

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

FUNCTIONAL CHARACTERIZATION OF HUMAN CRY1
INTERACTOME



BUKET SÜNBÜL

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ADVISOR: PROF. DR. NURİ ÖZTÜRK

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

İNSAN CRY1 İNTERAKTOMUNUN FONKSİYONEL
KARAKTERİZASYONU

BUKET SÜNBÜL

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI: PROF. DR. NURİ ÖZTÜRK

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ABSTRACT

The circadian clock is a genetically programmed mechanism that regulates daily rhythms in biological systems. The clock is generated by a transcription translation feedback loop (TTFL). In the positive arm of TTFL, the transcription factors CLOCK and BMAL1 activate the expression of *circadian clock-regulated genes (CCGs)*. Translated products of two of the *CCGs*, the *Cryptochrome* and *Period* genes, translocate into the nucleus to inhibit the activities of CLOCK:BMAL1 proteins. This activity and inhibition in the TTFL generates oscillations in *CCGs*. Moreover, secondary loops and post-translational modifications also regulate this oscillation to approximately 24 hours. Oscillations in *CCGs* are reflected as rhythms in physiology and metabolism.

Our laboratory has focused on studying the circadian clock network to better understand its functions. In this context, our laboratory has conducted extensive studies to define the interactome of PER2 and BMAL1, as well as CRY1, using proximity-dependent labeling techniques. In this thesis, we particularly focused on the effects of CRY1 interactors on the circadian oscillation and the stability of the CRY1 protein.

We initially used the luciferase reporter assay to follow the effect of selected CRY1 interactors on CLOCK:BMAL1 transactivity. We found that six of 23 CRY1-interacting proteins dose-dependently reduced Per1 promoter activity. This resulted in an increased suppressive capacity of CRY1 on CLOCK:BMAL1 activity. In addition, real-time bioluminescence experiments revealed that some interactors affect critical parameters of the circadian rhythm, such as amplitude and period. Moreover, western blot analysis showed dose-dependent effects of these interactors on CRY1 protein stability. Specifically, GLYR1 was found to negatively affect CRY1 stability compared to the control group.

Further investigation of untested interactors in addition to GLYR1 may provide insight into the circadian clock mechanism and pave the way for the development of new treatment strategies. This research may deepen our understanding of the complex interactions underlying biological clock systems and open new avenues for future studies.

Keywords: Cryptochrome 1, Circadian Rhythm, Luciferase Reporter Assay, Protein-Protein Interactions, Interactor, Immunoblot.

ÖZET

Sirkadiyen saat, biyolojik sistemlerdeki günlük ritimleri düzenleyen, genetik olarak programlanmış bir mekanizmadır. Moleküler saat, bir transkripsiyon çeviri geri bildirim döngüsü (TTFL) tarafından üretilir. TTFL'nin pozitif kolunda, CLOCK ve BMAL1 transkripsiyon faktörleri *sirkadiyen saatçe düzenlenen genlerin (CCG'ler)* ekspresyonunu aktive eder. CCG'lerden ikisi olan *Cryptochrome* ve *Period* genlerinin anlatım ürünleri, CLOCK:BMAL1 proteinlerinin aktivitelerini inhibe etmek için çekirdeğe göç ederler. TTFL'deki bu aktivite ve inhibisyon, CCG'lerdeki salınımları üretir. Ayrıca ikincil döngüler ve transkripsiyon sonrası modifikasyonlar da bu salınımı yaklaşık 24 saate şeklinde düzenler. CCG'lerdeki salınımlar fizyoloji ve metabolizmaya ritimler olarak yansır.

Laboratuvarımız, işlevlerini daha iyi anlamak için sirkadiyen saat ağının incelenmesine odaklanmıştır. Bu bağlamda laboratuvarımız, yakınlığa bağlı etiketleme tekniklerini kullanarak CRY1'in yanı sıra PER2 ve BMAL1'in interaktomunu tanımlamak için kapsamlı çalışmalar yürütmüştür. Bu tezde özellikle CRY1 etkileşimcilerinin sirkadiyen salınım ve CRY1 proteininin stabilitesi üzerindeki etkilerine odaklandık.

Başlangıçta seçilen CRY1 etkileşimcilerinin CLOCK:BMAL1 işlemi üzerindeki etkisini takip etmek için lusiferaz raportör testini kullandık. CRY1 ile etkileşime giren 23 proteinden altısının Per1 promotör aktivitesini doza bağlı olarak azalttığını bulduk. Bu, CRY1'in CLOCK:BMAL1 aktivitesi üzerindeki baskılayıcı kapasitesinin artmasına neden oldu. Ek olarak, gerçek zamanlı biyoluminesans deneyleriyle, bazı etkileşimcilerin genlik ve periyot gibi sirkadiyen ritmin kritik parametrelerini etkilediğini ortaya çıkardık. Ayrıca western blot analizini kullanarak, bu etkileşimcilerin CRY1 protein stabilitesi üzerindeki doza bağımlı etkilerini gösterdik. Spesifik olarak GLYR1'in, kontrol grubuyla karşılaştırıldığında CRY1 stabilitesini olumsuz etkilediğini gözlemledik.

GLYR1'e ek olarak test edilmemiş etkileşimcilerin daha fazla araştırılması sirkadiyen saat mekanizması hakkında fikir verebilir ve yeni tedavi stratejilerinin geliştirilmesinin önünü açabilir. Bu araştırma, biyolojik saat sistemlerinin altında yatan karmaşık etkileşimlere ilişkin anlayışımızı derinleştirebilir ve gelecekteki çalışmalar için yeni yollar açabilir.

Anahtar kelimeler: Cryptochrome 1, Sirkadiyen ritim, Lusiferaz raportör testi, Protein-Protein Etkileşimleri, İnteraktör, İmmünblotlama.

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LIST OF SYMBOLS AND ABBREVIATIONS

BCA	: Bicinchoninic Protein Assay
CCGs	: Clock-controlled genes
CCT2	: Chaperonin containing TCP1 subunit 2
CHX	: Cycloheximide
CRY	: Cryptochrome
dCRY	: Drosophila cryptochrome
DMEM	: Dulbecco's Modified Eagle Medium
FBS	: Fetal Bovine Serum
Glu	: Glutamate
GLYR1	: Glyoxylate reductase 1
h-CTZ	: Coelenterazine
ipRGCs	: Intrinsically photosensitive retinal ganglion cells
LB	: Luria-Bertani liquid medium
LIMS1	: LIM zinc finger domain containing 1
LPCAT1	: Lysophosphatidylcholine acyltransferase 1
MSI CRC	: Microsatellite Instability Colorectal Cancer
NEAA	: Non-essential amino acids
ng	: Nanogram
NNMT	: Nicotinamide N-methyl transferase
P/S	: Penicillin/Streptomycin
PACAP	: Pituitary adenylate cyclase-activating protein
PEI	: Polyethyleneimine
PHL	: Photolyase
PHR	: Photolyase homology region
PPIs	: Protein-protein interactions
PURG	: Purine rich element binding protein G
RHT	: Retinohypothalamic tract
RORE	: Retinoic acid-related orphan receptor response elements
SCN	: Suprachiasmatic nucleus
SEM	: Standard Error of Mean
TIM	: Timeless protein
TTFL	: Transcription-translation feedback loop
VIP	: Vasoactive intestinal polypeptide
°C	: Celsius
µg	: Microgram
µL	: Microliter

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1. INTRODUCTION

The circadian clock is a genetically encoded mechanism found in almost all living organisms, from cyanobacteria to humans. While its disruption may not be lethal, it has been associated with numerous diseases, aside from developmental disorders. Therefore, this mechanism is a basic biological process like apoptotic cell death and DNA repair mechanisms. The circadian regulation of approximately %10 of the transcriptome or %50 of protein coding genes in any given cell in humans and mice indicates the significance of this system in homeostasis [de Assis and Oster, 2021].

The circadian clock essentially consists of four main types of proteins and is regulated through a transcriptional feedback loop. However, beyond this regulation, protein-protein interactions (PPIs) enable the circadian clock to influence other pathways, while other pathways provide feedback to the circadian clock [Kavakli et al., 2022]. CRY1 is one of the key proteins responsible for the circadian clock. Beyond its interactions with the core clock proteins, the interactions with various other proteins and the effects of these interactions have been a subject of curiosity. Therefore, understanding the roles of various PPIs in the circadian clock, the pathways they participate in, and the potential outcomes they can cause is crucial for gaining insights into the circadian clock system.

Section 2 will provide more specific information regarding the purpose of this study. Important topics such as the circadian clock, transcriptional regulation of the circadian clock, importance of PPIs will be elaborated in more detail in Section 3 Literature Review.

2. AIM OF THE THESIS

This thesis study was conducted to investigate the functions of new CRY1 interacting proteins or CRY interactors, which were previously discovered in our laboratory using proximity-dependent labeling and mass spectrometry identification. The effects of hitherto unknown CRY1 interactor proteins on the circadian clock or the effects of the circadian clock on them will be analyzed, and research will be conducted on the unknown functions of the circadian clock through the functions of interactomes. It is envisaged to discover the basic reaction mechanisms, such as CRY's direct blocking of CLOCK:BMAL1 and PER-mediated removal of CLOCK:BMAL1 from DNA via CRY1, or to obtain information on how CRY1 interacts with other pathways. In summary, the functional analysis of the interactions of previously unknown and newly identified proteins that interact with CRY1 is the main aim of this thesis study.

In this direction, the effects of interactors on CRY1's blocking of CLOCK:BMAL1 activity will be analyzed using the dual luciferase reporter assay, how interactors affect variables such as period and amplitude in the circadian rhythm will be shown with bioluminescence experiments, and the effects of overexpression of interactors on the stability of CRY1 will be tested using immunoblotting analysis.

3. LITERATURE

3.1. Overview of Biological Rhythms

Most organisms use biological rhythms to adapt to the daily environmental changes that result from the daily rotation of the earth. The biological clock is generally defined as a mechanism by which organisms regulate the timing of their biological activities. The period length of these rhythms is important for identifying them. Biological rhythms are categorized into three groups based on the length of their periodicity. For example, circadian rhythms regulate the oscillation of activities that take place over a period of about 24 hours, and the sleep-wake cycle is an example of a circadian rhythm. Ultradian rhythms repeat over less than 24 hours and regulate activities such as breathing and urination, while infradian rhythms repeat over periods longer than 24 hours and control activities such as menstruation and molting [Aschoff, 1981].

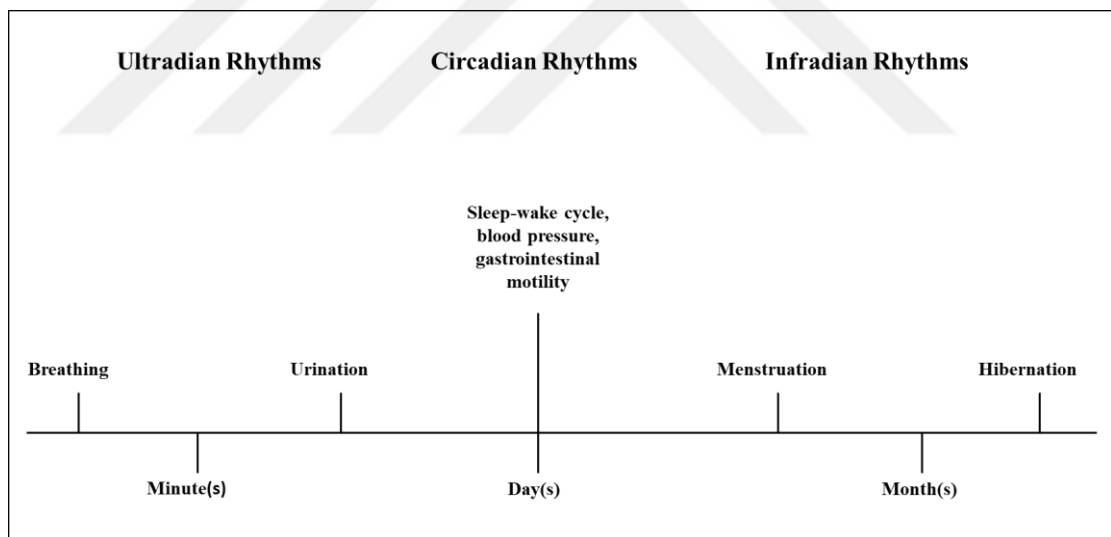


Figure 3.1: A visual depiction illustrating the biological rhythms schematically. Examples of ultradian, circadian, and infradian rhythms respectively.

3.2. Circadian Rhythms

The majority of living organisms exhibit behavioral and biochemical oscillations that occur as 24-hour cycles. These oscillations, known as circadian rhythms (from the Latin words "circa," meaning "approximately," and "diem," meaning "day"), are conserved across taxa [Robinson and Reddy, 2014]. Daily biological cycles are a result of an intrinsic circadian clock that exists in organisms. The transition between light and darkness generates a multitude of cues, including light, temperature, and resource availability, among others. These cues, often referred to as "zeitgebers," have the capacity to synchronize an organism's internal timing systems with the external environment [Roenneberg et al., 2007]. These zeitgebers include scheduled sleep, activity patterns, temperature changes, and social signals such as mealtimes and the presence of clocks. Nevertheless, the most influential Zeitgeber by a significant margin is the natural light-dark cycle, often referred to as the solar light/dark cycle [Duffy and Wright, 2005].

The capacity to anticipate and adapt to environmental fluctuations has been demonstrated to offer a distinct evolutionary advantage to organisms. For instance, cyanobacteria exhibiting oscillations with internal periods that align with the 24-hour light-dark cycle tend to have superior reproductive success when compared to colonies with asynchronous rhythms [Ouyang et al., 1998]. Likewise, *Arabidopsis thaliana* plants whose rhythms are synchronized with the external light cycle exhibit accelerated growth compared to those with mismatched rhythms [Dodd et al., 2005]. Additionally, wild-type *Drosophila melanogaster*, possessing intact circadian clocks, display higher reproductive fitness in contrast to counterparts with circadian clock mutations [Beaver et al., 2002]. These examples underscore the significant evolutionary benefits of aligning biological processes with the predictable rhythms of the external environment.

3.3. Mammalian Circadian Rhythms

The circadian system plays a crucial role in the regulation of a broad spectrum of physiological and behavioral rhythms [Lowrey and Takahashi, 2004]. In mammals, this circadian system is structured as a hierarchy of oscillators, with the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus at the top of this hierarchy. The SCN serves as the central pacemaker, responsible for harmonizing the independent peripheral oscillators within the body [Yamazaki et al., 2000]. This hierarchical organization is mirrored at the systems level, where signals, like light detected by the retina (external environmental input), are conveyed to the SCN, often referred to as the central clock. Peripheral clocks become synchronized by the SCN after the SCN is reset by light [Albrecht, 2012]. This light input helps the SCN align an organism's internal biological clock with the natural 24-hour light-dark cycle.

Light information is primarily perceived through intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina, which contain the light-sensitive photopigment melanopsin. These specialized cells transmit photic information to the SCN via the retinohypothalamic tract (RHT). The RHT fibers, which are monosynaptic, terminate in the ventrolateral region of the SCN, where they connect with neurons expressing vasoactive intestinal polypeptide (VIP) [Ecker et al., 2010].

When the retina is exposed to light at the end of the subjective night, it triggers the release of neurotransmitters like glutamate (Glu) and pituitary adenylate cyclase-activating protein (PACAP) at the synaptic terminals of the RHT [Ecker et al., 2010]. This signal is then relayed to the SCN, initiating a cascade of signaling pathways that result in chromatin remodeling, the activation of immediate early genes, and clock genes [Golombek and Rosenstein, 2010]. Consequently, the phase of the circadian clock is changed or shifted.

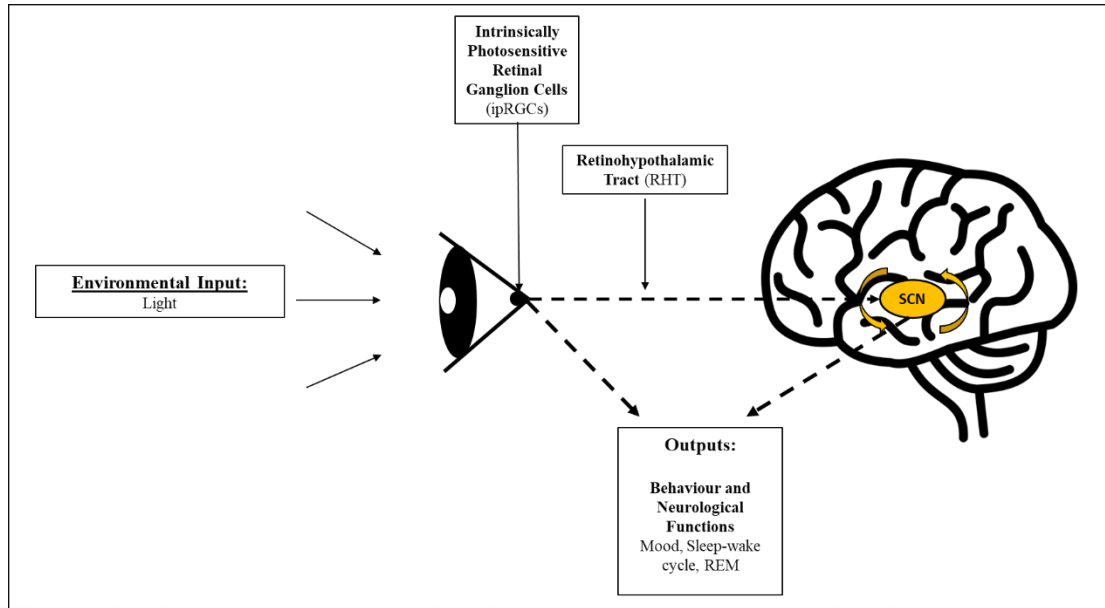


Figure 3.2: The suprachiasmatic nucleus (SCN) is a pivotal brain region responsible for perceiving and transmitting light signals. Positioned within the brain, it serves as a hub for receiving inputs and subsequently relaying this information to regulate circadian rhythms.

3.4. Molecular Mechanism of Mammalian Circadian Clock

The clock mechanism in the SCN and peripheral oscillators mentioned in Section 3.3 are similar at the molecular level, and the core molecular clock is generated by a transcription-translation feedback loop (TTFL). The molecular clock components are genes whose protein products are indispensable for the production and regulation of circadian rhythms in individual cells throughout the organism [Ko and Takahashi, 2006]. The genes recognized as 'core' clock genes include *Clock*, *Bmal1*, *Period* (*Per1*, *Per2*, *Per3*), and *Cryptochrome* (*Cry1*, *Cry2*). The 24-hour rhythmic mechanism is meticulously regulated through the expression of these genes [Jonathan et al., 2022].

The positive arm of the TTFL consists of *CLOCK* and *BMAL*, while the negative arm consists of *CRY* and *PER*. *BMAL1* and *CLOCK* function as transcription factors and form a heterodimer complex that binds to the E-box motifs (CACGTG) of *clock-controlled genes* (CCGs) and travels to the nucleus to induce their expression [Takahashi, 2017; Sancar, 2004]. Negative feedback within the circadian clock system is established through the action of *PER:CRY* heterodimers. After their formation, these heterodimers are transported back into the cell nucleus. There, they serve to

suppress their own transcription by interacting with the CLOCK:BMAL1 complex [Ko and Takahashi, 2006].

In addition to the primary TTFL, there exists a secondary feedback loop that operates alongside it. In this secondary loop, the CLOCK:BMAL1 complex plays a central role. This complex stimulates the transcription of orphan nuclear receptor genes REV-ERB α/β and ROR α/β through the activation of E-box-mediated mechanisms. The protein products of these genes, REV-ERB and ROR, then engage in a regulatory competition. They compete for binding to retinoic acid-related orphan receptor response elements (RORE) located within the promoter of the *Bmal1* gene. When REV-ERB proteins occupy the RORE binding sites, the transcription of *Bmal1* is inhibited. Conversely, when ROR proteins bind to these sites, they promote the transcription of *Bmal1*. This interplay between REV-ERB and ROR proteins constitutes an additional layer of circadian regulation, further contributing to the precise control of the circadian clock [Jonathan et al., 2022].

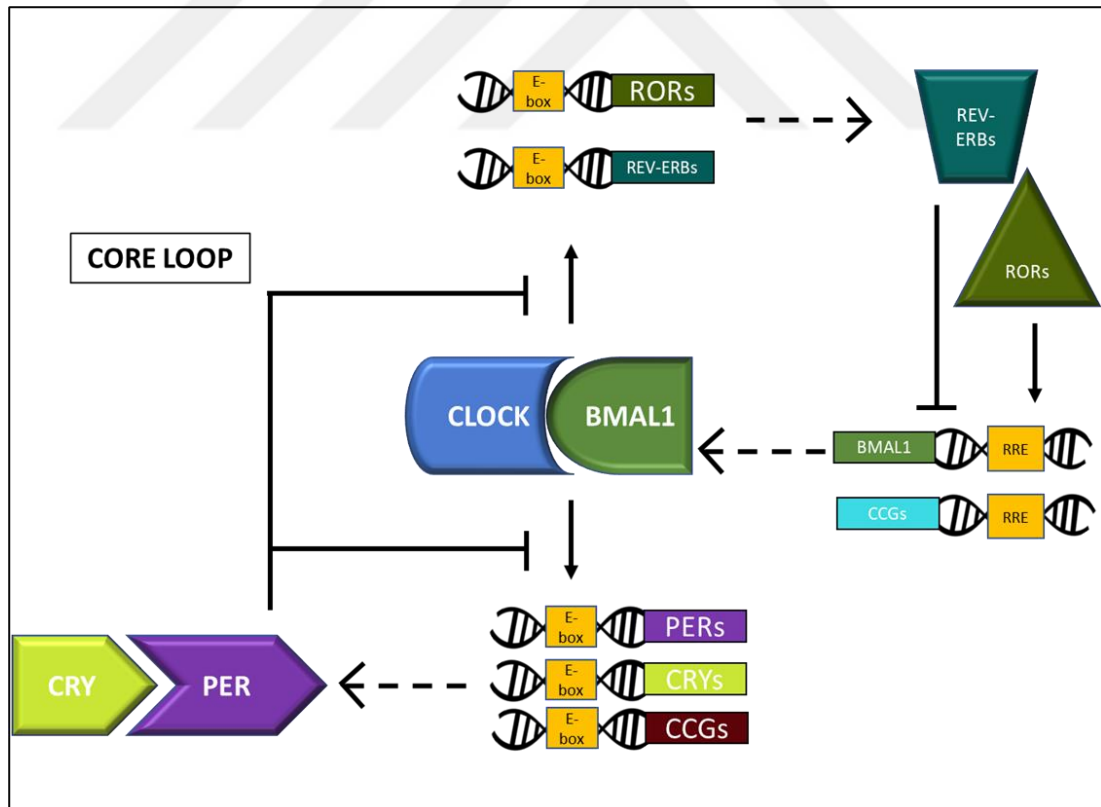


Figure 3.3: Scheme of the molecular mechanism of circadian transcription-translation feedback loop (TTFL).

Indeed, circadian clock proteins are subject to more than just feedback loops. They are also regulated through posttranslational and posttranscriptional modifications. These modifications play a crucial role in fine-tuning and precisely regulating the circadian clock [Harms et al., 2004].

3.5. CRY Protein

Cryptochromes belong to a large group of proteins called the photolyase/cryptochrome (PHL/CRY) family [Ozturk et al., 2017]. This family is structurally similar and contains a highly conserved core domain, the photolyase homology region (PHR), at the N terminus. However, evolutionary differences in the C terminus distinguish CRYs functionally [Lin and Todo, 2005; Öztürk et al., 2007]. For example, bacterial photolyases are enzymes that carry out DNA repair to preserve genetic integrity by converting UV-induced photoproducts into normal bases [Sancar, 2004]. CRY proteins do not show photolyase activity and function as photoreceptors in plants and *Drosophila* [Ahmad and Cashmore, 1993; Cashmore, 2003]. While CRYs in plants can affect phototropism, growth, and development *Drosophila* cryptochrome (dCry) plays roles related to the light entry pathway in setting the circadian clock [Emery et al., 1998; Stanewsky et al., 1998]. Mammalian CRYs (CRY1 and CRY2) do not function as photolyases or photoreceptors but instead function as transcriptional repressors independent of light [Khan et al., 2012].

CRY may be photochemically similar to the PHR, but variable C-terminus extensions of the CRY protein play an important role in protein-protein interactions, modifications, and signaling [Busza et al., 2004; Dissel et al., 2004]. For example, studies have found that the C-terminus of CRY proteins in *A. thaliana* interact with COP proteins involved in blue light signaling [Wang et al., 2001; Yang et al., 2000]. The interaction of dCRY with the TIMELESS (TIM) protein also plays an important role in blue light signal transduction [Ceriani et al., 1999]. Also, it is known that CRY and PER proteins, which are involved in the TTFL mechanism that regulates the circadian clock in mammals, inhibit CLOCK:BMAL1 activity by interacting at the C-terminus of CRY [Chaves et al., 2006]. CRYs have been noted to suppress the CLOCK:BMAL1 complex more strongly than PERs. All these interactions place CRYs at the basis of the core clock mechanism and reveal that they are the main clock components [Griffin et al., 1999; Horst et al., 1999]. In addition to interacting with

central clock proteins via the C-terminus and regulating the oscillation, mammalian CRYs also interact with FBXL3 and FBXL21 ubiquitin ligases, which plays an important role in determining its half-life [Parico et al., 2020]. FBXL3 regulates the degradation of CRY within the nucleus, while FBXL21 facilitates the ubiquitination of CRY in the cytoplasm. Moreover, FBXL21 plays a role in ensuring the proper degradation of CRY in the nucleus by counteracting the activity of FBXL3 on CRY. Additionally, the loss of functionality of FBXL3 causes the stabilization of CRY proteins and extends the period. Unlike mutations in FBXL3, mutations in FBXL21 cause the period to shorten. As a result, *Per* and *Cry* genes are transcriptionally repressed. All these post-translational modifications of CRY proteins clearly demonstrate the importance of circadian clock regulation [Siepka et al., 2007; Hirano et al., 2013; Yoo et al., 2013].

The two CRY proteins found in mammals are CRY1 and CRY2, and at least one is required for effective circadian clockwork; Losing both proteins can cause disruption of circadian rhythms and arrhythmias. Loss of CRY1 results in short circadian periods, while loss of CRY2 results in long periods. This suggests that these two proteins may have opposing functions in the correct adjustment of the circadian period [Vitaterna et al., 1999]. CRYs are not only associated with sleep/wake states but also play an important role in many important metabolic processes. For example, it has been observed that the absence of *Cry1* and *Cry2* genes causes shortening and lengthening of menstrual periods in mice [Horst et al. 1999]. In addition, CRYs play important roles in processes such as the differentiation of brown adipose tissue and hormone-based gluconeogenesis [Zhang et al., 2010; Lamia et al., 2011; Miller et al., 2020]. All these examples related to circadian rhythm and the CRY protein occur through many important protein-protein interactions (PPIs) [Kavakli et al., 2022].

Protein-protein interactions (PPIs) are fundamental to nearly all biological processes, and the timing of these interactions is paramount for their regulatory functions. In the circadian oscillator, many of the established PPIs occur predominantly at specific times of the day. For instance, the PER/CRY complexes bind to CLOCK/BMAL1 during the late night, effectively inhibiting transactivation [Lee et al., 2001]. Importantly, the temporal pattern of these interactions closely aligns with the abundance profiles of PER and CRY proteins. These instances underscore the critical significance of precisely timed PPIs in orchestrating the intricate machinery of the

circadian clock, where the coordinated timing of these interactions is pivotal for the clock's function and regulation [Wallach et al., 2013].

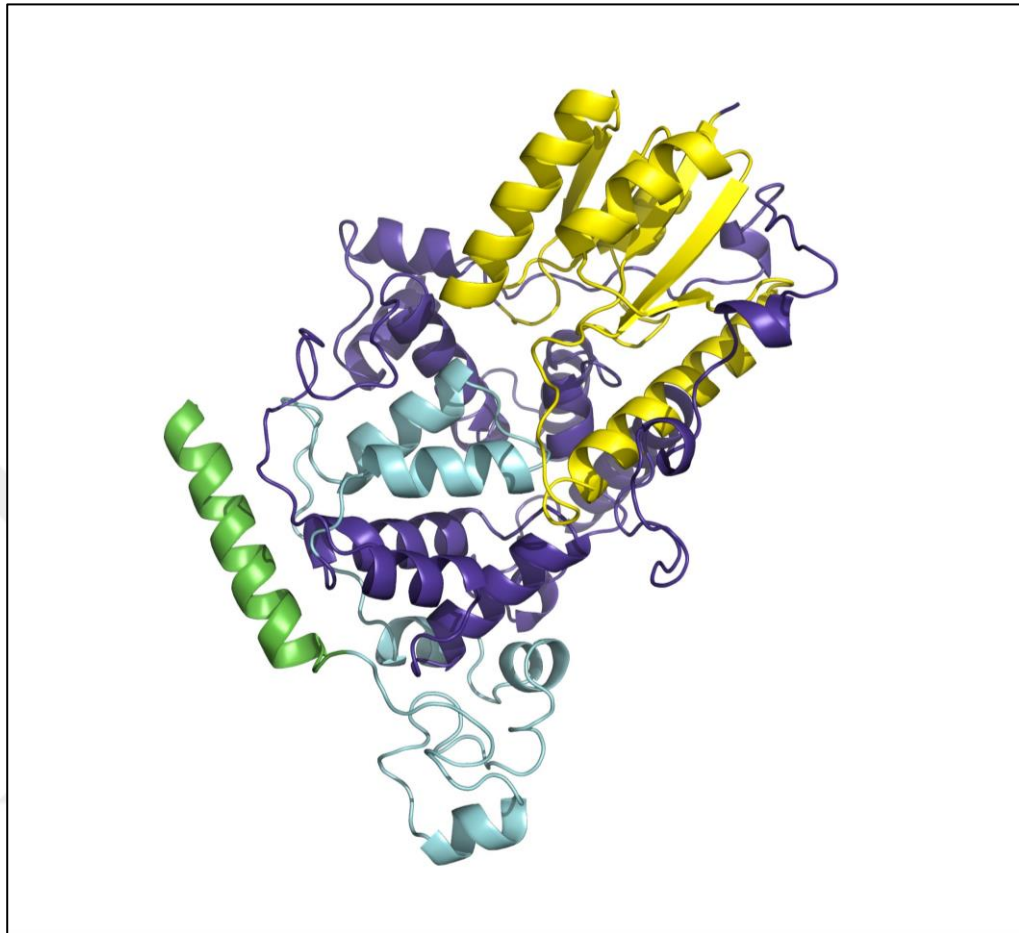


Figure 3.4: The human CRY1 protein structure was modeled using the SWISS-MODEL instead of mouse CRY1 in the crystal structure and then superimposed with PyMOL (github.com/schrodinger/pymol-open-source). CRY is shown in violet. Residues of interaction with the PER region are colored green. Residues of the photolyase region are colored as yellow. Residues of interaction with the CLOCK:BMAL1 region are colored blue.

4. MATERIALS

4.1. Kits and Reagents

Table 4.1: Kits and Reagents.

Nucleospin Plasmid Kit	740588.50 (Macherey-Nagel, Germany)
Pierce™ BCA Protein Assay Kit	23225 (Thermo Fischer, USA)
Protease Inhibitor Cocktail Tablets	S8830-2TAB (Sigma-Aldrich, USA)
Protein Molecular Weight Marker	S24052,(iNtRON Biotechnology, Korea)
Coelenterazine h	301-500 (NanoLight Technology, USA)
Chemiluminescent HRP Substrate	K-12043-D10 (Advansta, USA)
Luciferin	115144-35-9 (Sigma, USA)
Acrylamide (%40)	AB191100 (Ecotech, Turkey)
Tetramethylethylenediamine (TEMED)	T7024 (Sigma, USA)
β-Merkaptoethanol	A1108 (PanReac AppliChem, Italy)
Trypsin	25300054 (Thermo Fischer, USA)
Polyethylenimine	9002-98-6 (Sigma, USA)
Cycloheximide	66819 (Sigma, USA)

4.2. Buffers and Solutions

Table 4.2: List of buffers and solutions.

LB Agar	LBP04 (Caisson Lab, USA)
0.05% Trypsin-EDTA	25300-054 (Gibco-Invitrogen, USA)
Dulbecco's Modified Eagle Medium (DMEM)	41965-039 (Gibco-Invitrogen, USA)
Fetal Bovine Serum	10270-076 (Gibco-Invitrogen, USA)
100x Penicillin/Streptomycin	15140-122 (Gibco-Invitrogen, USA)
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄
5X RIPA Lysis Buffer	250 mM Tris pH: 8.0 750 mM NaCl 0.1% SDS 8% IGEPAL 1% NP40
Triton Lysis Buffer	150 mM Tris pH: 6.8 75 mM NaCl 3 mM MgCl ₂ 0.25% Triton X Autoclaved Water
3X Firefly Assay Buffer	15 mM DTT 0.6 mM COENZYME-A 0.45 mM ATP 0.4 mM Potassium-Luciferin
Renilla Salts	45 mM Na ₂ EDTA 30 mM Na Pyrophosphate (Na ₄ P ₂ O ₇) 1.425 M NaCl Water to 50 ml
3X Renilla Assay Buffer	0.01 mM h-CTZ Renilla Salts
10X SDS-PAGE Running Buffer	250 mM Tris-Base 190 mM Glycine 2% SDS
10X Tris-Buffered-Saline (TBS)	150 mM NaCl 20 mM Tris Base

Table 4.2: Continued.

4x Protein-Loading-Dye (4x Laemmli Buffer)	0.25 M Tris-HCl (pH 6.8) 8% SDS 40% Glycerol 20% β -mercaptoethanol 0.008% Bromophenol Blue
5% Blocking Solution	5% non-fat dry skim milk in 1X TBS-T
Tris Buffers for SDS-PAGE	1.5 M pH: 8.8 1 M pH: 6.8
1X Transfection Buffer	1X Tris-EDTA 150 mM NaCl
1X Transfer Buffer	10% 10X SDS-PAGE Running Buffer 20% Methanol 70% Double Distilled Water
1X TBS-T	10% 10X TBS 0.1% Tween-20 90% dH ₂ O
1X SDS-PAGE Running Buffer	10% 10X SDS-PAGE Running Buffer 90% dH ₂ O

Table 4.3: List of SDS-PAGE Preparation.

10% Separating Gel	10 ml	8% Separating Gel	10 ml	5% Stacking Gel	3 ml
Autoclaved ddH ₂ O	4.8 ml	Autoclaved ddH ₂ O	5.3 ml	Autoclaved ddH ₂ O	2.19 ml
40% Acrylamide-Bisacrylamide	2.5 ml	40% Acrylamide-Bisacrylamide	2 ml	40% Acrylamide-Bisacrylamide	0.375 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	1.5 M Tris-HCl, pH 8.8	2.5 ml	1.5 M Tris-HCl, pH 6.8	0.375 ml
10% SDS	0.1 ml	10% SDS	0.1 ml	10% SDS	0.03 ml
10% APS	0.1 ml	10% APS	0.1 ml	10% APS	0.03 ml
TEMED	0.004 ml	TEMED	0.006 ml	TEMED	0.003 ml

4.3. Antibodies

Table 4.4: Antibodies.

Name	Dilution	Source, Catalog No
Anti-CRY1	1:2000	Bethylabs, #A302-614A
Anti-HA	1:3000	Cell Signalling Technology, #C29F4
Anti- β -Actin	1:5000	Cell Signalling Technology, #C49675
Anti-Rabbit, HRP	1:1000	Thermo Fisher Scientific (#31460)

4.4. Equipment

Table 4.5: Equipment.

Electrophoresis System	Thermo Fisher Scientific, USA
Incubator (CO ₂)	1658004, Mini-PROTEAN [®] Bio-Rad, USA
Inverted Microscope	Nikon, Eclipse, E100
Laminar Flow Cabinet	MN 09/120 Class II Cabinet, Thermo Fisher, Scientific, USA
Centrifuges	521-1647 refrigerated, Micro Star, 17R, VWR, USA 521-1646 Micro Star 17, VWR, USA 521-2844 Mini Star, VWR, USA NF400-Nuve, 02-1827
Chemiluminescence Imaging System	ChemiDoc [™] XRS+ System, Bio-Rad, USA
Power Supply	Bio-Rad, USA
Varioskan Microplate Reader	Thermo Fischer Scientific, USA
Transblot Transfer System	1704155, Bio-Rad, USA
Shaker	Standard Analog shaker, VWR, USA
Vortex	Scientific Industries, USA
Water bath	Wisd, Germany

5. METHODS

5.1. Transformation

CRY1 interactor DNAs previously obtained in our laboratory and all plasmid DNAs required for the dual luciferase reporter assay were provided by Prof. Dr. Halil Kavaklı (Koç University). Ready to use competent DH5 α cells were used for transformation. 50 μ L of bacteria and 1 μ L of plasmid DNA were mixed in a microcentrifuge tube and incubated on ice for 23 minutes. Facilitation of DNA uptake by competent bacteria after 23 minutes was achieved by applying a heat shock. Heat shock was carried out by taking the centrifuge tubes from the ice to 42°C for 1 minute and then again on ice for 1 minute. After these procedures, 980 μ L of Luria Broth (LB) was added to the tubes and incubated at 37°C at 200 rpm for 50 minutes for bacteria to grow and show antibiotic resistance. After incubation, approximately 50 μ L of sample was taken from the tubes and spread on an LB petri dish containing 100 μ g/ μ L ampicillin. The Petri dish was left to incubate at room temperature for 10-15 minutes, then incubated overnight at 37°C. The next day, the petri dish was taken and the colonies formed were selected and liquid culture was started. Selected colonies were seeded in 10 mL of liquid LB containing 100 μ g/ μ L ampicillin. The liquid culture was incubated at 37°C at 200 rpm overnight to induce production of the transformed plasmid.

5.2. Plasmid DNA Isolation

For plasmid isolation, the Macherey-Nagel Nucleospin Plasmid Isolation kit was employed, adhering to the guidelines outlined by the manufacturer. After 22 hours of incubation, bacteria growing in 10 mL were precipitated at 4000 rpm for 10 minutes. The resulting pellet was dissolved in 250 μ L suspension buffer and transferred to microcentrifuge tubes. It was then incubated with 250 μ L of lysis buffer for 4 minutes. After 4 minutes, lysis was stopped with 300 μ L of neutralization buffer, and the tubes were centrifuged at 13,300 rpm for 10 minutes at room temperature. The supernatant was carefully transferred to a miniprep DNA binding column, and a brief centrifugation of 30 seconds at 11,000 x g facilitated the binding of plasmid DNA to the column, allowing the solution to pass through. Subsequently, the column was subjected to 650 mL washing buffer to eliminate impurities. To eliminate any

remaining ethanol, a final centrifugation of 1 minute at 11,000 x g was carried out, resulting in the elution of plasmid DNA into a solution containing 10 mM Tris pH 8.5 and 0.5 mM EDTA.

The plasmid DNA's concentration was determined using a Thermo NanodropLite Spectrophotometer.

5.3. Mammalian Cell Culture

5.3.1. Maintenance of the Cell Line

HEK293T cells, which derived human embryonic kidney 293 cells expressing the simian virus 40 (SV40) large T antigen, were grown and kept in Dulbecco's Modified Eagle Medium (DMEM) containing high (4.5 g/L) glucose and supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S), and 1% non-essential amino acids (NEAA). The cells were incubated in a 37°C incubator containing 5% CO₂ and humidity.

Cells were passaged once a week (80-90% confluency) and the medium was changed every 3-4 days. DMEM was aspirated from cells for passage and washed with 1 mL of 1x PBS. Then 400 µL of 0.05% trypsin-EDTA was added to the cells and incubated at 37°C for 5 minutes. After incubation, 1.2 mL of fresh DMEM was added to the cells and the cells were suspended. After the cells were collected, centrifuged at 1200 x g for 5 minutes and the cells were precipitated. The medium was aspirated without touching the pellet, then the pellet was dissolved with 1 mL of fresh DMEM. 3 mL of fresh DMEM was added to a new 60 mm petri dish and cells were seeded at a 1:10 dilution.

5.3.2. Forward Transfection

Transfection was performed to see the effects of overexpression of CRY1 interactors on CRY1 stability. Transfection was performed with a positively charged transfection agent, Polyethyleneimine (PEI) (Baker et al., 1997).

Within the scope of the experiments of this thesis, the transfection process was carried out with the determined CRY1 interactors in 12-well plates or 96-well plates. Cells

harvested from 60 mm and 100 mm dishes were seeded at 3×10^5 for 12-well plates and transferred to a 37°C incubator. After approximately 18-20 hours, 75 μ L of transfection buffer (containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA (pH 8)) was mixed with 500 and 1000 ng of interactor DNA and 3 μ L of PEI in a centrifuge tube. After incubation at room temperature for 15 minutes, fresh DMEM was added, mixed slowly, and transferred into the wells in equal amounts.

Transfection for the Luciferase assay was performed in 96-well and the amounts of transfection buffer and DNA were changed according to the 12-well plates. 20 μ L of transfection buffer was used for 96-well. Interactor DNAs were used in amounts of 25, 50 and 100 ng, while plasmids 10 ng mPer1-Luc (Experimental reporter), 1 ng pGL4-Renilla (Control of the reporter), 25 ng pCMV-Sport6-Clock, 10 ng pCMV-Sport6-Bmal1, 0.5 ng of pcDNA3-CRY1 and up to 110 ng of empty pCMV-sport6 plasmid were used to bring in total DNA.

5.4. Dual Luciferase Reporter Assay

Before the Luciferase assay, 3x firefly assay buffer and 3x renilla assay buffer were prepared according to the protocol (Their contents are in Table 4.2.). However, luciferin, the substrate of firefly buffer, and Coelenterazine (h-CTZ), the substrate of renilla buffer, were added 1-2 hours before starting the experiment.

Luciferase assay was performed according to the protocol in a previously published article [Cal-Kayitmazbatir et al., 2021]. 4×10^3 cells were seeded into 54 wells of a 96-well plates in 100 μ L of DMEM (3 replicates for the 0, 25, and 50 ng DNA dose ratios of 6 different interactors). After 18 hours, 25 ng and 50 ng interactor DNAs, 10 ng mPer1-Luciferase (Experimental reporter), 1 ng pGL4-Renilla (Control of the reporter), 25 ng pCMV-Sport6-Clock, 10 ng pCMV-Sport6-Bmal1, 0.5 ng pcDNA3-CRY1 and up to 110 ng of pCMV-sport6-empty plasmid were used to bring in total DNA.

The plate was taken from the incubator 24 hours after transfection. 50 μ L of 2x Firefly assay buffer (Diluted from 3X by luciferin addition) was added to each well (Final concentration: 5mM DTT, 0.2mM Coenzyme, 0.15mM ATP, 1.4mg/mL Luciferin, 0.03M Tris-HCl, 0.01M Tris-base, 25Mm NaCl, 1mM MgCl₂, 0.08%Triton X-100). Cells were thoroughly lysed with buffer and incubated for 10 minutes at room

temperature. After incubation, samples in each well were transferred to a 96-well opaque plate (Nunc, 96-well plates) and luminometric measurement was taken on a Varioskan microplate reader (Thermo Fischer Scientific, USA). After Firefly measurement, 75 μ L of 3x Renilla Assay Buffer (to dilute to 1x) was added to each well (0.01mM h-CTZ (in ethanol), 45mM Na₂EDTA, 30mM Na₄P₂O₇, 1.425 M NaCl) and luminometric measurement was taken again. Firefly luciferase activity results were calculated by normalizing according to Renilla activity results.

5.5. Real-Time Bioluminescence Assay

In this experiment, the U2OS-pLV7-Bsd-p(Bmal1)-dLuc cell line was used for real-time monitoring of circadian oscillation. Cells were seeded in an amount of 5×10^4 into a 96-well opaque plate with reverse transfection. Reverse transfection was performed as follows: 25, 50, and 100 ng of interactor DNAs were mixed in 20 μ L transfection buffer containing 100 mM NaCl, and 10 mM Tris.Cl pH 7.5 and 0.5 mM EDTA, respectively. Then, the required amount of PEI was added to each well and each tube was incubated at room temperature for 15 minutes. Meanwhile, the cells were harvested from the 100 mm dish. Then, the 5×10^4 cells in DMEM for each sample were added to each tube in equal amounts after 15 minutes of formation of the transfection mixture. The tubes were mixed gently without shaking and the mixture was transferred as 200 μ l per well.

After approximately 16-18 hours, the medium in the wells was aspirated and washed with 1X PBS. Then, a medium containing 0.1 μ M dexamethasone was added to each well and the cells were reset. Two hours after the resetting process, the medium in the wells was aspirated again, washed with 1X PBS, and this time homemade recording medium (low glucose DMEM powder (with L-glutamine) supplemented with 10 mM HEPES, 0.35 mg/ml of sodium bicarbonate, 3.5 mg/ml of d-(+)-glucose powder, 5% fetal bovine serum, 0.1 mM NEAA, 100 units/ml of penicillin and 100 mg/mL of streptomycin) containing 1 mM luciferin was added to each well as 200 μ L. The plate was covered with a transparent film to cover all wells and placed in Synergy H1 for luminescence reading. Luminescence readings were recorded every 10 min for 120 h at 32 °C.

5.6. Inhibition of Protein Synthesis with CHX

To stop protein synthesis and determine protein amounts, 3×10^5 HEK293T cells were seeded in each well of 12-well plates at 80% confluency. Approximately 18 hours later, the selected interactors were transfected at 500 and 1000 ng. 24 hours after transfection, cells were exposed to cycloheximide (CHX; Sigma, catalog no.: 66819) to a final concentration of 20 $\mu\text{g/mL}$. Cells were harvested every 4 hours for 12 hours after treatment of cycloheximide. The first moment of cycloheximide treatment was determined as timepoint hour 0. Collected samples were subjected to RIPA lysis and Bicinchoninic Protein Assay for Western Blot analysis.

5.7. RIPA Lysis and Bicinchoninic Protein Assay

Tubes from which cells will be collected for RIPA lysis and 12-well plates containing cells were placed on ice. Working on ice to prevent the degradation of proteins was a key point for this step.

Cells in the 12-well plates were harvested 48 h after transfection. For this, the cells were scraped with a scraper with their own medium, transferred to microcentrifuge tubes, and precipitated at 5500 rpm for 4 minutes. After centrifugation, the supernatant in the tubes was removed without touching the pellet, and the cell pellets were washed 2 times with 500 μL of 1X PBS. Cell pellets were lysed with 1.5X RIPA buffer after 2 washes.

1.5X RIPA buffer was prepared from the master stock of 5X RIPA buffer containing 350 μL of 5X, 100 μL of Protease Inhibitor, and 650 μL ddH₂O water. Cell pellets were lysed with minimum 40 and maximum 100 μL 1.5X RIPA buffer according to their density. It was then incubated on ice for 20 minutes, provided that it vortexed every 5 minutes. After 20 minutes, it was centrifuged at 13,300 rpm for 30 minutes at +4 °C and the supernatant, protein lysate, was transferred to new microcentrifuge tubes.

To determine the protein concentration, the Pierce™ BCA Protein Assay Kit (Thermo Fischer, 23225) was utilized. The A and B solutions were combined in a 49:1 ratio, followed by transferring 5 μL of each sample and RIPA buffer (blank) to 96-well plates. Next, 200 μL of BCA mix was added to the plate and briefly mixed, which was

then incubated at 37 °C for 30 minutes. If there were bubbles on the surface of the wells after the incubation, they were eliminated using a needle. A photometric measurement was performed on the Varioskan microplate reader (Thermo Fischer Scientific, USA) at 562 nm. To equalize the protein amount and total volume of each sample, except the lowest one, each sample was diluted by 1,5X RIPA buffer and standardized. Finally, 4X laemmli buffer with β -mercaptoethanol was added to each sample to 1,5X final concentration, and the samples were examined using SDS-PAGE (SDS-Polyacrylamide gel electrophoresis).

5.8. Western Blotting

To prepare the SDS-PAGE gel, the glasses were cleaned with 40% alcohol and placed in cassettes. The SDS-PAGE gel was prepared in two steps, consisting of top gel (stacking) and bottom gel (separating). Separating gel was prepared to be 10% or 12% according to the size of the proteins. After pouring the separating gel, isopropanol was poured on it and the gel was waited for freezing. After the acrylamide polymerized and the gel frozen, it was removed with isopropanol dH₂O. Stacking gel (5%) was prepared and poured, and 1.5 mm combs with 15 wells were attached to it without polymerization.

After the stacking gel was polymerized and frozen, the cassette was taken to the tank, and the inside of the tank was filled with 1X Running Buffer. At this time, the samples were taken from -20 °C and after thawing, they were boiled at 95 °C for 10 minutes. After boiling of the samples, a very short spin was thrown and the samples were ready for loading. Samples lysed with Laemmli Buffer were loaded into the wells as 20 μ L and the gel was run at 80 volts. After the samples came out of the wells and the marker became visible, the gel was taken to 120 volts and its walking was accelerated. After the bromophenol blue came out of the gel, the gel was stopped and for transfer, nitrocellulose membrane and Whatman papers were soaked for 10 minutes with 1X transfer buffer (700 μ l ddH₂O, 100 μ L 10X Running Buffer, 200 μ L Methanol).

The gel was removed from the tank and cassette until the membrane and Whatman papers were ready. After the comb on was carefully removed and thoroughly washed with dH₂O, the glasses were separated from each other, and the gel was placed in 1X transfer buffer. Transfer of the gel to the membrane was performed by placing 2

Whatman papers, membrane, gel, and 2 Whatman papers on top of each other, respectively. The transfer was done with the Trans-blot Turbo Transfer System (Serial No: 690BR011760, Biorad). The device was operated at 12 Volts for 60 minutes.

At the end of the transfer process, the membrane was removed from the transfer device and blocked with skim milk powder dissolved in 20 mL 1X TBS-T (1X TBS with 0.1% Tween-20) for 60 minutes. Blocking was done on a shaker at room temperature. At the end of 1 hour, the membrane was taken to the primary antibodies prepared as 10 mL in 50 mL falcons. Antibodies were prepared with 1% sodium azide and 5% skimmed milk powder. During this thesis study, anti-CRY1, anti-Actin, and anti-HA antibodies were used. The membrane was incubated in the antibody at +4 °C overnight on a rotater.

The day after incubation, the membrane was removed from +4 °C and washed in TBS-T for half an hour, changing every 5, 15, 5, and 5 minutes. After washing, the membrane was incubated with secondary antibodies on a shaker and at room temperature for 1 hour. Secondary antibodies were determined according to primary antibodies. After 1 hour of incubation, the membrane was washed with TBS-T for 30 minutes as in the first wash.

After the washing of the membrane was completed, 700 µL of chemiluminescent agent (WesternBright Sirius HRP substrate, Advansta Inc., CA, USA) was prepared to image the protein bands. For this, 350 µL of solution A and 350 µL of solution B was mixed and spread on the membrane after mixing well. The membrane was then incubated for 3.5 minutes, excess agent was removed, and the membrane was imaged with the Chemidoc™ XRS+ System with ImageLab™ (#1708265, Biorad).

5.8.1. Analysis of CRY1 Stability

To assess the impact of CRY1 interactor overexpression on CRY1 stability, the analysis of specific bands obtained from western blotting (immunoblotting) was conducted. The bands corresponding to CRY1 and the loading control (β-Actin) were selected from images captured with a linear exposure time, using the ImageLab™ Software. Subsequently, the software calculated the intensity of the band signals while removing background noise. The intensity values of the CRY1 bands were normalized against the intensities of the loading control bands. For the control group, a value of

100 was assigned, and all other normalized intensities were divided by the intensities of the control group and multiplied by 100, enabling the evaluation of the change in CRY1 bands as a percentage.

5.9. Statistical Analysis

The data was presented in the format of mean $\pm$ SEM (Standard Error of Mean). Statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software, California, USA). Group differences were assessed using either Student's t-test or one-way analysis of variance (ANOVA), followed by Tukey post hoc tests. The specific statistical tests applied in each experiment, along with their respective p-values, were clearly outlined in the figure legends.

6. RESULTS

6.1. Effects of CRY1 Interactors on *Per1* Promoter

CRY1 protein, known to interact with PER, is a protein that has an important place in the regulation of the biological clock. For this reason, proteins previously known to interact with CRY1 were subjected to dual-luciferase assay in our laboratory, and their dose-dependent effects were observed under the *Per1* promoter. As a result of the optimization experiments, the most appropriate CRY1 dose was determined to be 0.5 ng, and the amount of CRY1 was kept constant at 0.5 ng in dose-dependent experiments (**Figure 6.1**). Dose-dependent experiments to which the interactors were subjected were performed with 25-50 or 50-100 ng of DNA. The control group is labeled as 0 ng and contains all plasmids except interactor DNAs.

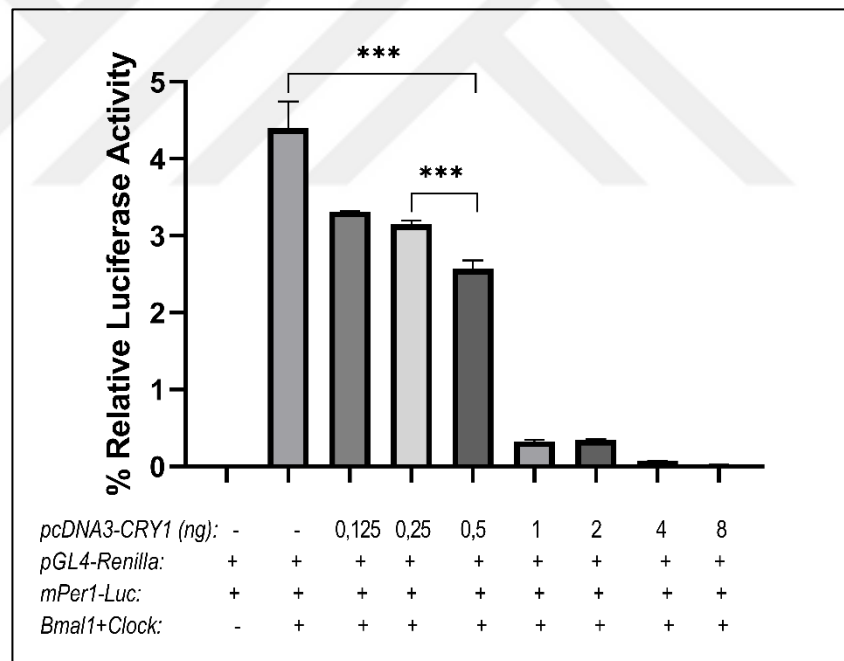


Figure 6.1: CRY1 dose determination chart. (one-way ANOVA and Tukey post-hoc test was used for analysis. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****)).

As a result of all dual-luciferase experiments, 6 interactors that decreased relative luciferase activity dose-dependently were selected as candidates for further experiments (**Figure 6.2**).

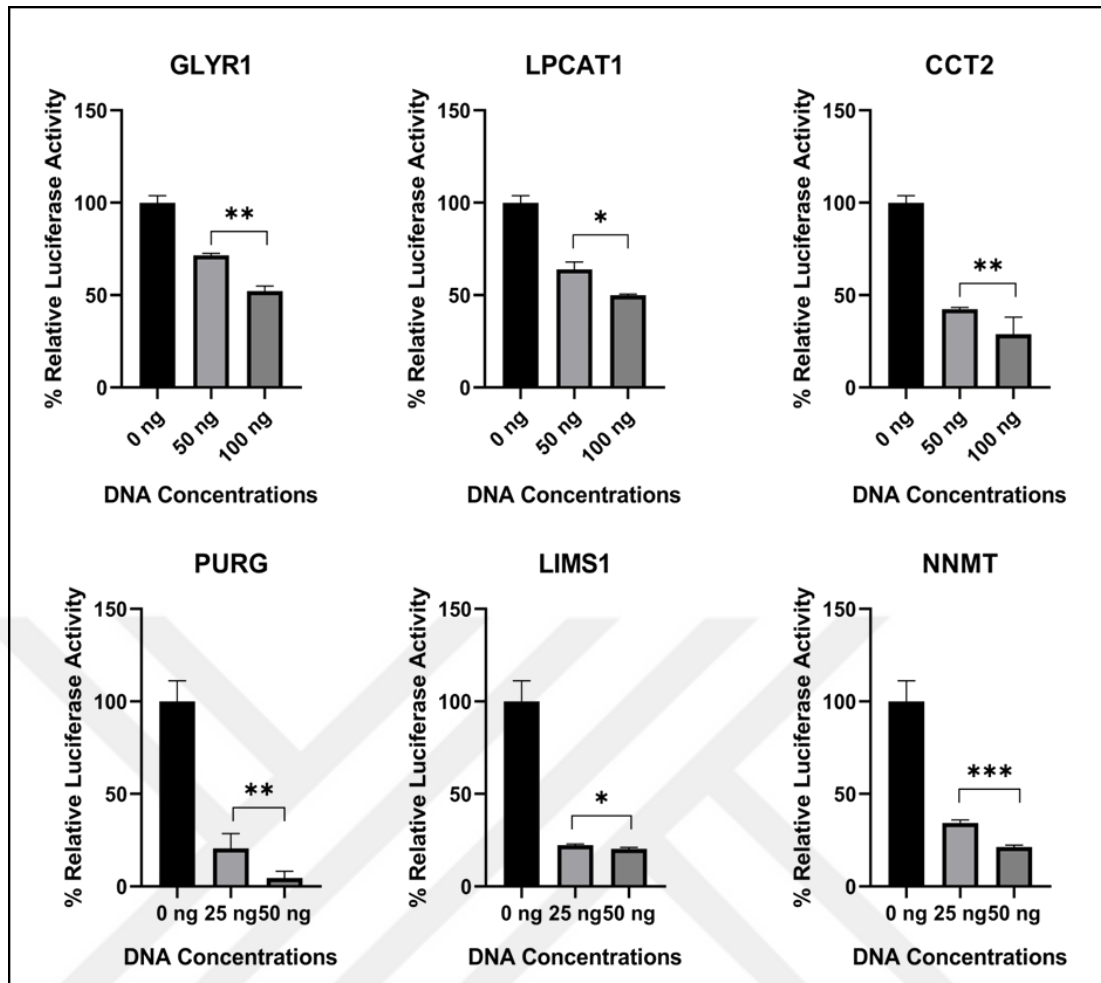


Figure 6.2: Six selected interactors that showed a significant dose-dependent decrease as a result of the dual luciferase reporter assay. (Unpaired t test was used for analysis. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****)).

6.2. Effects of Six Selected CRY1 Interactors on the Circadian Rhythm

It was a matter of curiosity how the six interactors selected as a result of dual-luciferase experiments affected the circadian rhythm. For this, the U2OS-pLV7-Bsd-p(BMAL1)-dLuc cell line was used and the selected interactor DNAs were transfected into this cell line at 50 and 100 ng. As a result of luminometric readings taken every 10 minutes for 120 hours, it was clarified how the interactors affect the circadian rhythm. Data such as period and amplitude were calculated in line with the reading results, and it was observed which interactor had what effect on the rhythm (**Figure 6.3**).

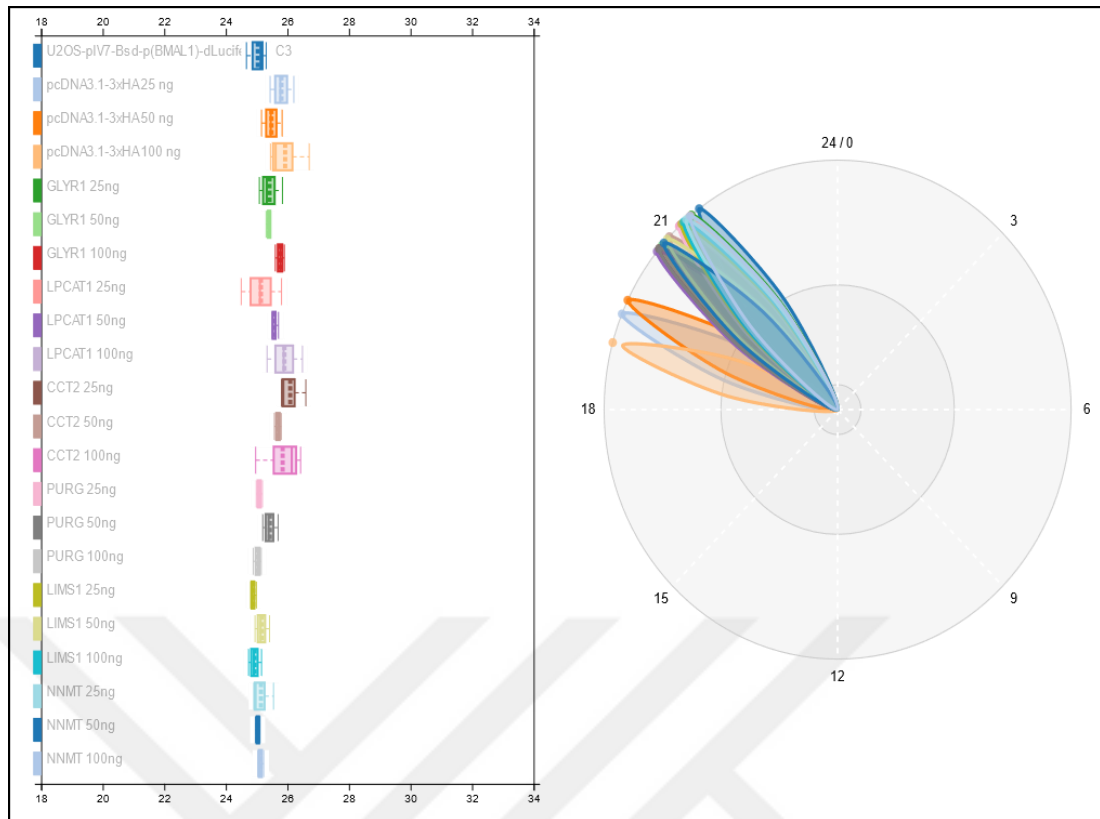


Figure 6.3: CRY1 interactors dose-dependent effect on the circadian rhythm of U2OS Bmal1-dLuc cells. pcDNA3.1-3xHA (empty vector) was used as a control. Analysis was performed in Biodare2.

The determination of period and amplitude values was conducted by utilizing the web tool Biodare2 (accessible at biodare2.ed.ac.uk) and Chronostar, as outlined in Maier et al. (2021). Interactors had different effects on the circadian rhythm (**Figure 6.4**), and (**Figure 6.5**).

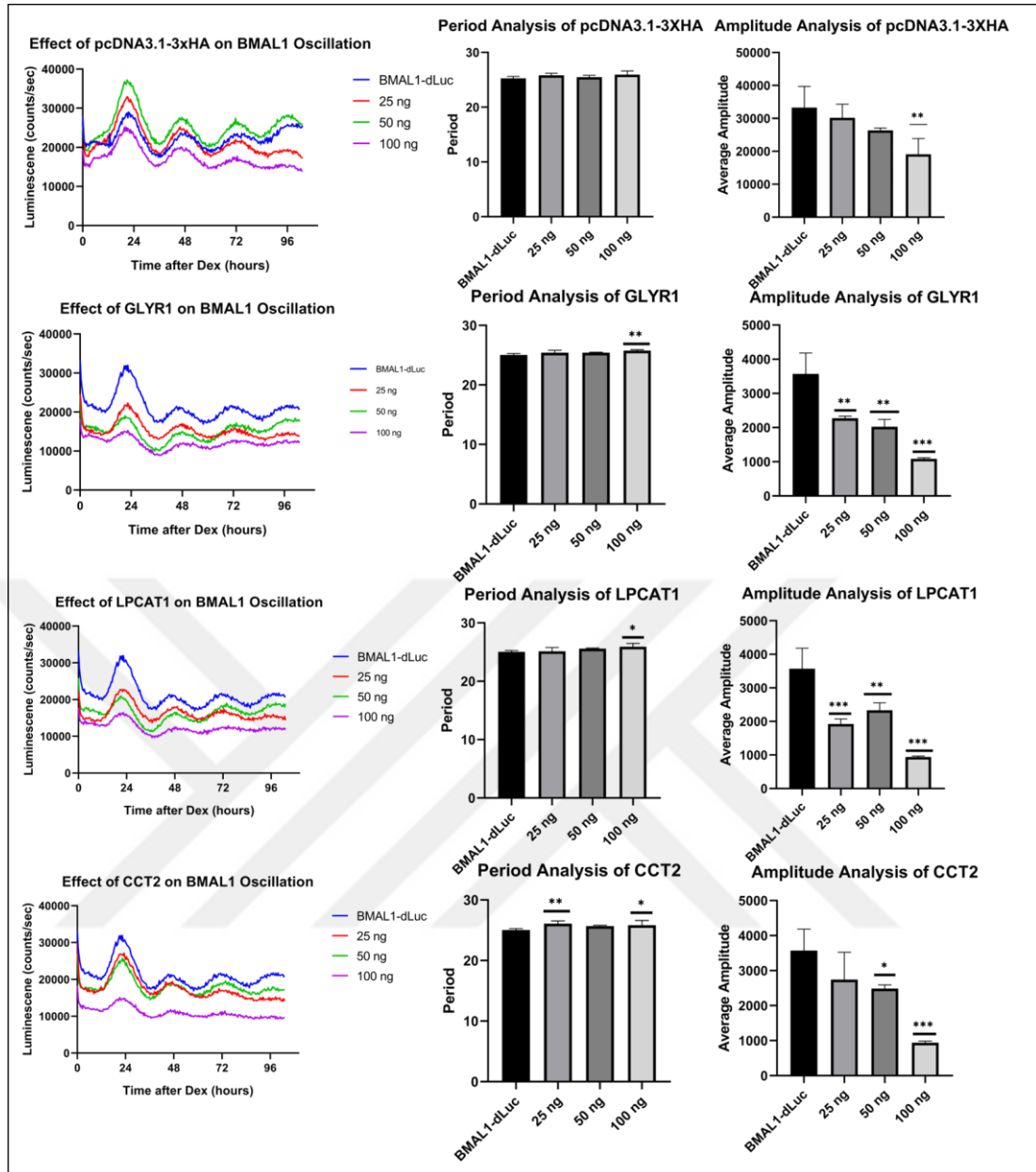


Figure 6.4: Dose-dependent effect of CRY1 interactors GLYR1, LPCAT1, and CCT2 on circadian rhythm in U2OS Bmal1-dLuc cells. pcDNA3.1-3xHA (empty vector) was used as a control. Analysis was performed in Chronostar. (one-way ANOVA and Tukey post-hoc test was used for analysis. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****)).

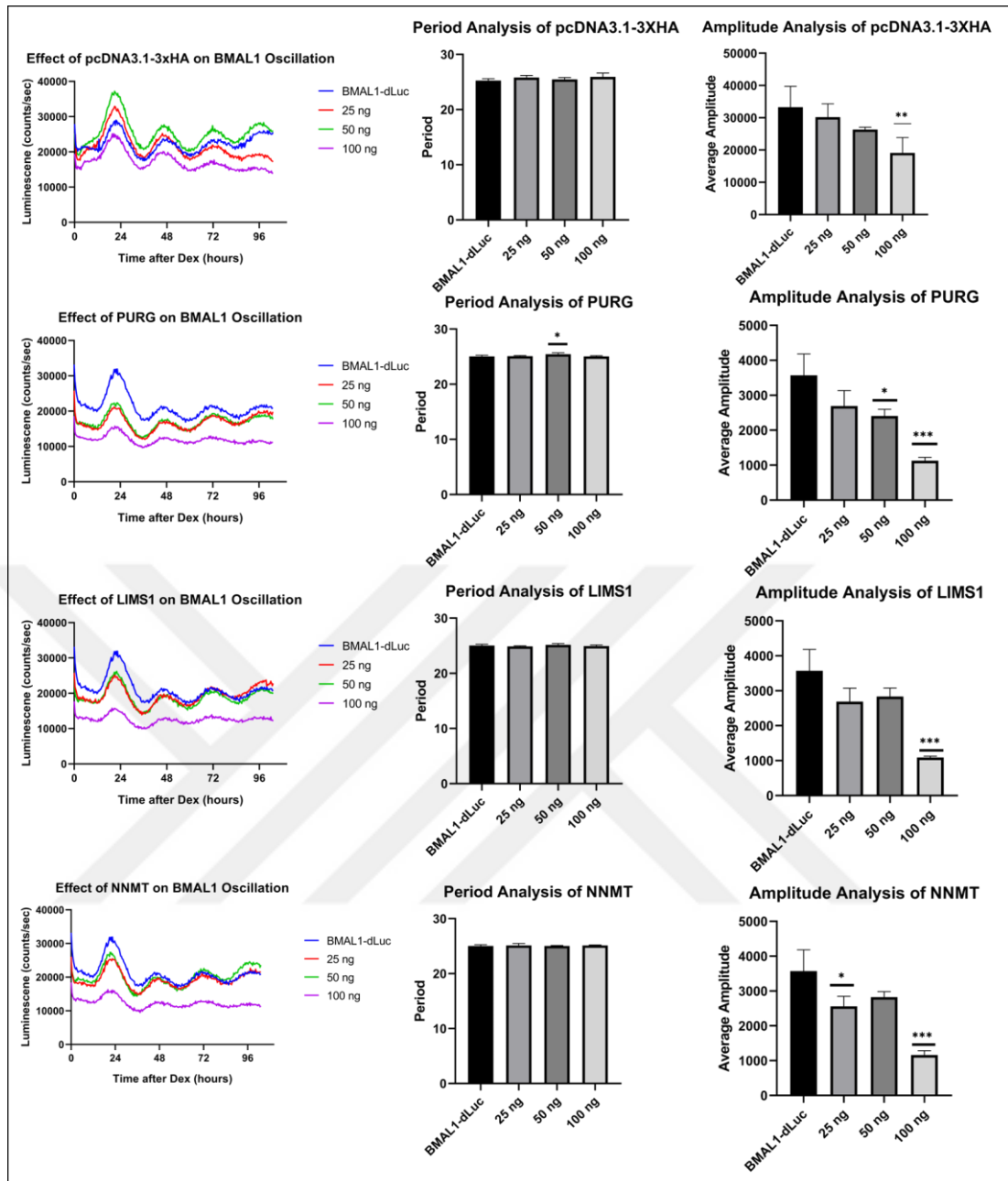


Figure 6.5: Dose-dependent effect of CRY1 interactors PURG, LIMS1, and NNMT on circadian rhythm in U2OS Bmal1-dLuc cells. pcDNA3.1-3xHA (empty vector) was used as a control. Analysis was performed in Chronostar. (one-way ANOVA and Tukey post-hoc test was used for analysis. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****)).

6.3. Determination of CRY1 Stability

The dose-dependent effect of the six selected interactors on CRY1 stability was determined with the previously mentioned (Section 5.6) cycloheximide (CHX) treatments. Interactors were transfected into HEK293T cells at doses of 500 and 1000 ng and treated with CHX after 24 h of growth. Samples were first collected for all interactors at the end of the 12th hour and the CHX effect was observed. All samples were subjected to RIPA lysis as previously stated and BCA analysis to determine protein amount (Section 5.7). After the proteins were obtained and their amounts equalized, western blot analyses were performed (Section 5.8). CRY1 stability was determined with anti-CRY1, and β -Actin was used for normalization. 3xHA-miniTurbo-NLS_pcDNA3 (Catalog number: 107172) was used as a control. Dose-dependent effects of all interactors at the end of 12 hours were determined by western blot analysis (**Figure 6.6**) and (**Figure 6.7**).

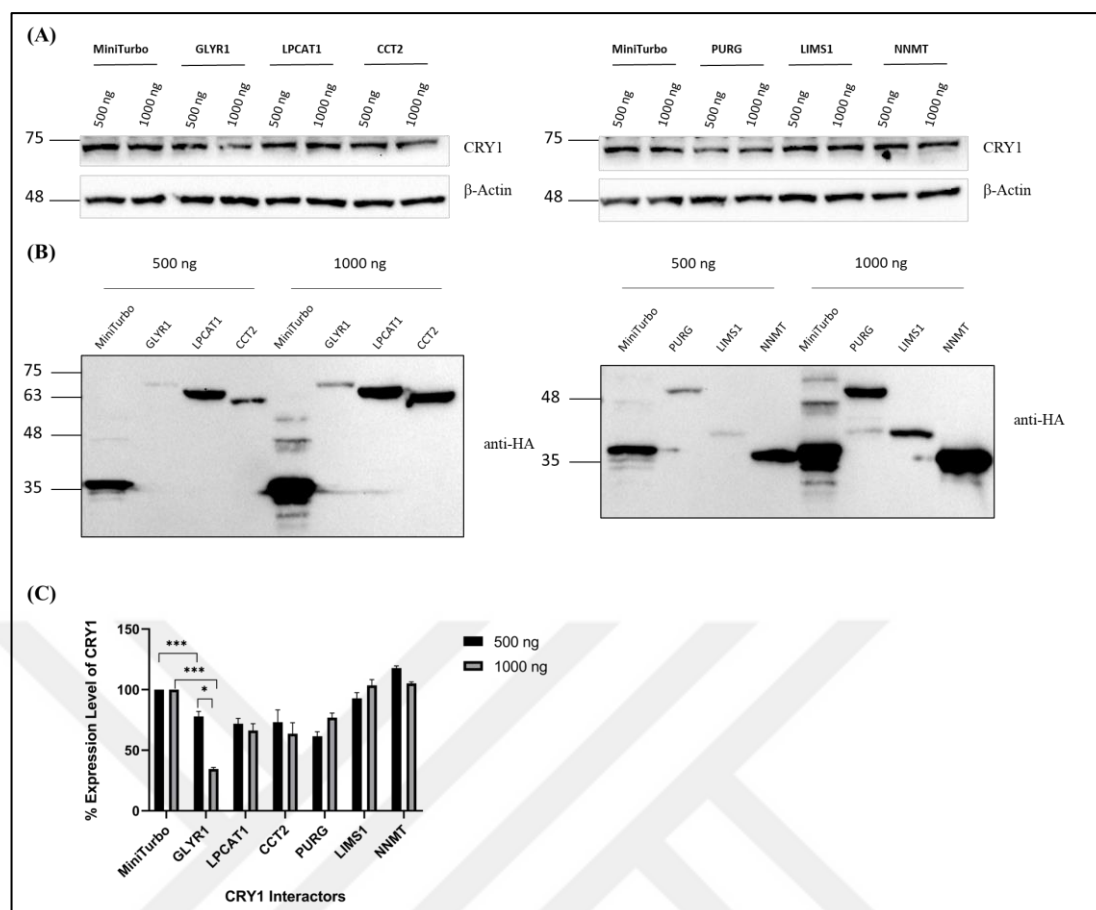


Figure 6.6: Dose-dependent effects of selected six CRY1 interactors at the end of 12 hours were determined by western blot analysis. A) Effect of different doses of interactors on CRY1 level in HEK293T cells. B) Expression of CRY1 interactors with HA tag at different doses on HEK293T cells. C) Effect of interactors and their different doses on CRY1 levels on HEK293T cells (one-way ANOVA with host-doc Tukey, *** $p = 0.001$ ** $p < 0.01$, $n=3$).

After 12 hours, one of the interactors, GLYR1, which clearly showed a decrease in CRY1 stability both time and dose-dependent, was selected for a more detailed screening (Figure 6.6C). GLYR1 interactor was transfected into HEK293T cells in the same way at 500 and 1000 ng and treated with CHX 24 hours later. The first moment CHX was given was considered hour 0, and then samples were collected every 4 hours for 12 hours. All collected samples were analyzed after going through the same RIPA lysis, BCA, and western blot stages, and clearer results were obtained.

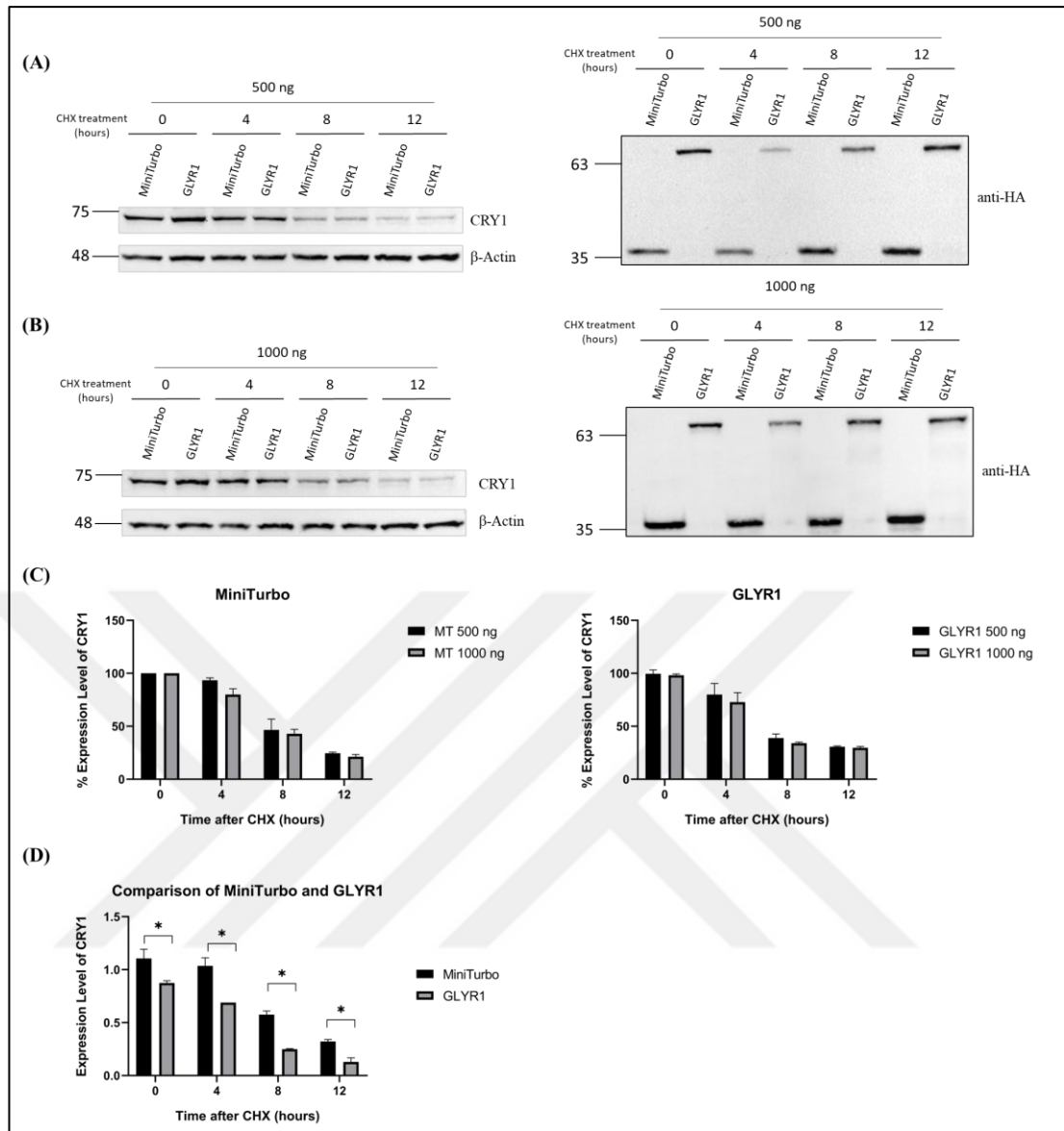


Figure 6.7: Dose-dependent effects of interactor GLYR1 on CRY1 stability with CHX treatment. A) Screening of the effect and expression of interactor GLYR1 and MiniTurbo on CRY1 stability as a result of 500 ng transfection dose. B) Screening of the effect and expression of interactor GLYR1 and MiniTurbo on CRY1 stability as a result of 1000 ng transfection dose. C) Effect of MiniTurbo and GLYR1 and their different doses on CRY1 levels on HEK293T cells (one-way ANOVA with host-doc Tukey). D) Comparison of MiniTurbo and GLYR1 on CRY1 stability as a result of 1000 ng transfection dose. (Unpaired t test was used for analysis. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****)).

When comparing the transfection doses of 500 and 1000 ng for GLYR1, no significant decrease was observed between the two doses (Figure 6.7C); however, a significant decrease in CRY1 stability was obtained compared to the control group MiniTurbo at the 1000 ng transfection dose (Figure 6.7D).

7. DISCUSSION

The circadian clock is a mechanism consisting of a series of molecular interactions and feedback loops, usually a protein-based system, that helps organisms to adapt to the Earth's 24-hour day-night cycle. Although the role of individual protein-protein interactions on the cycle is great, research has shown that clock proteins serve as a part of macromolecular protein complexes and facilitate the control of the cycle. For this reason, protein-protein interactions are of great importance in regulating the circadian clock, and studies continue to be carried out in this context [Mosier and Hurley, 2021].

This thesis was conducted to better understand the roles of PPIs in circadian rhythm. Because better elucidation of circadian PPIs and access to more information may contribute to the discovery of new treatments for diseases and exploring new biological processes in which biological clock is involved. It is very important to know these interactions for many other metabolic diseases other than circadian rhythm-related diseases [Ruan et al., 2021].

The main aim of the study was to investigate the effect of CRY1 interactors on the circadian rhythm and CRY1 stability. These interactors were previously identified in our laboratory by a graduate student using the BioID method; here, CRY1 was fused to a biotin ligase, allowing biotin affinity-tagged proteins to be identified via mass spectrometry analysis [Olfaz, 2020].

In our research, we primarily examined how 23 candidate interactor proteins change the relative luciferase activity under a constant CRY1 dose at the DNA level and in case of overexpression of the proteins. Since the dual luciferase reporter assay was performed under the *Per1* promoter, relative luciferase activity actually allowed us to reach a conclusion about PER1 levels. This allowed us to comment on how *Per1* promoter activity changes at constant CRY1 level and how overexpression of these interactors affects the clock. According to the results, we determined that six of the interactors significantly reduced luciferase activity in a dose-dependent manner (Figure 6.2). It can be inferred that increasing doses of CRY1 interactors decrease *Per1* promoter activity, consequently enhancing the suppressive capacity of CRY1 on CLOCK:BMAL1.

The six interactors, which were determined to enhance CRY1's suppressive capacity under the *Per1* promoter in a dose-dependent manner, were subsequently subjected to real-time bioluminescence experiments under the *Bmal1* promoter. Through real-time bioluminescence, we examined how these interactors affected concepts such as circadian rhythm, period, and amplitude. Real-time bioluminescence experiments were performed with interactor DNAs at doses of 25, 50, and 100 ng, and the empty vector pcDNA3-3xHA without interactor DNA was used as a control. The obtained results were analysed using Biodare2 and Chronostar tools. Data normalization was performed for Biodare2 using the Amp&Baseline trend reduction method. To find the period, the FFT-NLS method was employed, and the wave with the lowest error rate was selected. Average amplitudes were used for the amplitude values. While it was observed that interactors did not have any effect on the period, it was observed that they changed the amplitude depending on the dose increase (Figure 6.4 and Figure 6.5). Although the impact of some was significant while others were not, stability experiments continued with the same six interactors.

The impact of the interactors on CRY1 was examined using Western Blot. Interactors were tested at dose-dependent concentrations of 500 and 1000 ng. Protein synthesis was halted for 24 hours with cycloheximide after transfection. The moment cycloheximide was administered was designated as 0-hour, and initial samples were collected. The final samples were collected and analyzed at the 12-hour mark. The pcDNA3-3xHA-MiniTurbo was used as the control group. When compared to the control group, GLYR1, which showed the most significant dose-dependent decrease at the end of 12 hours, was selected for a more detailed analysis (Figure 6.6).

According to the Western Blot analysis of GLYR1, it was observed that both dose and CHX conditions negatively affected the stability of CRY1 compared to the control group MiniTurbo (Figure 6.7).

Considering all the obtained results; it was observed that the interactors increased the suppression capacity of CRY1 in the dual luciferase reporter assay, negatively affected the stability of CRY1 as the dose increased in Western blot experiments, and in bioluminescence experiments, these interactors did not alter the period but affected the amplitude. If the period remains unchanged but the amplitude decreases, this situation may indicate that the molecular-level clock is weaker or suppressed compared to normal. The decrease in amplitude in circadian oscillation is attributed to the reduction

in *Bmal1* promoter activity. In this scenario, it is speculated that GLYR1, at increasing doses, negatively affects *Bmal1* promoter activity. Variations in *Bmal1* promoter activity may arise from differences in the system responsible for BMAL1 transcription, referred to as the secondary loop. These differences could potentially block *Bmal1* promoter activity at the gene level or suppress the function of positive transcription factors for the *Bmal1* promoter at the protein level.

Furthermore, looking at all these results, it has been determined that the interactor of GLYR1 has a more pronounced effect on the stability of CRY1 at the 1000 ng transfection dose. The lack of such a noticeable effect at the 500 ng transfection dose might be due to the insufficiency of the transfection dose. Although it is observed that this interaction has a negative effect on CRY1 stability compared to the control group, perhaps better results can be obtained at higher transfection doses. Thus, a dose-dependent effect can be more effectively observed.

Even though these interactors negatively affect the stability of CRY1, the fact that they increase its suppression capacity actually indicates a strong influence on CRY1. Despite the decreased protein stabilization in the environment, the increase in the inhibition activity of CRY1 demonstrates the significant impact of these interactors on CRY1.

GLYR1, one of the interactors that most notably negatively affects CRY1 stability, not only plays a role in chromatin modification and the regulation of gene expression but also participates in transcriptional activation, acting as a nucleosome destabilizer [Fang et al., 2013; Marabelli et al., 2019; Fei et al., 2018]. Epigenetic changes, such as nucleosome destabilization involving GLYR1, are also subject to the concept of the epigenetic clock. When the situation is like this, the intervention of the biological clock in the epigenetic clock mechanism is an undeniable fact. Considering the results obtained from this study, it suggests the potential for CRY1 -GLYR1 interaction partner- to play a significant role in the regulation of the epigenetic clock. Additionally, it is involved in recognizing and binding to Histone H3 and plays a role in DNA binding [Vermeulen et al., 2010; Marabelli et al., 2019]. The connection between Histone H3 and GLYR1, and the MS analysis results obtained in our laboratory, suggest that the interaction between GLYR1 and CRY1, there may be an indirect interaction between CRY1 and Histone H3. In an indirect mechanism, when

CRY1 interacts with GLYR1, it could potentially alter the functional capacity of GLYR1 and, Histone H3 as well.

In addition, GLYR1 is assumed to have a high mutation frequency of microsatellite instability in colorectal cancer (MSI CRC) and is considered a novel tumor suppressor [Hu et al., 2020]. The first documentation of GLYR1's role in tumors in 2012 revealed a mutation frequency of 51% in MSI CRC, highlighting its potential as a new tumor suppressor [Alhopuro et al., 2012]. Some clinical studies have revealed that the disruption of circadian rhythm accelerates the progression of CRC [Lévi et al., 2014; Innominato et al., 2009]. Abnormal expression levels of clock genes have been observed in CRC tissues, and current research suggests that clock genes, especially *Per*, *Cry*, *Bmal1*, and *Clock*, may influence the progression of CRC [Stokes et al., 2021]. Specific genetic variations or mutations in the *Cry1* gene may contribute to the disruption of the circadian rhythm, potentially playing a role in the development of CRC. Additionally, given that CRY1 may have tumor suppressor properties, its interaction with GLYR1 could play a significant role in CRC.

The exploration of untested interactions of GLYR1 and CRY1 can contribute to a better understanding of the circadian clock mechanism and potentially facilitate the development of new treatment strategies. This research could deepen our scientific understanding by uncovering the complex interactions underlying biological clock systems, paving the way for future studies. Specifically, focusing on the interactions of GLYR1 and CRY1 has the potential to illuminate significant mechanisms in the regulation of circadian rhythms. This could not only enhance our understanding of the functioning of circadian clocks but also lead to the discovery of new therapeutic targets associated with various health issues, such as sleep-wake disorders. Therefore, investigating other factors interacting with GLYR1 and CRY1 may yield important implications for both basic science and clinical application. In this regard, comprehensive experimental studies as well as bioinformatics and genomic analyses could contribute to understanding interaction mechanisms. Thus, a thorough understanding of the molecular and cellular mechanisms underlying circadian rhythms could be a crucial step toward the development of new treatment strategies and the improvement of existing ones.

REFERENCES

- Ahmad M., Cashmore A. R., (1993), "HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor.", *Nature*, 366(6451), 162-166.
- Albrecht U., (2012), "Timing to perfection: the biology of central and peripheral circadian clocks.", *Neuron*, 74(2), 246-260.
- Alhopuro P., Sammalkorpi H., Niittymäki I., Biström M., Raitila A., Saharinen J., ... Aaltonen L. A., (2012), "Candidate driver genes in microsatellite-unstable colorectal cancer. ", *International journal of cancer*, 130(7), 1558-1566.
- Aschoff J., (1981), "*A survey on biological rhythms.*", (pp. 3-10). Springer US.
- Baker A., Saltik M., Lehrmann H., Killisch I., Mautner V., Lamm G., ... Cotten M., (1997), "Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery.", *Gene therapy*, 4(8), 773-782.
- Beaver L. M., Gvakharia B. O., Vollintine T. S., Hege D. M., Stanewsky R., Giebultowicz J. M., (2002), "Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*.", *Proceedings of the National Academy of Sciences*, 99(4), 2134-2139.
- Busza A., Emery-Le M., Rosbash M., Emery P., (2004), "Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception.", *Science*, 304(5676), 1503-1506.
- Cal-Kayitmazbatir S., Kulkoyluoglu-Cotul E., Growe J., Selby C. P., Rhoades S. D., Malik D., ... Kavakli I. H., (2021), "CRY1-CBS binding regulates circadian clock function and metabolism.", *The FEBS journal*, 288(2), 614-639.
- Cashmore A. R., (2003), "Cryptochromes: enabling plants and animals to determine circadian time.", *Cell*, 114(5), 537-543.
- Ceriani M. F., Darlington T. K., Staknis D., Más P., Petti A. A., Weitz C. J., Kay S. A., (1999), "Light-dependent sequestration of TIMELESS by CRYPTOCHROME.", *Science*, 285(5427), 553-556.
- Chaves I., Yagita K., Barnhoorn S., Okamura H., van der Horst G. T., Tamanini F., (2006), "Functional evolution of the photolyase/cryptochrome protein family: importance of the C terminus of mammalian CRY1 for circadian core oscillator performance.", *Molecular and cellular biology*, 26(5), 1743-1753.
- De Assis L. V. M., Oster H., (2021), "The circadian clock and metabolic homeostasis: entangled networks.", *Cellular and Molecular Life Sciences*, 78(10), 4563-4587.

Dissel S., Codd V., Fedic R., Garner K. J., Costa R., Kyriacou C. P., Rosato E., (2004), "A constitutively active cryptochrome in *Drosophila melanogaster*.", *Nature neuroscience*, 7(8), 834-840.

Dodd A. N., Salathia N., Hall A., Kévei, E., Tóth R., Nagy F., Webb A. A., (2005), "Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage.", *Science*, 309(5734), 630-633.

Duffy J. F., Wright Jr K. P., (2005), "Entrainment of the human circadian system by light.", *Journal of biological rhythms*, 20(4), 326-338.

Emery P., So W. V., Kaneko M., Hall J. C., Rosbash M., (1998), "CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity.", *Cell*, 95(5), 669-679.

Fang R., Chen F., Dong Z., Hu D., Barbera A. J., Clark E. A., ... Shi Y. G., (2013), "LSD2/KDM1B and its cofactor NPAC/GLYR1 endow a structural and molecular model for regulation of H3K4 demethylation.", *Molecular cell*, 49(3), 558-570.

Fei J., Ishii H., Hoeksema M. A., Meitinger F., Kassavetis G. A., Glass C. K., ... Kadonaga J. T., (2018), "NDF, a nucleosome-destabilizing factor that facilitates transcription through nucleosomes.", *Genes & development*, 32(9-10), 682-694.

Golombek D. A., Rosenstein R. E., (2010), "Physiology of circadian entrainment.", *Physiological reviews*, 90(3), 1063-1102.

Griffin Jr E. A., Staknis D., Weitz C. J., (1999), "Light-independent role of CRY1 and CRY2 in the mammalian circadian clock.", *Science*, 286(5440), 768-771.

Harms E., Kivimäe S., Young M. W., Saez L., (2004), "Posttranscriptional and posttranslational regulation of clock genes.", *Journal of biological rhythms*, 19(5), 361-373.

Hirano A., Yumimoto K., Tsunematsu R., Matsumoto M., Oyama M., Kozuka-Hata H., ... Fukada Y., (2013), "FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes.", *Cell*, 152(5), 1106-1118.

Horst G. T. V. D., Muijtjens M., Kobayashi K., Takano R., Kanno S. I., Takao M., ... Yasui A., (1999), "Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms.", *Nature*, 398(6728), 627-630.

Hu Z., Long T., Ma Y., Zhu J., Gao L., Zhong Y., ... Li Z., (2020), "Downregulation of GLYR1 contributes to microsatellite instability colorectal cancer by targeting p21 via the p38MAPK and PI3K/AKT pathways.", *Journal of Experimental & Clinical Cancer Research*, 39(1), 1-15.

Innominato P. F., Focan C., Gorlia T., Moreau T., Garufi C., Waterhouse J., ..., Chronotherapy Group of the European Organization for Research and Treatment of Cancer, (2009), "Circadian rhythm in rest and activity: a biological correlate of quality of life and a predictor of survival in patients with metastatic colorectal cancer.", *Cancer research*, 69(11), 4700-4707.

Jonathan S. Y., Díaz N. M., D'Souza S., Buhr E. D., (2022), "The molecular clockwork of mammalian cells.", In *Seminars in Cell & Developmental Biology* (Vol. 126, pp. 87-96). Academic Press.

Kavakli I. H., Ozturk N., Baris I., (2022), "Protein interaction networks of the mammalian core clock proteins.", *Advances in Protein Chemistry and Structural Biology*, 131, 207-233.

Khan S. K., Xu H., Ukai-Tadenuma M., Burton B., Wang Y., Ueda H. R., Liu A. C., (2012), "Identification of a novel cryptochrome differentiating domain required for feedback repression in circadian clock function.", *Journal of Biological Chemistry*, 287(31), 25917-25926.

Ko C. H., Takahashi J. S., (2006), "Molecular components of the mammalian circadian clock.", *Human molecular genetics*, 15(suppl_2), R271-R277.

Lamia K. A., Papp S. J., Yu R. T., Barish G. D., Uhlenhaut N. H., Jonker J. W., ... Evans R. M., (2011), "Cryptochromes mediate rhythmic repression of the glucocorticoid receptor.", *Nature*, 480(7378), 552-556.

Lee C., Etchegaray J. P., Cagampang F. R., Loudon A. S., Reppert S. M., (2001), "Posttranslational mechanisms regulate the mammalian circadian clock.", *Cell*, 107(7), 855-867.

Lévi F., Dugué P. A., Innominato P., Karaboué A., Dispersyn G., Parganiha A., ... ARTBC Chronotherapy Group, (2014), "Wrist actimetry circadian rhythm as a robust predictor of colorectal cancer patients survival.", *Chronobiology international*, 31(8), 891-900.

Lin C., Todo T., (2005), "The cryptochromes.", *Genome biology*, 6, 1-9.

Lowrey P. L., Takahashi J. S., (2004), "Mammalian circadian biology: elucidating genome-wide levels of temporal organization.", *Annual Review of Genomics and Human Genetics*, 5, 407-441.

Maier B., Lorenzen S., Finger A. M., Herzel H., Kramer A., (2021), "Searching novel clock genes using RNAi-based screening.", *Circadian Clocks: Methods and Protocols*, 103-114.

Marabelli C., Marrocco B., Pilotto S., Chittori S., Picaud S., Marchese S., ... Mattevi A., (2019), "A tail-based mechanism drives nucleosome demethylation by the LSD2/NPAC multimeric complex.", *Cell Reports*, 27(2), 387-399.

Miller S., Son Y. L., Aikawa Y., Makino E., Nagai Y., Srivastava A., ... Hirota T., (2020), "Isoform-selective regulation of mammalian cryptochromes.", *Nature chemical biology*, 16(6), 676-685.

Mosier A. E., Hurley J. M., (2021), "Circadian interactomics: how research into protein-protein interactions beyond the core clock has influenced the model of circadian timekeeping.", *Journal of biological rhythms*, 36(4), 315-328.

Olfaz S., (2020), "Exploring the interactome of recombinant CRY1 protein by proximity labeling.", MSc. Thesis, Gebze Technical University.

Ouyang Y., Andersson C. R., Kondo T., Golden S. I., Johnson C. H., (1998), "Resonating circadian clocks enhance fitness in cyanobacteria.", *Proceedings of the National Academy of Sciences*, 95(15), 8660-8664.

Ozturk N., (2017), "Phylogenetic and functional classification of the photolyase/cryptochrome family.", *Photochemistry and photobiology*, 93(1), 104-111.

Öztürk N., Song S. H., Özgür S., Selby C. P., Morrison L., Partch C., ... Sancar, A., (2007), "Structure and function of animal cryptochromes.", In *Cold Spring Harbor Symposia on Quantitative Biology* (Vol. 72, pp. 119-131). Cold Spring Harbor Laboratory Press.

Parico G. C. G., Partch C. L., (2020), "The tail of cryptochromes: an intrinsically disordered cog within the mammalian circadian clock.", *Cell Communication and Signaling*, 18, 1-9.

Robinson I., Reddy A. B., (2014), "Molecular mechanisms of the circadian clockwork in mammals.", *Federation of European Biochemical Societies letters*, 588(15), 2477-2483.

Roenneberg T., Kuehnle T., Juda M., Kantermann T., Allebrandt K., Gordijn M., Meroow M., (2007), "Epidemiology of the human circadian clock.", *Sleep medicine reviews*, 11(6), 429-438.

Ruan W., Yuan X., Eltzschig H. K., (2021), "Circadian rhythm as a therapeutic target.", *Nature Reviews Drug Discovery*, 20(4), 287-307.

Sancar A., (2004), "Regulation of the mammalian circadian clock by cryptochrome.", *Journal of Biological Chemistry*, 279(33), 34079-34082.

Siepkha S. M., Yoo S. H., Park J., Lee C., Takahashi J. S., (2007), "Genetics and neurobiology of circadian clocks in mammals.", In *Cold Spring Harbor symposia on quantitative biology* (Vol. 72, p. 251). National Institute of Health Public Access.

Stanewsky R., Kaneko M., Emery P., Beretta B., Wager-Smith K., Kay S. A., ... Hall J. C., (1998), "The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*.", *Cell*, 95(5), 681-692.

Stokes K., Nunes M., Trombley C., Flôres D. E., Wu G., Taleb Z., ... Karpowicz P., (2021), "The circadian clock gene, *bmal1*, regulates intestinal stem cell signaling and represses tumor initiation.", *Cellular and molecular gastroenterology and hepatology*, 12(5), 1847-1872.

Takahashi J. S., (2017), "Transcriptional architecture of the mammalian circadian clock.", *Nature Reviews Genetics*, 18(3), 164-179.

Vermeulen M., Eberl H. C., Matarese F., Marks H., Denissov S., Butter F., ... Mann M., (2010), "Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers.", *Cell*, 142(6), 967-980.

Vitaterna M. H., Selby C. P., Todo T., Niwa H., Thompson C., Fruechte E. M., ... Sancar A., (1999), "Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2.", *Proceedings of the National Academy of Sciences*, 96(21), 12114-12119.

Wallach T., Schellenberg K., Maier B., Kalathur R. K. R., Porras P., Wanker E. E., ... Kramer A., (2013), "Dynamic circadian protein-protein interaction networks predict temporal organization of cellular functions.", *PLoS genetics*, 9(3), e1003398.

Wang H., Ma L. G., Li J. M., Zhao H. Y., Deng X. W., (2001), "Direct interaction of Arabidopsis cryptochromes with COP1 in light control development.", *Science*, 294(5540), 154-158.

Yamazaki S., Numano R., Abe M., Hida A., Takahashi R. I., Ueda M., ... Tei H., (2000), "Resetting central and peripheral circadian oscillators in transgenic rats.", *Science*, 288(5466), 682-685.

Yang H. Q., Wu Y. J., Tang R. H., Liu D., Liu Y., Cashmore A. R., (2000), "The C termini of Arabidopsis cryptochromes mediate a constitutive light response.", *Cell*, 103(5), 815-827.

Yoo S. H., Mohawk J. A., Siepka S. M., Shan Y., Huh S. K., Hong H. K., ... Takahashi J. S., (2013), "Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm.", *Cell*, 152(5), 1091-1105.

Zhang E. E., Liu Y., Dentin R., Pongsawakul P. Y., Liu A. C., Hirota T., ... Kay S. A., (2010), "Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis.", *Nature medicine*, 16(10), 1152-1156.

BIOGRAPHY

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Sunbul Buket, Ozturk Nuri, “Functional Characterization of Human CRY1 Interactome”, 7th Graduate Research Symposium in Gebze Technical University-Kocaeli, Turkey, 2023.



APPENDICES

APPENDIX A: PROTEIN MARKER

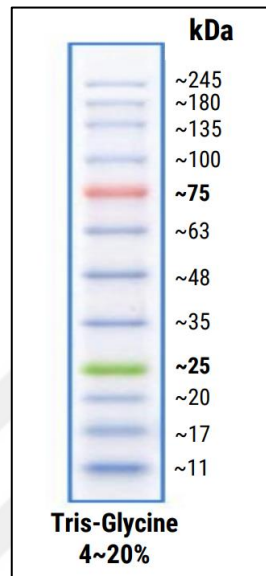


Figure A1.1: Gangnam-Stain™ Prestained Protein Ladder

APPENDIX B: OSCILLATION RAW DATA

Table A1.1: Period and Amplitude values of GLYR1 transfected HEK293T cell line.

Data Label	Amplitude	Period
Bmal1-dLuc	4227.0	24,67
Bmal1-dLuc	2950.0	25,13
Bmal1-dLuc	3276.0	25,19
Bmal1-dLuc	4533.0	24,82
Bmal1-dLuc	3605.0	24,87
Bmal1-dLuc	3145.0	25,21
Bmal1-dLuc	3162.0	24,97
Bmal1-dLuc	3023.0	25,19
Bmal1-dLuc	4250.0	25,31
pcDNA3.1-3XHA-25 ng	2923.0	25,44
pcDNA3.1-3XHA-25 ng	2666.0	25,77
pcDNA3.1-3XHA-25 ng	3471.0	26,21
pcDNA3.1-3XHA-25 ng	2713.0	25,83
pcDNA3.1-3XHA-25 ng	2570.0	25,46
pcDNA3.1-3XHA-25 ng	2621.0	25,16
pcDNA3.1-3XHA-25 ng	1465.0	26,71
pcDNA3.1-3XHA-25 ng	1847.0	25,62
pcDNA3.1-3XHA-25 ng	2414.0	25,45
GLYR1 25NG	2332.0	25,84
GLYR1 25NG	2278.0	25,09
GLYR1 25NG	2205.0	25,33
GLYR1 50NG	1790.0	25,46
GLYR1 50NG	2218.0	25,43
GLYR1 50NG	2065.0	25,31
GLYR1 100NG	1100.0	25,9
GLYR1 100NG	1053.0	25,61