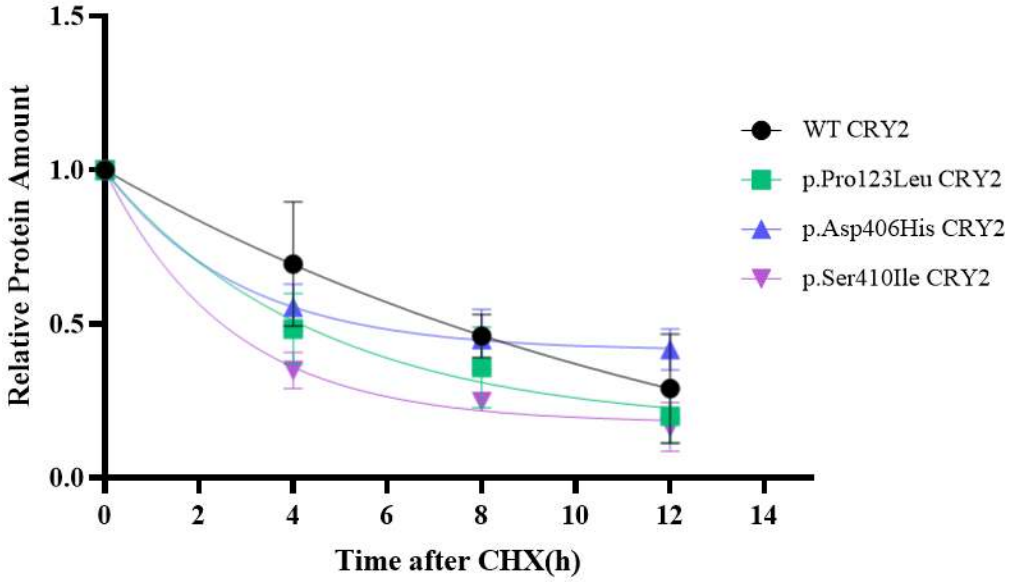

Figure E1. Quantification of western blots(n=3) and non-linear best fit curve between time points for protein degradation assay. HEK293T cells were transfected with 500 ng *Cry2* WT and variants, CHX was used to stop protein expression.

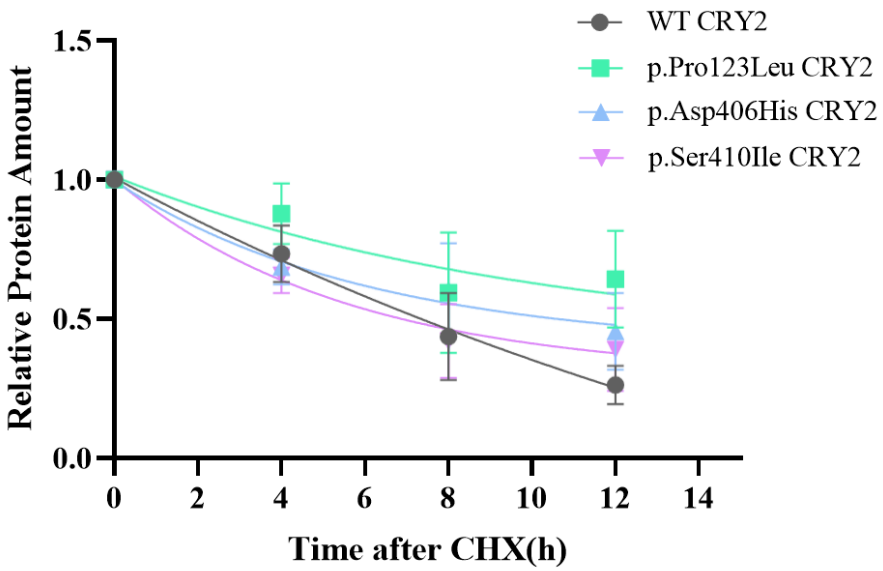

Figure E2. Quantification of western blots(n=3) and non-linear best fit curve between time points for protein degradation assay. HEK293T cells were transfected with 500 ng *Cry2* WT and variants and 500 ng *Per2*, 20 µg/ml CHX was used to stop protein expression.

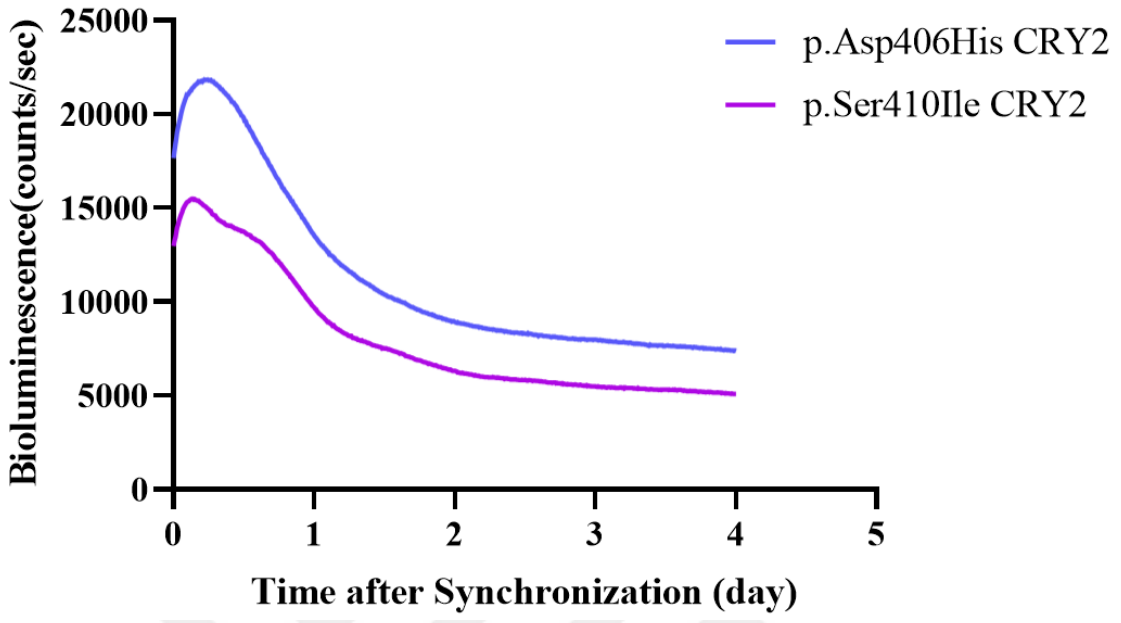

Figure E3. Bioluminescence rhythm of *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ MEF cells expressing p.Asp406His CRY2 and p.Ser410Ile CRY2 variants endogenously. DKO MEF cells transiently transfected with 2000 ng of pGL3-Per2-dLuc and 600 ng of wild type (WT) Cry2, Cry1 and Cry2 variants in rescue plasmids. After 72 h, cells were synchronized with dexamethasone and luminescence values were recorded for 4 days.

APPENDIX F Experimental Data

Table F1. Bioluminescence data of repression activities of the CRY2 variants in 0.25 ng and 0.5 ng. (n≥3)

	1 rep	2 rep	3 rep	4 rep
PLc	0.00041	0.00035	0.00037	
BCmPLc	1	1	1	
cry2-0.25	0.823842	0.754339	0.933333	0.800386
cry2-0.5	0.6082	0.667557	0.771053	0.759884
G162W-0.25	0.879271	0.936578	0.891032	
G162W-0.5	0.665148	0.784661	0.692382	
R275C-0.25	0.909643	0.744838	0.97107	
R275C-0.5	0.709188	0.628319	0.798457	
R440H-0.25	0.854973	0.794985	0.949855	
R440H-0.5	0.544419	0.671091	0.779171	
R29H-0.25	1.096431	1.325959	1.302455	
R29H-0.5	1.006074	1.026372	0.900675	
C278Y-0.25	1.173121	1.078171	0.962392	
C278Y-0.5	1.11997	1.115044	0.929605	
S280T-0.25	1.192352	1.18024	1.090351	
S280T-0.5	1.539977	1.064085	1.17807	
S280C-0.25	1.219003	1.138852	1.121053	
S280C-0.5	1.31518	1.013351	0.980702	
L283P-0.25	1.177677	1.195829	1.026037	
L283P-0.5	1.274867	1.317497	0.99325	
W358C-0.25	1.075319	0.722296	0.862281	
W358C-0.5	1.035921	1.024032	0.898246	
V380M-0.25	0.937428	0.910547	1.114912	
V380M-0.5	1.008111	0.696929	0.999123	
V380G-0.25	1.130939	1.010681	0.998246	
V380G-0.5	1.158749	0.909212	0.925439	
D406-0.25	1.146067	0.914958	0.939845	1.115718
D406-0.5	0.920599	0.955961	1.019286	
S410-0.25	1.009738	1.153509	1.051817	
S410-0.5	0.955056	1.055429	1.064912	
S420F-0.25	1.077636	0.854473	0.931579	
S420F-0.5	1.172654	0.755674	0.950877	
P123L-0.25	0.85132	0.93211	1.116063	
P123L-0.5	0.87222	0.93715	0.840887	1.087265
empty	0.00041	0.00035	0.00037	

Table F2. Bioluminescence data of repression activities of the CRY2 variants for increasing doses; 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng and 50 ng (n≥3).

	1 rep	2 rep	3 rep	4 rep
PLc	0.012	0.0118	0.0155	0.00867
BCmPLc	1	1	1	1
cry2-0.25	0.823842	0.754339	0.933333	0.800386
cry2-0.5	0.6082	0.667557	0.771053	0.759884
cry2-1	0.51337	0.574587	0.484443	
cry2-2	0.31787	0.365678	0.346445	
cry2-20	0.03578	0.03578	0.034495	
cry2-50	0.00232	0.00554	0.00334	
p123-0.25	0.85134	0.93222	1.11287	
p123-0.5	0.87227	0.93786	0.840887	
p123-1	0.88326	0.925877	0.69854	
p123-2	0.305931	0.299407	0.65224	
p123-20	0.330862	0.200404	0.300408	
d406-0.25	1.146067	0.914958	0.939845	1.115718
d406-0.5	0.920599	0.955961	1.019286	
d406-1	0.875655	1.037965	0.880881	
d406-2	0.94382	0.909643	0.987493	
d406-20	0.869663	0.854973	0.78916	
d406-50	0.760572	0.860322	0.723091	
S410-0.25	1.009738	1.051817	1.153509	
S410-0.5	0.955056	1.055429	1.064912	
S410-1	1.10412	1.123766	1.484217	
S410-2	1.04794	1.050873	1.207266	
S410-20	1.169288	1.103265	0.897558	
s410-50	0.773134	0.8542169	0.873139	
empty	0.000111	0.0000146	0.00035	

Table F3. Bioluminescence data of repression activities of the CRY2 variants for increasing doses; 0.25 ng, 0.5 ng, 1 ng, 2 ng, 20 ng and 50 ng in the presence of PER2 (n≥3).

	1 rep	2 rep	3 rep
PLc	0.011245	0.024911	0.025111
BCmPLc	1	1	1
cry2-0.25	0.740189	1.019406	0.801941
cry2-0.5	0.723951	0.894653	0.895733
cry2-1	0.5318	0.802376	0.702426
cry2-2	0.460081	0.567129	0.621129
cry2-20	0.042097	0.044119	0.041319
cry2-50	0.028564	0.027564	0.027334
p123-0.25	0.903924	1.009901	1.008721
p123-0.5	0.817321	0.838416	0.800216
p123-1	0.699594	0.719208	0.691808
p123-2	0.585927	0.659802	0.634108
p123-20	0.215426	0.172673	0.189367
p123-50	0.083972	0.08404	0.086931
d406-0.25	1.050265	1.024357	1.050265
d406-0.5	1.032173	0.998647	1.031561
d406-1	0.973871	0.932341	0.988152
d406-2	0.784112	0.840325	0.799612
d406-20	0.442367	0.465494	0.455372
d406-50	0.356557	0.227064	0.30099
S409-0.25	0.998904	0.828146	0.812128
S409-0.5	0.893288	0.772666	0.872016
S409-1	0.798112	0.709066	0.709218
S409-2	0.797601	0.641407	0.689125
S409-20	0.442064	0.333018	0.453028
s409-50	0.374653	0.325981	0.364752
empty	0.000104	0.000224	0.000193

Table F4. Calculated half-lives of WT and mutants in the absence of PER2 (n=3).

	CRY2-WT	CRY2-P123L	CRY2-D406H	CRY2-S410I
1 rep	6.158	4.617	1.958	1.841
2 rep	8.962	3.341	2.297	2.068
3 rep	6.314	3.545	2.063	2.003

Table F5. Calculated half-lives of WT and mutants in the presence of PER2 (n=3).

	CRY2-WT	CRY2-P123L	CRY2-D406H	CRY2-S410I
1 rep	16.15	7.76	5.11	3.847
2 rep	15.14	7.972	4.22	4.945
3 rep	15.44	7.86	4.45	3.87

Table F6. Pulled protein amounts in the absence of PER2 (n=3).

	CLOCK		BMAL1	
	WT	P123L	WT	P123L
1 rep	0.185804	0.06254	0.356056	0.227701
2 rep	0.227806	0.041018	0.276671	0.232033
3 rep	0.255593	0.018849	0.304495	0.202592

Table F7. Pulled protein amounts in the presence of PER2 (n=3).

	PER2		CLOCK		BMAL1	
	WT	P123L	WT	P123L	WT	P123L
1 rep	0.234907	0.62786	0.007128	0.042046	0.005131	0.004599
2 rep	0.289315	0.41102	0.019979	0.057336	0.002095	0.009769
3 rep	0.439761	0.548713	0.02694	0.039576	0.004351	0.005029

Table F8. Pulled protein amounts in the absence of PER2 (n=3).

	CLOCK		BMAL1	
	WT	D406H	WT	D406H
1 rep	0.142076	0.04391	0.095284	0.069059
2 rep	0.23702	0.045554	0.078693	0.054219
3 rep	0.177925	0.027181	0.056517	0.031835

Table F9. Pulled protein amounts in the presence of PER2 (n=3).

	PER2		CLOCK		BMAL1	
	WT	D406H	WT	D406H	WT	D406H
1 rep	0.676709	0.257115	0.193068	0.194177	0.504993	0.313772
2 rep	1.079704	0.407551	0.20833	0.220807	0.402649	0.43063
3 rep	1.180615	0.962978	0.374456	0.329465	0.485494	0.31867

Table F10. Pulled protein amounts in the absence of PER2 (n=3).

	CLOCK		BMAL1	
	WT	S410I	WT	S410I
1 rep	0.147391	0.043602	0.106745	0.05352
2 rep	0.157307	0.052892	0.053903	0.040822
3 rep	0.192252	0.03382	0.046124	0.034222

Table F11. Pulled protein amounts in the presence of PER2 (n=3).

	PER2		CLOCK		BMAL1	
	WT	S410I	WT	S410I	WT	S410I
1 rep	0.541904	0.691057	0.421743	0.650943	0.118648	0.098236
2 rep	0.761103	0.817747	0.422467	0.646477	0.097792	0.074104
3 rep	0.515843	0.647692	0.328872	0.540152	0.073903	0.049867

Table F12. Bioluminescence rhythm of Cry1-/-/Cry2-/- MEF cells expressing wild type CRY2, CRY1, p.Asp406His CRY2 and p.Ser410Ile CRY2 variants endogenously.

https://drive.google.com/file/d/1FPE3ixy21CoxFl96GnMiYIBbfP_kA1Do/view?usp=sharing