

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EXPLORING NOVEL CRY1 INTERACTIONS

SABA SELVİ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
2021

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

EXPLORING NOVEL CRY1
INTERACTIONS

SABA SELVİ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

THESIS SUPERVISOR
PROF. DR. NURİ ÖZTÜRK

GEBZE

2021

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CRY1 İNTERAKTÖRLERİNİN
BELİRLENMESİ

SABA SELVİ
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

GEBZE
2021



YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 01/07/2021 tarih ve 2021/30 sayılı kararıyla oluşturulan jüri tarafından 30/07/2021 tarihinde tez savunma sınavı yapılan Saba SELVİ'nin tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE

(TEZ DANIŞMANI) : Prof. Dr. Nuri ÖZTÜRK

ÜYE : Prof. Dr. Halil KAVAKLI

ÜYE : Prof. Dr. Tamer YAĞCI

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun
...../...../..... tarih ve/..... sayılı kararı.

İMZA/MÜHÜR

SUMMARY

Circadian clock is an internal autonomous 24-hour oscillating system that mainly sustains the metabolic and genomic homeostasis of almost all living organisms. At the molecular level, four main proteins manage the circadian rhythms via a transcriptional-translational feedback loop mechanism. Two of these proteins (BMAL1 and CLOCK) are the activators, contrarily CRYPTOCHROME and PERIOD, the product of these activators, are the negative regulators of the oscillation. Disruption of the circadian clock is considerably associated with genomic malfunctions and metabolic disorders.

To identify the molecular interaction between circadian clock protein Cryptochrome 1 (CRY1) and DNA damage responses, the interactors of this protein were aimed to be validated by the co-immunoprecipitation method. An HA-tag was fused to the interactor candidates and the generated proteins were transfected into the 3xFLAG-CRY1 expressing stable HEK293T cell line for this purpose. After production of the interactors at the protein level by the stable cells, the protein lysate was loaded into the anti-FLAG antibody-coated magnetic beads. The presence of the interactor was confirmed by western blot analysis performed with an anti-HA antibody to detect the candidate proteins.

To conclude, the novel interactors of the CRY1 protein related to DNA damage and repair pathways were identified. The results were demonstrated that *CRY1* gene is an important protector of cells from DNA damages offering this gene can be targeted for drug therapies.

Key Words: Cryptochrome 1, DNA-Damage Response, Circadian Clock, Protein-Protein Interaction (PPI), Interactor, Co-Immunoprecipitation (co-IP).

ÖZET

Sirkadiyen saat neredeyse bütün canlı organizmaların metabolik ve genomik dengelerini düzenleyen, hücre içinde otomatik olarak oluşturulan 24 saatlik salınımlardan oluşan bir sistemdir. Moleküler düzeyde dört ana protein tarafından transkripsiyonel- translasyonel geribildirim döngüsü mekanizması ile kontrol edilir. Bu proteinlerden iki tanesi (BMAL1 ve CLOCK) aktivatör olarak görev alırken bu aktivatörlerin ürünü olan CRYPTOCHROME ve PERIOD proteinleri bu salınının negatif düzenleyicileridir. Sirkadiyen saatteki bozulmalar genomik işlev bozuklukları ve metabolik hastalıklar ile önemli derecede ilişkilidir.

Sirkadiyen saat proteini Cryptochrome 1 ve DNA hasar yanıtı arasındaki moleküler etkileşimi tanımlamak için bu proteinin interaktörlerinin ko-immünopresipitasyon metodu ile doğrulanması amaçlanmıştır. Bu amaç doğrultusunda aday olarak seçilen interaktörler HA etiketi ile birleştirilip kalıcı olarak 3xFLAG-CRY1 eksprese eden HEK293T hücre hattına transfekte edilmiştir. İnteraktörlerin stabil hücre hattı tarafından protein düzeyinde ekspresyonu gerçekleştikten sonra protein lizatı anti-FLAG antikoru ile kaplanmış manyetik boncuklara yüklenmiştir. Aday interaktör proteinlerin varlığı anti-HA antikoru kullanılarak gerçekleştirilen western blot analizi ile onaylanmıştır.

Sonuç olarak, CRY1 proteininin DNA hasar ve tamir yollarıyla ilişkili olan bilinmeyen interaktörleri belirlenmiştir. Elde edilen sonuçlar CRY1'in hücreleri DNA hasarından koruyan ve ilaç terapilerinde hedeflenebilecek önemli bir gen olduğunu göstermiştir.

Anahtar Kelimeler: Cryptochrome 1, DNA Hasar Yanıtı, Sirkadiyen Saat, Protein-Protein Etkileşimi, İnteraktör, Ko-İmmünopresipitasyon.

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to thesis supervisor Prof. Dr. Nuri ÖZTÜRK for his thoughtful guidance and help. I would like to thank my thesis jury members Prof. Dr. İbrahim Halil KAVAKLI and Prof. Dr. Tamer YAĞCI for spending their valuable time to examining this thesis.

Also, I would like to offer my special thanks to Gözde ÖZÇELİK, who has given endless countenance, and encouragement to me whenever I needed, and other laboratory mates Fatma YILMAZ, Büşra TİRYAKİ, Barış Can ÜLKÜ, Şeyma YAZAN, Serdar KOCA, Satı PALTACI, Kevser ÇİLENK and Feride DEMİRHAN who are always very helpful and friendly.

Lastly, I am indebted to my dear parents Mehmet SELVİ and İnci KARŞI SELVİ, my dear sisters, and my dear best friends for the endless supports and loves.

This study was supported by the Turkish Scientific and Technical Research Council (TUBITAK) grant 118Z085 (to N.O.).

TABLE of CONTENTS

	<u>Page</u>
SUMMARY	v
ÖZET	vi
ACKNOWLEDGEMENTS	vii
TABLE of CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	x
LIST of FIGURES	xii
LIST of TABLES	xiii
1. INTRODUCTION	1
1.1. The Purpose, Contribution and Content of Thesis	1
2. LITERATURE REVIEW	3
2.1. Circadian clock	3
2.2. Transcription Translation Feedback Loop (TTFL)	3
2.3. CRY1: A Member of Photolyase/Cryptochrome Family	6
2.4. CRY1 and DNA Damage Response (DDR)	8
2.5. Co-Immunoprecipitation (co-IP)	10
3. MATERIALS	12
3.1. General Kits and Reagents	12
3.2. Buffers and Solutions	13
3.3. Antibodies	14
3.4. Oligonucleotide Sequences	15
3.5. Equipment	16
4. METHODS	17
4.1. Molecular Cloning	17
4.1.1. PCR Amplifications of Constructs with Cloning Primers	17
4.1.2. Digestion of Plasmid and Oligos with Restriction Enzymes	18
4.1.3. Agarose Gel Electrophoresis	19
4.1.4. Ligation of Inserts into the Plasmid	19
4.1.5. Transformation	19
4.1.6. Plasmid Isolation	20

4.1.6. Confirmation of the Transformation	20
4.2. Cell Culture	21
4.2.1. Maintenance of the Cell Line	21
4.2.2. Transfection	21
4.2.3. Generation of a Stable Cell Line	22
4.3. Co-IP	22
4.3.1. Cell Collection	22
4.3.2. Cell Lysis and Sample Preparation	23
4.3.3. Immunoprecipitation of Interactomes with Magnetic Beads	23
4.4. Western Blot	23
4.4.1. SDS-PAGE Gel Preparation	23
4.4.2. Sample Preparation for SDS Polyacrylamide Gel	24
4.4.3. SDS-PAGE Gel Running	24
4.4.4. Transfer of Proteins from Gel to Membrane	25
4.4.5. Blocking and Immunoblotting	25
4.4.6. Imaging	26
5. RESULTS	27
5.1. Generation of Interactor Expression Constructs	27
5.1.1. Determination of the Candidates	27
5.1.2. Preparation of Plasmids for Cloning	28
5.1.3. Amplification of the Interactors	30
5.1.4. Ligation of the Interactors into the 3xHA-NLS_pcDNA3 Plasmid	31
5.2. Generation of HEK293T-CRY1-3xFLAG Stable Cell Line	32
5.3. Confirmation of the Generation of a FLAG Expressing Stable Cell Line	33
5.4. Expression of the Interactors in the Stable Cell Line	36
5.5. co-IP of Interactors from HEK293T-CRY1-3xFLAG Cell Line	36
6. DISCUSSION	39
REFERENCES	41
BIOGRAPHY	45
APPENDICES	46

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
β -TrCP1	: β transducin-repeat containing protein 1
$^{\circ}\text{C}$	: Celsius
μg	: Microgram
μl	: Microliter
APS	: Ammonium persulfate
ARNT	: Aryl hydrocarbon receptor nuclear translocator-like protein
ATR	: Ataxia-and Rad-Related
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle ARNT-like1
bp	: Base pair
CCG	: Circadian-controlled genes
CHK1	: Checkpoint kinase 1
CK1	: Casein kinase 1
co-IP	: co-immunoprecipitation
CRY	: Cryptochrome gene or protein
<i>Cry1^{-/-} Cry2^{-/-}</i>	: CRY double knockout
ddH ₂ O	: Double-distilled water
DD	: Constant darkness
DMEM	: Dulbecco's Modified Eagle's Medium
EDTA	: Ethylene-diamine-tetra-acetic acid
FBXL	: F-box/leucine-rich repeat protein
FAD	: Flavin adenine dinucleotide
GST	: Glutathione-S-transferase
HA	: Hemagglutinin
HEK293T	: Human embryonic kidney 293T cells
HR	: Homologous recombination
HRP	: Horseradish peroxidase
	:

kbp	: Kilobase pair
LB	: Luria Bertani
LD12:12	: 12 hour light-12 hour dark
LRR	: Leucine-rich repeat
MAPK	: Mitogen-activated protein kinase
NER	: Nucleotide excision repair
nm	: nanometer
<i>p53^{-/-}</i>	: p53 knockout
PCR	: Polymerase chain reaction
PER	: Period gene or protein
PHR	: Photolyase
PPI	: Protein-protein interaction
Pry<>Pry	: Pyrimidine dimer
RE	: Restriction enzyme
RER	: RORE-RevRE
REV-ERB	: Retinoic acid orphan nuclear receptors
ROR	: Retinoic acid-related orphan receptor
RPM	: Revolution per minute
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T _m	: Melting temperature
TTFL	: Transcription Translation Feedback Loop
UV	: Ultraviolet
XP	: <i>Xeroderma pigmentosum</i>
XPA	XP group A

LIST of FIGURES

<u>Figure No:</u>	<u>Page</u>
2.1: Transcription-Translation Feedback Loop (TTFL) control of the circadian clock at molecular level	6
2.2: 3-D crystal structure of the CRY1 protein.	7
2.3: Steps of co-immunoprecipitation method.	111
5.1: Interaction map of CRY1 and DNA damage and repair response (DDR) pathway proteins.	28
5.2: 3xHA-NLS-miniTurbo_pcDNA3 (#107172) plasmid map.	29
5.3: Illustration of the Cloning Strategy of 3xHA-NLS-miniTurbo_pcDNA3 plasmid.	30
5.4: Accuracy of the PCR reaction of the candidate interactors.	31
5.5: Confirmation of the cloning of the interactors into the 3xHA-NLS_pcDNA3 vector.	32
5.6: hCRY1-FLAG-Myc-6xHis_pcDNA4 plasmid map.	33
5.7: Western blot analysis of co-IP performed with anti-FLAG antibody to confirm generation of the stable FLAG expressing HEK293T cell line.	34
5.8: hCRY1-3xFLAG-Myc-6xHis_pcDNA4 plasmid map.	35
5.9: Verification of the generation of a stable 3xFLAG-CRY1 expressing HEK293T cell line.	35
5.10: Validation of the expression of interactors in a stable HEK293T-CRY1-3xFLAG cell line.	36
5.11: Western blot analysis of co-IP sample of BUB3 and SNIP1 proteins.	37
5.12: Western blot analysis of co-IP sample of BMAL1 and CBX3 proteins.	38

LIST of TABLES

<u>Table No:</u>	<u>Page</u>
3.1: List of kits and reagents.	12
3.2: List of buffers and solutions.	13
3.3: List of SDS-PAGE recipe.	14
3.4: List of antibodies.	14
3.5: List of PCR primer oligos.	15
3.6: List of equipment.	16
4.1.1: PCR reaction contents.	17
4.2: PCR conditions of thermocycler.	18

1. INTRODUCTION

The subject of this thesis is to test of the interaction between CRY1 and its novel interactors which was identified in our laboratory using proximity-dependent biotinylation and identification by mass-spectrometry (Bio-ID) method. We concentrated on the candidates which are involved in DNA damage pathway(s) because circadian clock components were already associated with DNA damage. Even though, we confirmed one interaction with CRY1 and novel interactor candidate, further functional assays are required to validate the co-immunoprecipitation results. In summary, we aimed to show that some of the novel interactors identified by Bio-ID method can be confirmed with co-immunoprecipitation assay. Exploration of the CRY1 interactors can give new aspect of view to protect cells from DNA damages, pathological indications and disorders.

1.1. The Purpose, Contribution and Content of Thesis

The aim of this study is to validate new CRY1 interactors associated with DNA damage and repair responses. All possible interactors of the CRY1 protein were determined by a mass-spectrometry analysis in a previous study in our laboratory. The candidates were specified and selected regarding the purpose and easy cloning properties in the context of this thesis. The experimental design was as follows: 1) the production of an HA-tag expressing interactors, 2) generation of a stable 3xFLAG-CRY1 expressing human embryonic kidney 293T (HEK293T) cell line, 3) transfection of the interactors into the stable cell line, 4) co-immunoprecipitation of the CRY1 and interactor with an anti-FLAG antibody-coated magnetic beads, and 5) validation of the interactor(s) by western blot analysis using an anti-HA antibody. The strategy of the co-IP is the immunoprecipitation of FLAG-tag-expressing CRY1 and the interactor protein (which is in the same complex with CRY1 protein) with anti-FLAG antibody, and then the confirmation of HA-tagged interactor in the precipitated complex by immunoblotting using an anti-HA antibody. At the end of the study, we aimed to identify novel CRY1 interactors and contribute new information about CRY1-related pathways.

The literature data including circadian clock, core clock gene *CRY1*, CRY1-photolyase, and DNA damage response relevance, and co-immunoprecipitation are given in Chapter 2. The detailed experimental procedures are explained in Chapters 3 and 4. Chapter 5 demonstrates the results by figures and tables. Finally, the thesis is accomplished by the conclusion and discussion part in Chapter 6.

2. LITERATURE REVIEW

2.1. Circadian clock

Most of living organisms, from bacteria to humans, arrange their metabolisms according to environmental changes such as temperature, light availability mostly because of earth's rotation from itself. The day and night cycles regulate behaviors, metabolisms, and even the gene expressions of an organism. Daily oscillations in metabolism and physiology are controlled by an endogenous system called the circadian clock (or biological clock in a general term) [Takahashi, 2016].

Literally, the circadian clock is a 24-hour endogenous oscillating rhythm. The word "circadian" is derived from the Latin word *circa diem* meaning "about a day". These rhythms are essential for the balance of cellular pathways and the survival of the organism. Even though, the circadian clock generated autonomously it can be affected by external factors (called *zeitgeber*). The clock can be synchronized even in the absence of visual photoreceptors. Lockey and his colleagues proved this by experiments on blind people. They and other researchers demonstrated that half of the blind people have a normal circadian rhythm even though these individuals cannot sense the light by eyes [Parsch et al., 2014], [Lockey et al., 1997].

The pacemaker of the circadian rhythms is the suprachiasmatic nucleus (SCN) in mammals. The SCN is localized within the hypothalamus in brain [Reppert and Weaver, 2002]. The light is sensed by the melanopsin receptor within the retina of the eye, and the photic signal is transmitted to the SCN by neuronal pathways. Once the circadian clock is reset in the SCN (which is called master clock), it in turn synchronizes the peripheral clocks in the organs [Allen, 2019].

2.2. Transcription Translation Feedback Loop (TTFL)

The circadian clock contributes to the regulation of physiological and biochemical pathways in mammals by altering the levels of gene expressions and protein processing. Approximately, 10% of the transcriptome and 20% of the proteome are under the control of the circadian clock. Daily circadian rhythms are generated at

molecular circadian clocks which are based on the transcription-translation feedback loop (TTFL). The core of the TTFL is highly conserved through species including fungi, bacteria, drosophila, and mammals by emphasizing the functional importance of the regulation [Brown et al., 2012].

The TTFL has two opposite arms and four components of the arms generating circadian oscillations. Brain and muscle ARNT-like1 (BMAL1; also known as MOP3, ARNT3 and JAD3) is the master regulatory protein of the circadian synchronization and composes the positive arm with its partner, the Circadian Locomotor Output Cycles Protein Kaput (CLOCK). These proteins are basic-helix-loop-helix (bHLH) and PER-ARNT-SIM (shortly PAS) domain containing transcription factors. BMAL1 and CLOCK proteins are activated and heterodimerized [Takahashi, 2016]. The complex binds to the E-boxes in front of *CRYPTOCHROME* (*CRY1*, *CRY2*), *PERIOD* (*PER1*, *PER2*, *PER3*), and circadian clock-controlled genes (CCGs). *CRY* and *PER* genes are the members of the negative arm and suppress the expressions of their activators *BMAL1* and *CLOCK*. *CRY* and *PER* have high transcript levels in the afternoon, later on, the protein levels of these genes are increased in the evening [Ozturk, 2009]. Shortly after the production of *CRY* and *PER* proteins, they heterodimerize within the cytoplasm with the help of the casein kinase 1 δ and ϵ (CK1 δ and CK1 ϵ) proteins [Reppert and Weaver, 2002]. *PER/CRY* complex translocates back to the nucleus, following the heterodimerization, and downregulates the expression of their activators *BMAL1* and *CLOCK* by binding their E-boxes at night. The remaining *CRY* and *PER* proteins are degraded by E3 ubiquitin ligases during the night. This feedback mechanism resets the circadian clock approximately every 24-h [Takahashi, 2016].

The circadian rhythm also has a second feedback loop that controls the expressions of the core clock genes *BMAL1* and *CLOCK*. The *BMAL1/CLOCK* complex binds to E-boxes of retinoic acid orphan nuclear receptors (REV-ERB α & REV-ERB β) and retinoic acid-related orphan receptors α and β (ROR α & ROR β) by activating their expressions in the SCN [Akashi and Takumi, 2005]. ROR α & β are the inducers of the *BMAL1* oscillation and upregulates the *BMAL1* expression by binding RORE-RevRE (shortly, “RER”) region. On the other hand, “REV-ERB α and REV-ERB β ” are suppressors of the *BMAL1* gene. Even though REV-ERBs have a tissue-specific expression, they compete with ROR α for binding to the promoter region of *BMAL1*. REV-ERB α & β binding to *BMAL1* downregulates *BMAL1* expression and

also *CLOCK* gene in the morning, and helps accurate circadian clock synchronization [Duez and Steal, 2009], [Everet and Lazzar, 2014].

Post-translational modification is another significant regulator of circadian timing. Phosphorylation, ubiquitination, histone acetylation, and methylations orchestrate circadian clock genes' function, stability, and degradation. While *BMAL1* and *CLOCK* genes' histones should be acetylated for heterodimer activation, histone H3 methylation leads to transcriptional repression [Brown et al., 2012]. CK1 ϵ & δ contributes to the activation of BMAL1 by phosphorylation. Once BMAL1 is phosphorylated, BMAL1/CLOCK complex turns into an active state. On the other hand, phosphorylation of the BMAL1 on multiple regions by mitogen-activated protein kinase (MAPK) decreases the BMAL1/CLOCK heterodimer activity [Gallego and Virshup, 2007].

Phosphorylation is a crucial event for CRY/PER complex and allows their entry to nucleus [Chaves, et al., 2006]. CK1 ϵ & δ phosphorylate PER and generate a binding affinity for β transducin-repeat containing protein 1 (β -TrCP1). β -TrCP1 is a member of the E3 ubiquitin ligase family, which ubiquitinates hyperphosphorylated PER proteins for their further degradation in 26S proteosomes [Akashi, 2002]. F-box/leucine-rich-repeat (LRR) protein 3 (FBXL3) leads to the ubiquitination-dependent degradation of CRY, whereas FBXL21, an E3 ubiquitin ligase, increases the half-life of CRY within the cytoplasm [Yoo et al., 2013]. These ubiquitination and degradation events have great importance for the synchronization of the circadian timing every 24-h. Beyond the mutation in clock genes, the problems arising in the phosphorylation may affect the circadian clock which may cause severe abnormalities and diseases.

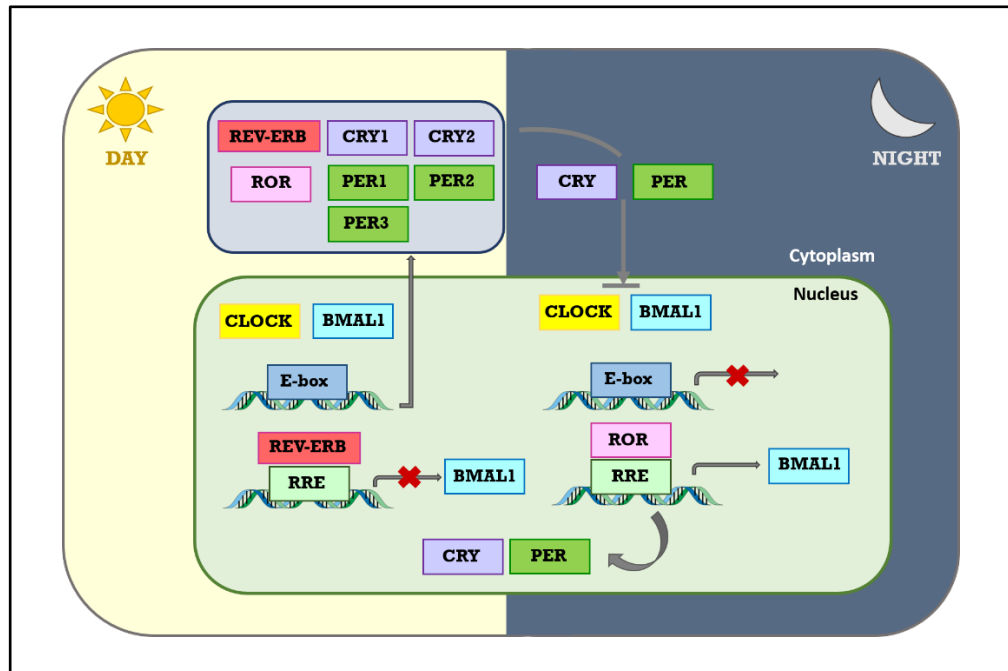


Figure 2.1: Transcription-Translation Feedback Loop (TTFL) control of the circadian clock at molecular level. Induction and repression of the circadian proteins during light and dark cycles are shown by arrows and crosses.

2.3. CRY1: A Member of Photolyase/Cryptochrome Family

CRY genes belong to the photolyase/cryptochrome (PHR/CRY) family. Photolyases are the blue light sensitive DNA repair enzymes and use flavin adenine dinucleotide (shortly, FAD) as a cofactor. The main role of the photolyases is to repair of the ultraviolet (UV)-induced DNA damage [Ozturk, 2017]. Cyclobutane pyrimidine dimer (Pry<>Pry) and pyrimidine-primidone products (6-4 photoproducts) are two damages that can arise in the DNA upon exposure to UV radiation. PHRs have a high affinity for UV-damaged DNA, uses blue light as an energy source to catalyze the repair reactions. Primarily, the structural distortion caused by UV is recognized by a photolyase independently to light. Energy derived from the light is transferred to the pyrimidine dimer through FAD. Then, these bases are flipped out from the DNA double helix. Pyrimidine nucleotides of the dimer are split from each other by broken the bonds between them [Sancar, 2004]. PHRs show very low affinity to undamaged DNA so that the enzyme dissociates from the DNA upon repair of the lesion [Sancar, 2004], [Ozturk, 2017].

CRYs exhibit high sequence similarities (25% to 50%) to the PHRs [Sancar, 2004]. The main structural difference of *CRY* that distinguishes from PHRs is the extended C-terminal region [Ozturk, 2017]. CRYs are the photoreceptors of many living organisms, such as *Drosophila melanogaster* and *Arabidopsis thaliana*. The CRYs contribute to the blue light-dependent growth and development of some plants. Some of the *CRY* genes are found to be involved magnetoreception of flies and birds. [Mouritsen and Ritz, 2005]. Foley has suggested that the human CRYs also have magnetoreception ability. Even though mammalian CRYs do not act as photoreceptors, protein levels are found to be high in the inner section of the human retina [Mouritzen and Ritz, 2005], [Foley et al., 2011], [Ozturk, 2017], [Wan et al., 2021].

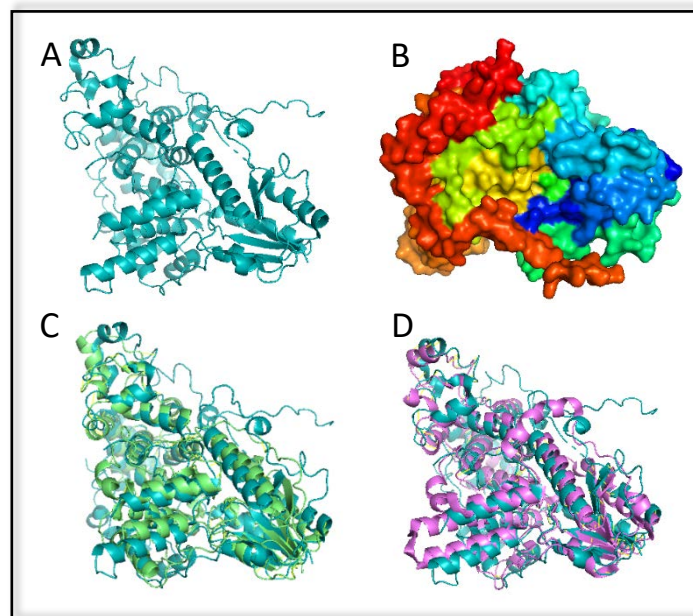


Figure 2.2: 3-D Crystal structure of the CRY1 protein. A) Ribbon model of human CRY1 (hCRY1), B) Surface structure of the hCRY1, C) Alignment of the crystal structures of hCRY1 (blue) and *Escherichia coli* photolyase (green), D) Alignment of the crystal structures of hCRY1 (blue) and *Arabidopsis thaliana* CRY1 protein; blue represents hCRY1, pink color shows *Arabidopsis thaliana* CRY1. The images were created with The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.

Two CRY genes, *CRY1* and *CRY2*, are widely expressed in mammals. The C-terminal regions of the *CRY* genes are characteristic features for functional determination. Mammalian CRY1 and CRY2 proteins exhibit structural differences (in terms of amino acid content and length) from *Drosophila* and plant CRYs, which may prove that mammalian CRYs do not function as a photoreceptor. The human

CRY1 gene is 586 amino acids in length, while the *CRY2* gene encodes a 593 amino acid long protein [Sancar, 2004]. Functional modifications and protein-protein interactions of the *CRY1* gene is significantly carried out by the C-terminus. *CRY1* interacts with the core clock components, BMAL1 and CLOCK transcription factors and PER proteins, through the C-terminal to maintain circadian oscillation. Generation of the PER/CRY heterodimer leads to nuclear localization, allowing association with the BMAL/CLOCK complex for negative feedback control of the circadian timing. Furthermore, the FBXL3 and FBXL21 ubiquitin ligases interact with the *CRY1*, which influences the half-life of the protein [Chaves et al., 2006], [Gao et al., 2013].

The stability of the CRY protein is controlled by post-translational modification. FBXL3 and FBXL21 are E3 ubiquitin ligases that have very similar sequences but work antagonistically from each other. FBXL3 resides within the nucleus, and the major role of *CRY1* is the stimulation of proteasomal degradation. The C-terminus of the FBXL3 protein has LRR and recognizes phosphorylated lysine residues of *CRY1*. Contrary to FBXL3, FBXL21 locates in the cytoplasm and ubiquitinates different lysine residues of the CRY proteins. FBXL21 activity increases *CRY1* stability [Godinho et al., 2007], [Hirano et al., 2013].

The protein level of *CRY1* is deterministic for circadian timing. *CRY1* gene has the highest mRNA level in the afternoon whereas, the protein amount peaks in the evening during 12 hours light-12 hours dark, LD12:12, period. Stabilization of the *CRY1* lengthens the circadian period, on the contrary, a reduction of the protein level shortens rhythmicity. The synchronization of the circadian timing is not affected in *CRY1* knockout (*Cry1*^{-/-}) mice under LD12:12 circumstances. On the contrary, it is shown that *Cry1*^{-/-} mice exhibit a slower period and lengthen timing in constant darkness (DD) conditions [Vitaterna et al., 1999], [Gao et al., 2013].

2.4. CRY1 and DNA Damage Response (DDR)

CRY genes participate in UV-damaged DNA repair beyond circadian synchronization. CRY levels affect the responses to DNA damages [Lee et al, 2012]. For example, CRY and XPA proteins are in opposite phases and XPA is involved in the repair of UV-induced damages through nucleotide excision repair [Kang et al., 2010]. These lesions stimulate the cell cycle checkpoint: Ataxia-and Rad-Related

(ATR), in humans. ATR phosphorylates and activates checkpoint kinase 1 (Chk1). The downstream signaling promotes the nucleotide excision repair (NER) and p53 pathways. Downregulation of *CRY1* in mouse embryonic fibroblasts with small interfering RNA, abrogates clock-dependent ATR activation. [Zhao and Piwnicka-Worms, 2001], [Niida and Nakanishi, 2005], [Sancar et al., 2010], [Kang and Leem, 2014].

The nucleotide excision repair is the essential DNA repair mechanism that mends UV lesions within the DNA helix in mammals. Defects in the NER lead to insensitivity against UV radiation, generation of *Xeroderma pigmentosum* (XP), and even skin cancer. The current researches have indicated that the *CRY1* gene is correlated with the NER, respectively [Sancar et al., 2010]. Several proteins are included in the NER system, but the most important one is XP group A (shortly, XPA). XPA protein is phosphorylated by ATR kinase on serine 196 residue and activated in response to UV-induced DNA damage. The activated form of XPA recognizes the break and recruits other components of the NER to the damaged site. The level of the XPA protein is under transcriptional control of the *CRY1* gene. XPA expression peaks at 5 pm, whereas *CRY1* peaks at 5 am in mouse skin, manifesting XPA has an anti-phase oscillation pattern with the *CRY1* protein. Low levels of *CRY1* expression give rise to increased XPA protein, causing abnormal DNA repair responses. Accordingly, *CRY1* regulates the rhythmicity of the XPA protein and controls the repairing capacity of the UV-damaged DNA [Sancar et al., 2010], [Gaddameedhi et al., 2011], [Lee et al., 2014], [Ozturk, 2017]. Additionally, Ozturk et. al. has been proved that *p53* and *CRY* double knockout (*p53*^{-/-}*Cry1*^{-/-} *Cry2*^{-/-}) cells are vulnerable to UV-induced apoptosis compared with the *p53*^{-/-} cells [Ozturk et al., 2009]. There are several researches that suggest the *CRY1* gene impresses the expression of p53 [Zhou et al., 2018], [Jia et al., 2020].

CRY1 is also demonstrated to be stabilized after double-stranded DNA breaks, which are repaired by homologous recombination mechanism (HR). *RAD51*, *BRCA1*, *BRCA2*, *CHEK1*, and *XRCC3* are the major components of the HR, and expressions of these genes were shown to be downregulated upon the knock-down of *CRY1* [Shafi et al., 2021].

2.5. Co-Immunoprecipitation (co-IP)

Possible factors that are associated with the protein of interest can be determined using a couple of assays such as yeast-two-hybrid assay, proximity-dependent biotinylation and identification (Bio-ID), or co-immunoprecipitation. Among them, the co-immunoprecipitation (co-IP) method is widely used for the identification of the PPI. In this study, we actually used co-IP method to confirm some possible PPIs which had been identified using Bio-ID technique from MiniTurbo hCRY1-U2OS cells by Senanur Olfaz [Olfaz, 2020] in our laboratory at Gebze Technical University. Co-IP method allows the purification of the protein of interest in its natural physiology and also it is a probe for the possible interactor in the complex with immunoblotting [Wang and Liu, 2011].

The co-IP method is based on protein-antibody interaction. The protein of interest (bait) is principally fused with a tag (the FLAG, HA, and GST tags commonly used) and precipitated with an antibody specific to this tag. On the other hand, specific antibodies can be used to immunoprecipitate the endogenous proteins without any tags. The system exploits immobilized antibodies attached to magnetic or other kind of beads. When cell lysate is loaded into these magnetic beads under non-denaturing conditions, the target protein and its partner (known as an interactome or interactor) through the target protein are linked to the antibodies. Other proteins cannot bind to the magnetic beads, and are removed during washing steps. Only the target protein remains attached to the beads, which is eventually used to elute or precipitate the interactor. Precipitated complexes are then run on a SDS-PAGE, and analyzed with immunoblotting using specific antibodies to probe the candidate interactors [Maccarone et al., 2016].

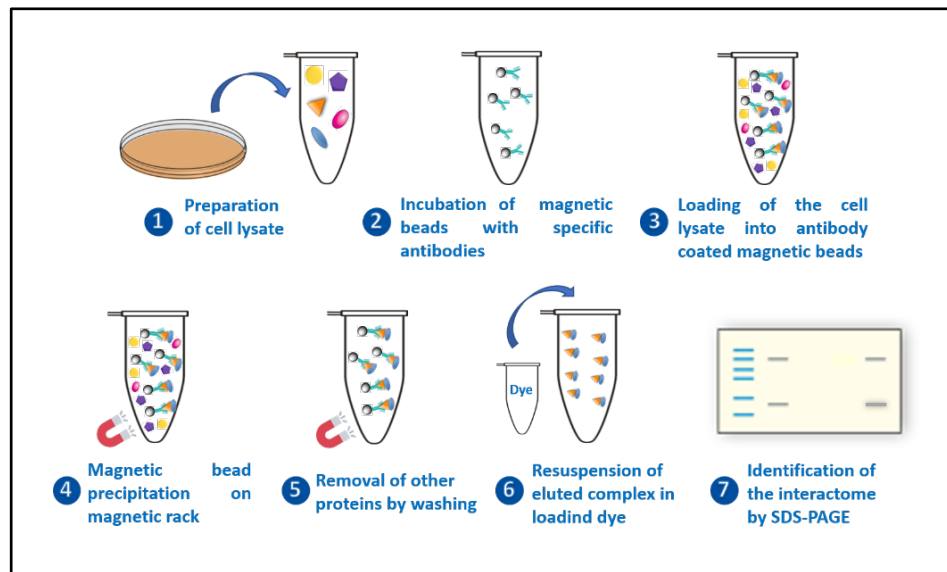


Figure 2.3: Steps of the co-immunoprecipitation method. 1) Cells are lysed with non-ionic detergent; 2) Protein G-coupled magnetic beads and specific antibodies (anti-FLAG antibody in our case) are incubated in a microcentrifuge tube for 1 hour to bind the anti-FLAG antibody to magnetic beads via Protein G; 3) Cell lysate is loaded into the antibody-coated magnetic bead containing tube to allow specific antibodies to bind the protein of interest; 4) Then tubes are placed into magnetic rack to precipitate the magnetic beads; 5) Tubes are washed three or four times with lysis buffer to remove unbound proteins, except the protein of interest bound by the specific antibody to magnetic bead and interactors that are bound to the protein of interest; 6) After removing excess liquid, the complex is resuspended in SDS loading dye; 7) Samples are loading onto SDS-PAGE and then analyzed with immunoblotting to probe the protein of interest and interactors.

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1: List of the reagents and kits.

Nucleospin Plasmid Kit	740588.50 (Macherey-Nagel, Germany)
PCR Clean-up Gel Extraction Kit	740609.50 (Macherey-Nagel, Germany)
PureLink Genomic DNA Mini Kit	K1820-02 (Invitrogen, USA)
Protease Inhibitor Cocktail Tablets	S8830-2TAB (Sigma-Aldrich, USA)
Protein Molecular Weight Marker	26616 (Thermo Fisher Scientific, USA)
Q5® High-Fidelity Polymerase	M0491S (New England Biolabs, NEB, USA)
T4 DNA Ligase	1527-4017 (Invitrogen, USA)
Chemiluminescent HRP Substrate	K-12043-D10 (Advansta, USA)
1 kb DNA Ladder	N3232S (New England Biolabs, NEB, USA)
100 bp DNA Ladder	N3231S (New England Biolabs, NEB, USA)
DNA Loading Dye (6X)	B7021S (New England Biolabs, NEB, USA)
MagnaBind Protein G Beads	21349 (Thermo Fisher Scientific, USA)

3.2. Buffers and Solutions

Table 3.2: List of Buffers and Solutions.

5x SDS- PAGE Running Buffer	125 mM Tris-Base (pH 8.3) 95 mM Glycine 1% SDS
5x Tris-Buffered-Saline (TBS)	100 mM Tris-HCl (pH 7.6) 750 mM NaCl
1x TBS-T	1x TBS 0.1% Tween-20
1x Transfer Buffer	10% 10x SDS-PAGE Running Buffer 20% Methanol
5% Blocking Solution	5% non-fat dry skim milk in 1x TBS-T
20x Tris-Borate-EDTA (TBE)	216 g Tris-Base 110 g Boric Acid 80 ml 0.5M EDTA (pH 8.0)
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
NP-40 Lysis Buffer (co-IP Lysis Buffer)	0.5 M Tris-HCl (pH 7.4) 0.25 M NaCl 5 mM EDTA (pH 8.0) 1% NP40 (IGEPAL)
Dulbecco's Modified Eagle Medium (DMEM)	41965-039 (Gibco-Invitrogen, USA)
0.05% Trypsin-EDTA	25300-054 (Gibco-Invitrogen, USA)
Fetal Bovine Serum	10270-076 (Gibco-Invitrogen, USA)
100x Penicillin/Streptomycin	15140-122 (Gibco-Invitrogen, USA)
LB Agar	LBP04 (Caisson Lab, USA)
Restriction Endonucleases	#ER0975, #ER0675, #FD0644; #R045S, #R0552S, #R0195S (Thermo Fisher Scientific, USA; New England Biolabs, USA)
Restriction Endonuclease Buffers	Thermo Fisher Scientific, USA

Table 3.3: List of SDS-PAGE recipe.

12% Separating Gel	10 ml	15 ml	20 ml
Autoclaved ddH ₂ O	4.3 ml	6.45 ml	8.6 ml
40% Acrylamide-Bisacrylamide	3 ml	4.5 ml	6 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	3.75 ml	5 ml
10% SDS	0.1 ml	0.15 ml	0.2 ml
10% APS	0.1 ml	0.15 ml	0.2 ml
TEMED	0.004 ml	0.006 ml	0.008 ml

5% Stacking Gel	2 ml	3 ml	6 ml
Autoclaved ddH ₂ O	1.46 ml	2.19 ml	4.38 ml
40% Acrylamide-Bisacrylamide	0.25 ml	0.375 ml	0.75 ml
1.5 M Tris-HCl, pH 8.8	0.25 ml	0.375 ml	0.75 ml
10% SDS	0.02 ml	0.03 ml	0.06 ml
10% APS	0.02 ml	0.03 ml	0.06 ml
TEMED	0.002 ml	0.003 ml	0.006 ml

3.3. Antibodies

Table 3.4: List of antibodies.

Name	Dilution	Source, Catalog No
Anti-CRY1	1:1000	Bethyl Laboratories-, #A302-6164A
Anti-FLAG	1:2000	Sigma-Aldrich, #F1804
Anti-HA	1:1000	Cell Signalling Technology, #C29F4
Anti-mouse-HRP	1:6000	Cell Signalling Technology, #7076S
Anti-rabbit-HRP	1:5000	Thermo Fisher Scientific, #31460

3.4. Oligonucleotide Sequences

Table 3.5: List of PCR primer oligonucleotides.

Oligo Name	Oligo Sequence (5' to 3')
DDX24_Foward	GCAGCTAGCATGAAGTTGAAGGACACAAA
DDX24_Reverse	GTCGTCGACTTAATTTGCACTTGTACTTG
SNIP1_Foward	GCAGCTAGCATGAAGGCGGTGAAGAGCGA
SNIP1_Reverse	ATGCTCGAGCTAGCTGTCAGACACTTCTT
CBX3_Foward	GCAGCTAGCATGGCCTCCAACAAAACACTAC
CBX3_Reverse	ATGCTCGAGTTATTGAGCTTCATCTTCTG
SF3B1_Foward	GCAGCTAGCATGGCGAAGATCGCCAAGAC
SF3B1_Reverse	ATGCTCGAGTCAAGCAGCAGAATAGAAGC
BUB3_Foward	GCAGCTAGCATGACCGGTTCTAACGAGTT
BUB3_Reverse	ATGCTCGAGTTAGGTGGACTTGGGTTTTG
INCENP_Foward	GCAGCTAGCATGGGGACGACGGCCCCAGG
INCENP_Reverse	ATGCTCGAGTCAGTGCTTCTTCAGGCTGT
CDCA8_Foward	GCAGCTAGCATGGCTCCTAGGAAGGGCAG
CDCA8_Reverse	ATGCTCGAGTCATTTGTGGGTCCGTATGC
CENPC_Foward	GCAGCTAGCATGGCTGCGTCCGGTCTGGA
CENPC_Reverse	ATGCTCGAGTCATCTTTTTATCTGAGTAA
MCM2_Foward	GTCGTCGACTCAGAACTGCTGCAGGATCA
MCM2_Reverse	ATGCTCGAGTCAGAACTGCTGCAGGATCA
TTK_Foward	GCAGCTAGCATGGAATCCGAGGATTTAAG
TTK_Reverse	ATGCTCGAGTCATTTTTTTTCCCCTTTTTTTTC

3.5. Equipment

Table 3.6: List of the equipment.

Agarose Gel Electrophoresis Apparatus	1704406, Bio-Rad, 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
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 ClassII Cabinet, Thermo Fisher Scientific, USA
Power Supply	Bio-Rad, USA
PCR Thermal Cycler	Bio-Rad, 41798
Transblot Transfer System	1704155, Bio-Rad, USA
Nanodrop	206-26300-48, Shimadzu Biotech
Shaker	Standard Analog shaker, VWR, USA
Vortex	Scientific Industries, USA
Waterbath	Wisd, Germany
Magnetic Rack	DynaMag™-2 Invitrogen

4. METHODS

4.1. Molecular Cloning

4.1.1. PCR Amplifications of Constructs with Cloning Primers

The PCR reactions were performed to amplify the candidate interactor's coding sequences (called interactor) using Q5® High-Fidelity Polymerase (#M0491S, New England Biolabs, NEB, USA). 500 ng of U2OS cDNA was added into the microcentrifuge tube containing a master mix: 5 µl 5x Q5 Reaction Buffer; 0.5 µl 10 mM dNTPs; 1.25 µl 10 µM Forward and Reverse Primers; 0.25 µl Q5 High-Fidelity Polymerase and ddH₂O final volume to 25 µl.

The PCR conditions were: initial denaturation at 98°C for 30 seconds; denaturation at 98°C for 10 seconds, annealing at 58°C for 15 minutes, elongation at 72°C for 1,5 minutes for 35 cycles; and final elongation at 72°C for 2 minutes, respectively.

Table 4.1: PCR reaction contents.

Stock Solutions' Concentrations and Cat. No	µl used for total 25 µl reaction
5x Q5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward and Reverse Primer	1.25 µl
Template DNA	0.5 µl (500 ng)
Q5 High-Fidelity DNA Polymerase	0.25 µl
Ampule Water	17.5 µl

Table 4.2: PCR conditions of thermocycler.

Temperature	Time	Step	Cycle
98°C	30 seconds	Initial Denaturation	
98°C	10 seconds	Denaturation	35 cycles
58°C	15 seconds	Annealing	
72°C	1.5 minutes	Extension	
72°C	2 minutes	Final Elongation	

4.1.2. Digestion of Plasmid and Oligos with Restriction Enzymes

The plasmid vector and the amplified oligo DNA were digested with NheI (#ER0975) and XhoI (#ER0695) or SalI, (#FD0644, all enzymes were purchased from Thermo Fisher Scientific, USA). Plasmid DNA or amplified products were incubated in suitable restriction enzyme setup. The reaction mixture contained 2 µl of suitable reaction buffer (final volume 1x in the reaction), 200 ng DNA (2 µl), 0.5 µl enzyme, and ddH₂O to 20 µl final volume in 1.5 ml microcentrifuge tubes. The interactor DNA were digested at 37°C for 1 hour with NheI and XhoI enzymes at 37°C for 4 hours. The plasmid DNA was digested with NheI and XhoI using the same conditions.

To generate a construct to express hCRY1, 3xFLAG coding sequence was inserted into pcDNA4.Myc.HisA plasmid (Thermo Fischer Scientific). Then amplified hCRY1 coding sequence was inserted to pcDNA4.3xFLAG-His (Myc was removed during the insertion of 3xFLAG) using T4 DNA Ligase mediated ligation reaction. The same enzymes, that were used for cloning, were used to analyze the candidate clones after ligation products were transformed into *E.coli* strain DH5- α .

4.1.3. Agarose Gel Electrophoresis

PCR amplified and double digested DNA samples were run on 1% agarose gel containing Redsafe™ Nucleic Acid Staining Solution. For this, 0.4 gram agarose was dissolved in 40 ml 1X TBE Buffer by boiling, and 2.3 µl of the staining solution was added to the gel. Gel was poured into a horizontal electrophoresis tank. After agarose gel was solidified, samples were mixed with 6X Loading Dye to a final volume 1X, the tank was filled with 1X TBE Buffer, and the samples were loaded onto the wells. 1 kb and 100 bp DNA ladders were used for an accurate evaluation of the band sizes. DNA was separated by gel electrophoresis at 120V for 45 minutes.

The interested fragments were visualized under black light (366 nm), and cut from the gel using a scalpel, and transferred into a 1.5 ml microcentrifuge tube. The DNA isolation was performed using Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit according to the manufacturer's protocol. The DNA was eluted in 50 µl double-distilled water. The concentration of eluted DNA was measured using Thermo NanodropLite Spectrophotometer.

4.1.4. Ligation of Inserts into the Plasmid

The interactomes and the 3xHA-NLS_pcDNA3; the hCRY1-FLAG-Myc-6xHis_pcDNA4 plasmid and the human CRY1 (hCRY1) DNA; and hCRY1-FLAG-Myc-6xHis_pcDNA4 and 3xFLAG tag plasmid were ligated overnight at 4°C using T4 DNA Ligase (#1522-4017, Invitrogen, USA). The reaction was set up as: 1X T4 Ligation Buffer, 1 unit of the T4 DNA Ligase, and 1:3 molar vector to insert ratio in 10 µl volume.

4.1.5. Transformation

Escherichia coli (*E. Coli*) DH5- α strain was used as a competent cell in this study. The competent cells were prepared using a previously published protocol [Baldi et al., 2008]. The competent cells were taken from a -80°C freezer and thawed on ice. 5 µl of ligation product was added into 100 µl of competent cells in a new microcentrifuge tube and were incubated on ice for 20 minutes. Following this

incubation, cells were exposed to /incubated at 42°C for 1 minute to generate a heat shock, then the tubes were placed back on ice for 1 minute. Then, 950 µl Luria Broth (LB) was added onto tubes and cells were cultured into a rotating shaker at 37°C for 50 minutes in order to properly express an antibiotic selection gene ampicillin. 120 µl of the cells were spread on LB agar plates containing 100 µg/µl ampicillin. Plates were kept at room temperature for 5 minutes, then placed at 37°C for overnight incubation in an inverted state. After the generation of single colonies that express the ligation products, colonies transferred into 3.5 ml liquid LB medium containing 100 µg/µl ampicillin and incubated overnight at 37°C on a shaker for further plasmid isolation.

4.1.6. Plasmid Isolation

Macherey-Nagel Nucleospin Plasmid Isolation kit was used to isolate the recombinant DNA from *E. coli* cells. Preliminarily, overnight bacterial cell culture was centrifuged at 4000 rpm for 10 minutes. The pellet was thawed with resuspension buffer, lysed, and transferred into a column. DNA was bounded to the column with the help of the filter, washed, and eluted according to the kit's protocol. The concentration of the eluted plasmid DNA was quantified using a Thermo NanodropLite Spectrophotometer.

Clones were confirmed by restriction enzyme digestion. Following, the single-colonies derived from the transformation were picked up with micropipette and transformed into 20 ml of LB medium containing 100 µg/µl ampicillin for the requirement of a large amount of DNA. The amplified bacteria were collected and plasmid DNA isolations were performed by Macharey-Nagel Nucleospin Plasmid Isolation kit as explained previously in this section.

4.1.6. Confirmation of the Transformation

Isolated DNA from transformants were digested with SalI restriction enzyme in order to confirm the accuracy of the transformation. 2 µl of 10x FastDigest Buffer, 2 µl of plasmid DNA, 0.5 µl of SalI FastDigest enzyme were mixed in 1.5 ml centrifuge tubes to a final volume 20 µl. The volume was completed with distilled water. Tubes

were incubated at 37°C for 20 minutes. After incubation, samples were run on 1% agarose gel.

4.2. Cell Culture

4.2.1. Maintenance of the Cell Line

HEK293T, human embryonic kidney 293 cells, were grown and maintained in Dulbecco's Modified Eagle medium (DMEM) containing 4.5 g/L high glucose and enriched with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (P/S), and 1% non-essential amino acids (NEAA). Cells were incubated at a 37°C 5% CO₂-containing humidified incubator.

HEK293T cells were passaged when their confluency reached 80%. The medium was discarded, cells were washed with 1x PBS. Following, cells were trypsinized with 0.05% trypsin-EDTA and incubated at 37°C for approximately 5 minutes. Detached cells were diluted by the addition of fresh medium, which is the two-fold volume of the trypsin. Cells were seeded into a new 60-mm petri dish by dilution 1:10 ratio and incubated at 37°C with 5% CO₂.

4.2.2. Transfection

Chemical transfection with polyethylenimine (PEI) was performed. PEI is a positively charged transfection agent that surrounds and stabilizes the negative charges of DNA (Baker et. al., 1997).

The interactor DNA amplified within the plasmid was transfected into a stable 3xFLAG- CRY1 expressing HEK293T cell line. Cells were seeded into 60-mm dishes at 60-70% confluency and incubated overnight at 37°C incubator supplied with 5 %CO₂. The next day, the confluency of the cells was screened. If the confluency was about 80%, transfection was performed. 150 µl of transfection buffer [composed of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA (pH 8.0)] was mixed with a 3:1 ratio of PEI and plasmid DNA. 1 µg plasmid DNA was used for the transfection of each interactome. The mixture was vortexed and spun, then incubated at room temperature for 15 minutes. The old medium was removed from dishes, and 1 ml of

fresh medium was added into the dishes. 2 ml of fresh medium was mixed with the transfection buffer solution, pipetting gently, and added into the 60-mm dishes as a final step. The dishes containing transfected cells were incubated for 2 days at a 37°C humidified incubator under 5% CO₂ conditions.

Transfection method as given conditions was also used to transfect plasmids for stable FLAG-CRY1 expressing HEK293T cell line.

4.2.3. Generation of a Stable Cell Line

Cells were seeded on 6-well culture plates approximately with 60% confluency and incubated overnight. The next day, transformation of pcDNA4-FLAG-Myc-6xHis and pcDNA4-3xFLAG-Myc-6xHis were performed as explained in the previous chapter to generate a stable cell line. The recombinant cells (expressing the transfected plasmid) were selected after 24 or 36 hours from transfection. After 2 days, cells were detached from the culture plate by trypsinization and seeded into a 96-well plate by serial dilutions. Following, cells were incubated at medium enriched with an addition of convenient volumes of an antibiotic to select the stable clones. Stable clones derived from single cells were picked from the old plate and were seeded into 6-, 12-well culture dishes as a duplicate for expression screening by western blot using an anti-FLAG antibody (#F1804, Sigma Aldrich).

4.3. Co-IP

4.3.1. Cell Collection

Transfected HEK293T cells were collected with their medium by a scraper. The cells were scraped from the surface of the dishes and transferred into a new microcentrifuge tube. The cells were pelleted by spinning in a centrifuge at 5000 rpm for 3 minutes. The pellets were washed two times with ice-cold 1x PBS solution to remove all possible remnants of the medium. The supernatant was discarded, and cells were put on ice for the lysis step.

4.3.2. Cell Lysis and Sample Preparation

The pellets were lysed using NP-40 Lysis Buffer (#FNN0021, Thermo Fisher Scientific, USA) enriched with a 1x protease inhibitor (PI). The pellets were thawed gently in 1 ml of the NP-40 lysis buffer supplemented with protease inhibitor cocktail, then incubated on ice for 30 minutes. Lysates were vortexed every 5 minutes to perform an effective lysis process. The lysates were centrifuged at 13300 rpm for 20 to 30 minutes at 4°C. The supernatants were transferred into new ice-cold 1.5 ml microcentrifuge tubes.

45 µl of supernatant was taken into new microcentrifuge tubes to be used as a positive control of transfection (also named as input). The lysate was mixed with an adequate volume of the 4x-protein-loading-dye to obtain a final volume of 1x, and kept at -20°C until loaded onto the SDS polyacrylamide gel.

4.3.3. Immunoprecipitation of Interactomes with Magnetic Beads

1.5 ml centrifuge tubes were placed into a magnetic rack and enriched with 10 µl of MagnaBind™ Protein G Beads (#88802, Thermo Fisher Scientific). The beads were washed using 500 µl of NP-40 lysis buffer three times. 1 µg of anti-FLAG antibody (#F1804, Sigma Aldrich) or IgG (as a control, #7076S, Cell Signalling Technologies) per sample was added into the tubes. After the addition of 200 µl of NP-40 lysis Buffer for each sample, tubes were incubated at 4°C for 1 hour. Following, 400 µl of cell lysate is loaded into the microcentrifuge tubes and incubated overnight at 4°C for proper attachment of protein complex to the antibodies.

4.4. Western Blot

4.4.1. SDS-PAGE Gel Preparation

SDS-PAGE glasses were cleaned using 70% ethanol and placed into a gel cassette in an appropriate order. Gel cassette was put onto a gel apparatus. There were two distinct gels namely separating gel (at the bottom) and stacking gel (on the top). Firstly, separating gel was prepared into a falcon as explained in table 3.3. Ammonium

persulfate (APS) and TEMED within the gel rapidly initiates the polymerization event (Swamy et al., 2006), so that gel was homogenized and poured expeditiously. Promptly after the gel was poured between glasses, isopropanol is added onto the gel to prevent air contact of the gel to remove bubbles and help to the generation of properly frozen smooth gel surface. When the gel was frozen (approximately 20 minutes), isopropanol was discarded and the gel was washed with dH₂O to remove isopropanol remnants. Wet areas between glasses were dried using Whatman paper. A 1.5 mm comb was cleaned with 70% ethanol. 5% stacking gel was prepared in a falcon, then poured onto the separating gel. A comb is gently placed onto the stacking gel and allowed to polymerize. The gel was incubated for 10 minutes at 4°C to ensure that the gel was completely polymerized.

4.4.2. Sample Preparation for SDS Polyacrylamide Gel

The samples were taken from -20°C and allowed for thawing at room temperature. After thawing, the microcentrifuge tubes were put into a heat block which is at 95°C, and heated for 10 minutes. The heating would help denaturation of the proteins and turned the proteins into their primary structure. By means, proteins could be separated according to their molecular weight in the SDS-PAGE system.

4.4.3. SDS-PAGE Gel Running

The comb is gently taken out and the wells were washed with dH₂O to generate a smooth surface. The gel was placed into a running cassette. Because the cassette required two gel for running, a plastic glass was put on the other part of the cassette. The cassette was placed into the running tank. The tank and the area between glasses were filled with 1x Running Buffer (prepared by diluting 10x Running Buffer with distilled water). PageRuler™ Prestained Protein Ladder (#26617, Thermo Fisher Scientific, USA) and the samples were loaded in into the wells as equal protein amount. The gel was run at 80V after the samples were entered into the separating gel, then the voltage was increased to 120V for 2 hours. Samples were run until the bromophenol blue dye within the 4x-protein-loading dye reached the end of the gel, then the running was stopped.

Glasses were taken out from the cassette, separated gently using a spatula. The wells were cut off and the gel was taken into transfer buffer to prepare the gel for transfer.

4.4.4. Transfer of Proteins from Gel to Membrane

Trans-blot Turbo Transfer System (Serial No: 690BR011760, Biorad) was used to perform a semi-dry transfer of the protein from gel to nitrocellulose membrane. 4 Whatman paper and 1 membrane with the same size of SDS gel were incubated at 1x transfer buffer for about 5 minutes. The glasses of the SDS gel were separated gently then the gel was taken into 1x transfer buffer. A transfer sandwich was prepared in the following order; 2 Whatman paper, membrane, gel, and 2 Whatman paper are put onto a transfer cassette, respectively. After the gel was placed onto the membrane, the bubbles were generated by the gap between membrane and the gel was removed using a roller. The cassette was locked, placed into the transblot appliance as (-) electrode (cathode) at the bottom and (+) electrode (anode) at the top. The transfer of proteins from gel to nitrocellulose membrane was performed at 12V for 1 hour.

4.4.5. Blocking and Immunoblotting

5% skim milk containing blocking solution was prepared by dissolving the skim milk in 1x TBS-T (composed of 10x TBS-T Buffer diluted with distilled water, and 0.1% Tween-20). After the transfer was accomplished, the membrane was taken into a blocking case filled with blocking solution and blocked at room temperature on shaker for 1 hour to avoid non-specific antibody attachment. Thereafter, the membrane was incubated overnight at 4°C on shaker with a primary antibody (diluted to recommended concentration in 5% blocking solution, %1 sodium azide was added to prevent bacterial contamination and prolong the antibody's half-life).

On the following day, the membrane was taken out from the primary antibody, washed with 1x TBS-T for four times (with 5-15-5-5 minute time intervals) to remove unbound antibodies. The membrane was incubated with specific secondary antibodies. The membrane was incubated for 1 hour at room temperature on a shaker with an anti-mouse secondary antibody (1:6000, #7076S, CST) against the anti-FLAG, and an anti-

rabbit secondary antibody (1:5000, #31460, Thermo Fisher Scientific) for the anti-HA primary antibody. When the incubation was completed, the membrane was washed four times with 1x TBS-T as explained above.

4.4.6. Imaging

The membrane was placed into a black imaging paper, following the fourth washing step, and dried well. The chemiluminescence substrate namely WesternBright Sirius HRP substrate (Advansta Inc., CA, USA) was applied onto the membrane. HRP molecules were conjugated to the secondary antibody. These molecules were performed a chemical reaction with the HRP substrate and were generated chemiluminescence signals. After 3 minutes of incubation, the excess amount of the substrate was discarded. Chemidoc™ XRS+ System with ImageLab™ Software (#1708265, Biorad) was used to capture the chemiluminescence signals of the membrane.

5. RESULTS

The major purpose of this thesis is to validate novel CRY1 interactomes associated with DNA damage and repair pathways. Candidate CRY1 interactors were identified in our laboratory previously [Olfaz, 2020]. For this aim, we developed an experimental approach based on co-immunoprecipitation. We generated stable cell line from HEK293T overexpressing human CRY1 with a 3xFLAG tag at the C-terminus (HEK293T-CRY1-3xFLAG) and used this cell line to immunoprecipitate the CRY1 complex. The interactor candidates which selected from mass-spectrometry data, were cloned into mammalian expression vector to express them with a 3xHA tag at N-terminus. The interactors were transiently expressed in the stable HEK293T-CRY1-3xFLAG cell line and the presence of the candidate in the CRY1-3xFLAG complex were analyzed by immunoblotting.

5.1. Generation of Interactor Expression Constructs

5.1.1. Determination of the Candidates

The possible interactor proteins named as candidates were selected from CRY1 mass-spectrometry analysis data that were described in a MSc thesis from our laboratory [Olfaz, 2020]. In that study, CRY1 interactome was identified by BioID technique. The proteins where in close of CRY1 were labeled with biotin and identified by mass-spectrometry after isolation of biotinylated proteins using streptavidin-magnetic beads [Go et al., 2021]. The candidates having higher Saint scores, 0.95 to 1 were chosen using the Saint Express Analysis tool [Teo et al., 2014]. Then, due to the close relation between DNA repair and circadian clock, we selected the candidates that are related to DNA repair and DNA damage response pathways (DDR). The significant interactors related directly to the DDR were classified with David Bioinformatic Resources 6.8. Maps representing the interactors of CRY1 protein associated directly or indirectly with DDR were created by Cytoscape 3.7.2 bioinformatic tool (Figure 5.1).

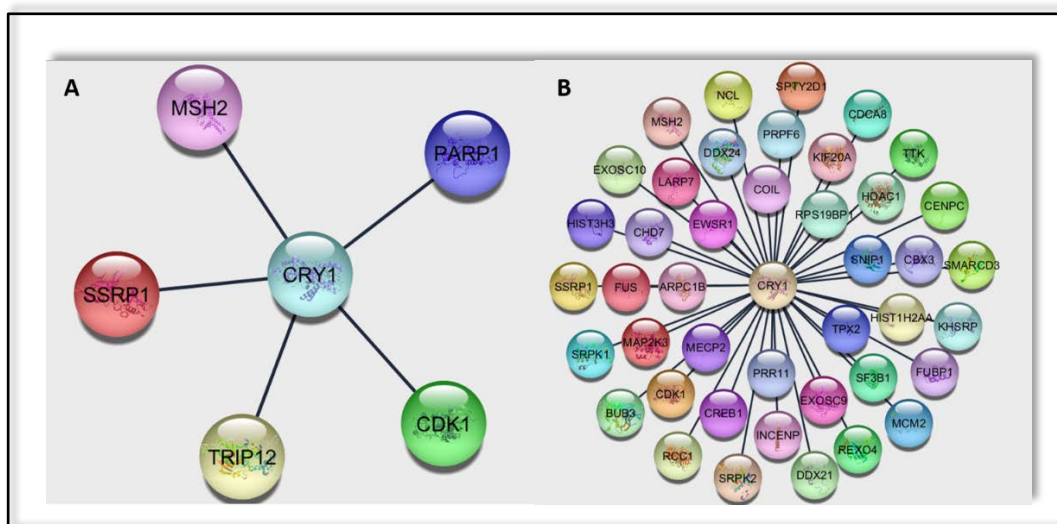


Figure 5.1: Interaction map of CRY1 and DNA repair and DNA damage response (DDR) pathway proteins. A) Interactors directly associated with DDR, B) Indirect interactors. The maps were generated using String App and Cytoscape 3.7.2 Bioinformatic tool.

5.1.2. Preparation of Plasmids for Cloning

3xHA-NLS-miniTurbo_pcDNA3 (#107172) plasmid purchased from Addgene was used as a vector in order to clone the interactors. The HA tag is widely used for tagging protein when specific antibodies are not available. This tag is a well-characterized that can easily be detected with an anti-HA antibody from different sources such as rabbit and mouse. Also, the HA tag can be inserted into the N or C terminus of a protein and does not interfere with the bioactivity of the recombinant protein. By means of these properties, the HA tag was decided to be fused with the interactors. The 3xHA-NLS-miniTurbo_pcDNA3 plasmid, shown in the Figure 5.2, was chosen regarding its 3xHA tag and convenience of cloning.

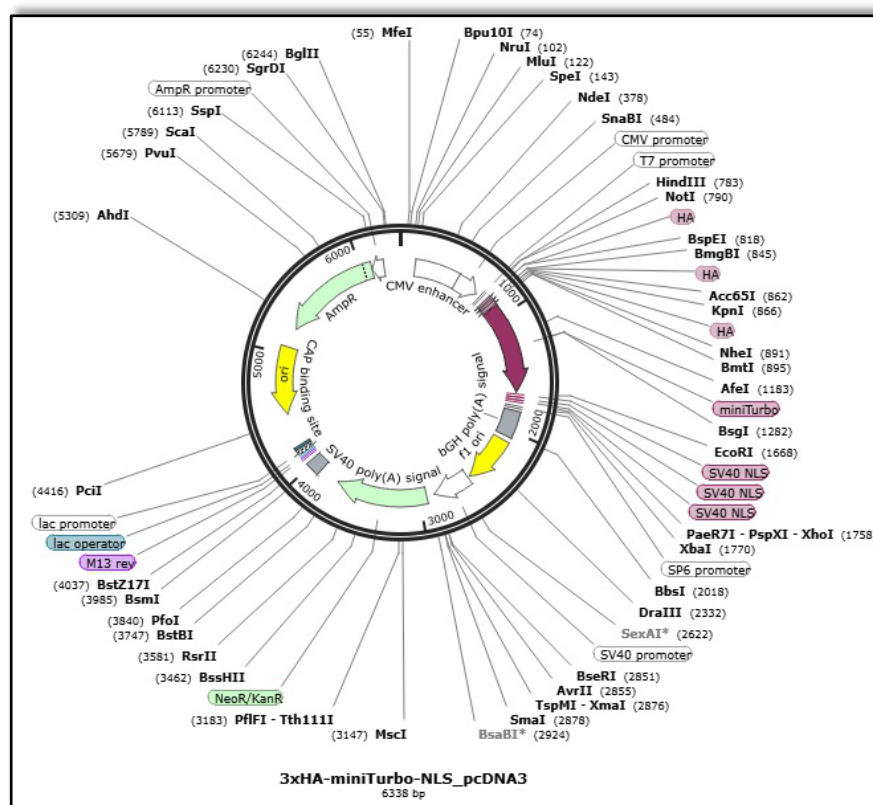


Figure 5.2: 3xHA-NLS-miniTurbo_pcDNA3 (#107172) plasmid map. The map was generated using SnapGene Viewer Program.

The cloning strategy for the interactors was as follow: The interactors were amplified from U2OS cDNA using high-fidelity polymerase using specific oligos with suitable enzyme restriction site close to the 5' ends (Figure 5.3). Amplified interactor candidates were isolated from agarose gel and then digested with *NheI* and *XhoI* restriction enzymes. In parallel, 3xHA-NLS-miniTurbo_pcDNA3 plasmid were digested with *NheI* and *SalI* enzymes and large fragment were isolated from agarose gel leaving miniTurbo expressing cassette out. The interactor candidates were ligated into pcDNA3-3xHA part of pcDNA3-3xHA-miniTurbo-NLS through compatible ends. Please note that *XhoI* and *SalI* generated overhangs are compatible and suitable for cloning. The strategy was described in Figure 5.3.

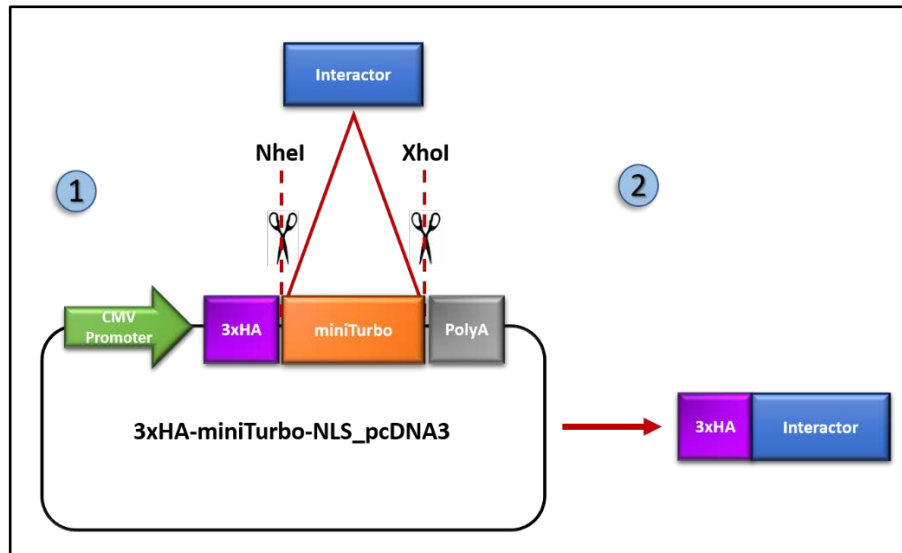


Figure 5.3: Illustration of the Cloning Strategy of the 3xHA- miniTurbo-NLS_pcDNA3 plasmid. 1) The strategy, 2) The protein that was expressed by the generated plasmid.

5.1.3. Amplification of the Interactors

The interactor DNA sequences were amplified using convenient oligos (ordered from Sentebiolab, Turkey) and Q5 High Fidelity Polymerase through human bone osteosarcoma epithelial cell line (U2OS) cDNA. Either NheI and XhoI enzyme recognition sites (6 bps) and another 3 bps to allow the restriction enzymes to bind DNA were added to the 5' ends of oligos. The reaction conditions were given in chapter 4.1.1. The accuracy of the amplification was controlled by running the PCR products on 1% agarose gel. Results were represented in Figure 5.4. The expected bands for interactors from interactor 1 (Int 1) to interactor 10 (Int 10) were: 980, 551, 842, 2831, 2579, 2726, 434, 1190, 2570 base pairs (bp), respectively. Candidate interactors were chosen according to base pairs compared to the 1 kb (N3232S, New England Biolabs, NEB, England) and 100 bp (N3231S, New England Biolabs, NEB, England) DNA ladders.



Figure 5.4: Accuracy of the PCR reaction of the candidate interactors. The lane numbers represent the followings; 1: 1 kb DNA ladder, 2-12: Candidate interactors, 12: 100 bp DNA Ladder. 1% agarose gel was used. The red rectangles indicate expected bands.

5.1.4. Ligation of the Interactors into the 3xHA-NLS_pcDNA3 Plasmid

The 3xHA-NLS_pcDNA3 vector and the PCR amplified interactors were digested with *NheI* and *SalI* or *NheI* and *XhoI* restriction enzymes, to generate compatible sticky ends for ligation. The 3xHA-miniTurbo-NLS_pcDNA3 were digested with *NheI* and *SalI* which removes miniTurbo cassette, and the remaining large fragment can be used for cloning the candidates which later can be used to express candidate with a 3xHA tag in mammalian cells such as HEK293T. The digestion products were loaded on 1% agarose gel to confirm the efficiency of the digestion. The plasmid was screened under 366 nm black light and excised from the gel. Then, the Macherey-Nagel Nucleospin Gel and PCR Clean-up kit was used in order to elute DNA from agarose gel.

T4 DNA Ligase was used to clone candidate interactors into a digested into a pcDNA3.3xHA-*NheI*-*SalI* fragment of 3xHA-NLS_pcDNA3 plasmid. 5 µl of ligation product was transformed into *E.coli* DH5- α strain. The next day, colonies were screened, two single colonies from each interactor were plated out into 3 ml LB media with ampicillin in 15-ml conical tubes. After overnight incubation, plasmid DNA was isolated from the bacterial cultures with Macherey-Nagel Nucleospin Plasmid Kit. To confirm the insertion of the interactomes into the plasmid, isolated plasmid DNA was

digested with the SalI enzyme. The clones that gave the expected DNA bands were chosen for further experiments (Figure 5.5).

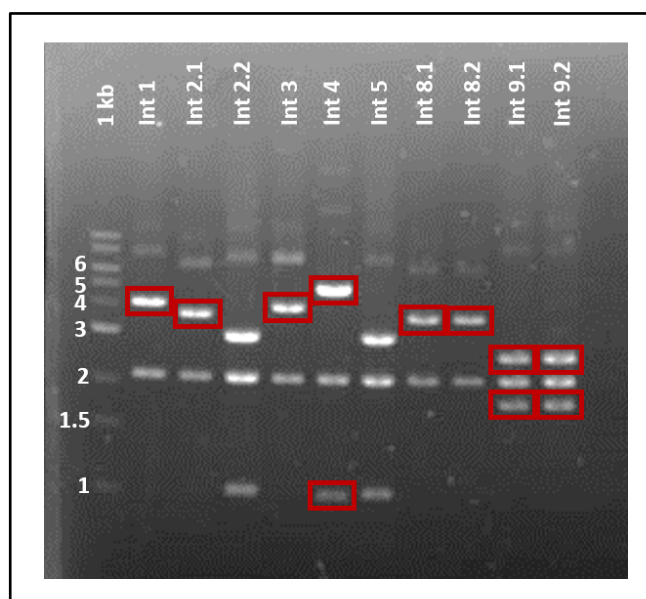


Figure 5.5: Confirmation of the cloning of the interactors into the 3xHA-NLS_pcDNA3 vector. The vector was cut with SalI enzyme. The lane numbers represent followings; 1: 1 kb DNA ladder, 2-11: Interactor 1 to Interactor 9.2. The samples were run on 1% agarose gel. The expected bands were shown with the red rectangles.

5.2. Generation of HEK293T-CRY1-3xFLAG Stable Cell Line

FLAG tag is commonly used for protein pull-down and purification experiments. The tag is a small molecular weight protein, only 1 kDa (8 amino acid residue), and has a hydrophilic nature. This hydrophilicity does not affect protein functions and antigenicity. Therefore, a FLAG tag does not increase the charge of the protein expression, besides it allows high purity yield [Zhao et al., 2013]. Because of these advantages, the FLAG tag was chosen to pull-down to protein of interest, in our case CRY1.

We have tried first to use one FLAG tag to immunoprecipitate human CRY1 (hCRY1) with anti-FLAG antibody-coupled magnetic beads. pcDNA4.CRY1-FLAG-MycHis plasmid (original name: pfmh-hCRY1, Addgene Plasmid #25843), obtained from Prof. Dr. Aziz SANCAR (The University of North Carolina at Chapel Hill, NYC, USA) and the map of the plasmid was shown in Figure 5.6. Because one FLAG did

not give efficient immunoprecipitation of CRY1, the co-IP of CRY1-FLAG from HEK293T cells were not successful.

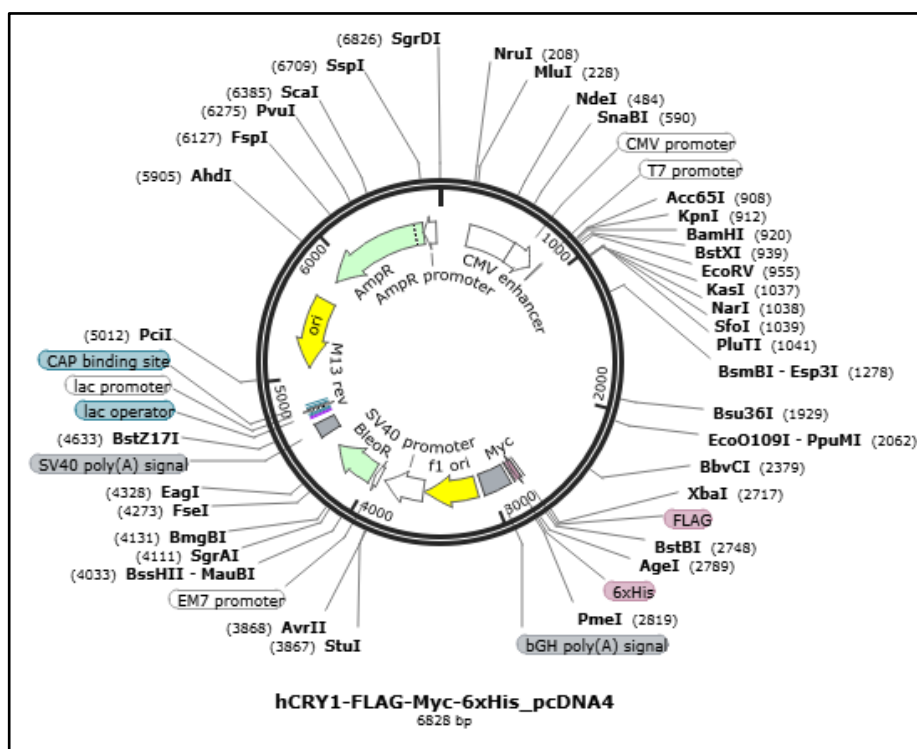


Figure 5.6: hCRY1-FLAG-Myc-6xHis_pcDNA4 map. The map was created using SnapGene Viewer Program.

5.3. Conformation of the Generation of a FLAG Expressing Stable Cell Line

Anti-CRY1 and anti-FLAG antibodies were used to detect the presence of the FLAG-CRY1 protein. The CRY1 band expressed with 1xFLAG could not be detected from western blot analysis. The result in Figure 5.7 was given solely non-specific bands arising from IgG attachment. The co-IP results were not significant until the presence of the CRY1 has been proved by anti-FLAG antibody, the cloning strategy was changed. A new plasmid construct was developed to express the 3xFLAG-CRY1 protein.

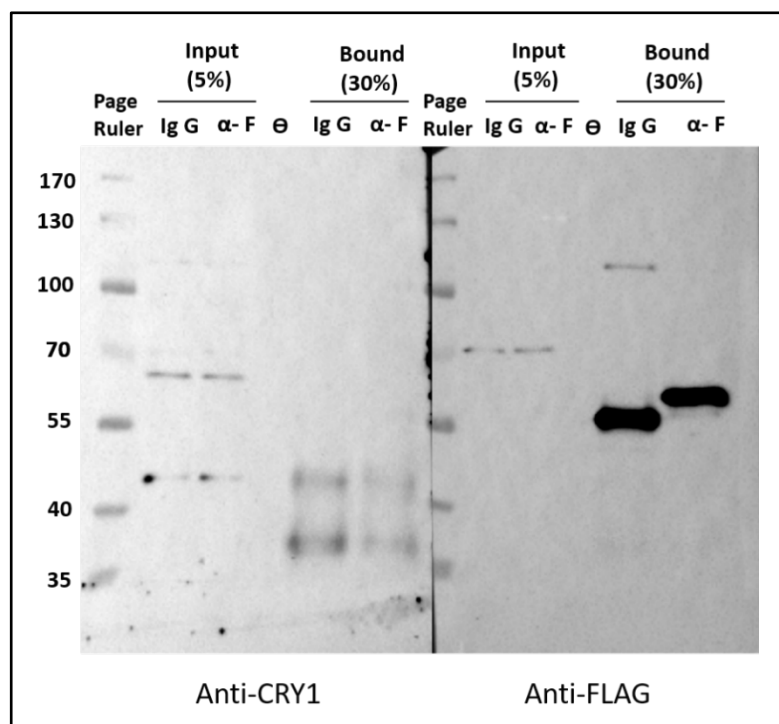


Figure 5.7: Western blot analysis of co-IP performed with anti-FLAG antibody to confirm generation of the stable FLAG expressing HEK293T cell line. Anti-CRY1 and anti-FLAG antibodies were used to detect the presence of the FLAG-CRY1 protein. Input demonstrates the cell lysate (which does not immunoprecipitated), bound shows the immunoprecipitated samples. IgG was used as a control, α -F represents the anti-FLAG antibody. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 12% SDS gel.

We decided to express human CRY1 with 3xFLAG tag, where the FLAG tags are expressed in triplicate sequentially. For this, we first inserted 3xFLAG coding sequence into XbaI and AgeI site of the pcDNA4.CRY1-FLAG-MycHis plasmid (Figure 5.8). Then, this construct was used to generate HEK293T-CRY1-3xFLAG as explained in Chapter 4.2.3. Endogenously expressed human CRY1 gene is 66.3 kDa in molecular weight, after the fusion of the 3xFLAG tag, 70 kDa band was expected in SDS polyacrylamide gel as demonstrated Figure 5.9.

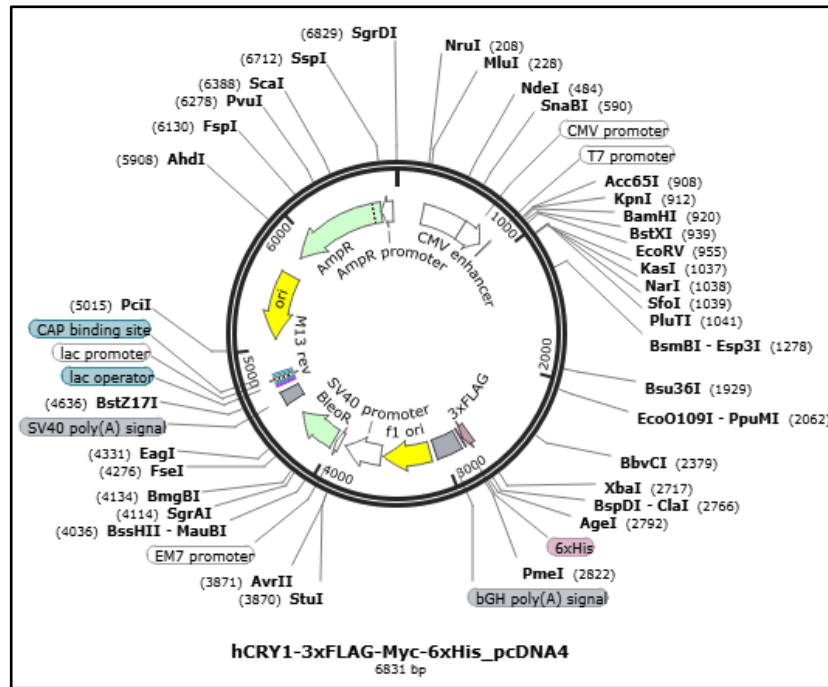


Figure 5.8: hCRY1-3xFLAG-Myc-6xHis_pcDNA4 plasmid map. The plasmid was developed from hCRY1-FLAG-Myc-6xHis_pcDNA4 plasmid by addition of 3xFLAG tag. The map was generated using SnapGene Viewer Program.

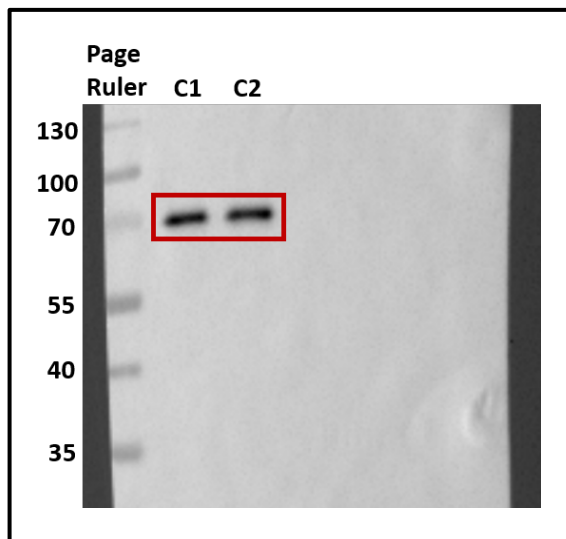


Figure 5.9: Verification of the generation of a stable 3xFLAG-CRY1 expressing HEK293 T cell line. PageRuler™ Prestained Protein Ladder (#26617, Thermo Fisher Scientific, USA) was used as a marker. Endogenous human CRY1 is a 66.3 kDa protein. After the addition of the 3xFLAG tag, a 70 kDa band was expected. 12% SDS polyacrylamide gel was used. The red rectangles demonstrate expected bands.

5.4. Expression of the Interactors in the Stable Cell Line

Before performing the co-immunoprecipitation assay, the selected clones were transfected into HEK293T-CRY1-3xFLAG cell line to ensure the interactors were efficiently expressed in this cell line. The expected bands from interactor 1 to interactor 9 were: 36, 20, 31, 104, 16, and 44, respectively (Figure 5.10).

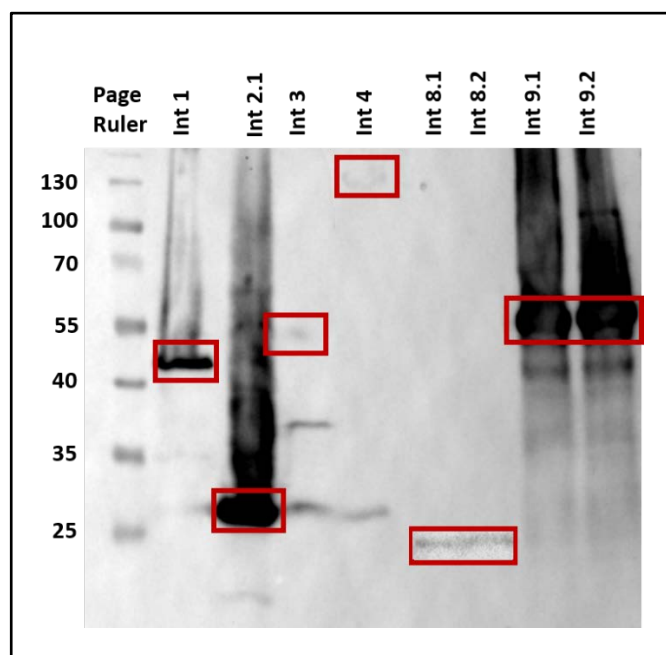


Figure 5.10: Validation of the expression of interactors in a stable HEK293T-CRY1-3xFLAG cell line. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 12% SDS gel. The expected bands were represented by the red rectangles.

5.5. co-IP of Interactors from HEK293T-CRY1-3xFLAG Cell Line

Co-immunoprecipitation was performed to verify the protein-protein interactions of CRY1 and DNA damage repair response pathway-related interactors. 3xFLAG-CRY1 protein was immunoprecipitated using an anti-FLAG antibody, other proteins within the cell lysates were removed by washing steps. The interactors were expected to co-immunoprecipitate with the 3xFLAG-CRY1 protein due to the protein-protein interaction. Western blot analysis was implemented to confirm the presence of the interactors. Anti-FLAG antibody was used to show CRY1 protein. Because the

interactors were fused with an HA-tag, the anti-HA antibody was also used in western blot experiments. It was expected to see FLAG and HA bands in both input lanes (incubated with non-specific IgG and anti-FLAG antibodies), and anti-FLAG bound lanes. In contrast, no band would be seen in the bound IgG lane, due to the absence of specific protein-antibody interaction (Figure 5.11).

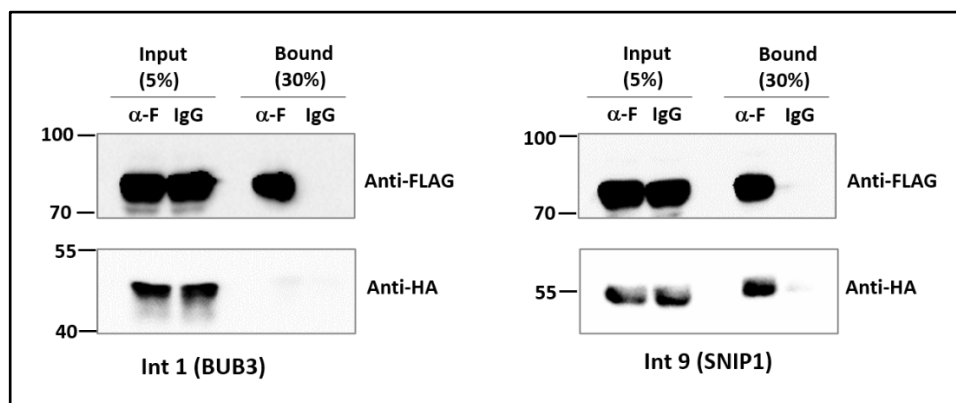


Figure 5.11: Western blot analysis of co-IP sample of BUB3 and SNIP1 proteins. Input represents the cell lysate (which does not immunoprecipitated), bound shows the immunoprecipitated samples. IgG was used as a control, α -F represents the anti-FLAG antibody. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 8% SDS gel.

The expected interaction signal could not be detected when the immunoprecipitated complex was blotted with an anti-HA antibody. To confirm the accuracy of the CRY1 and its interactor BMAL1, which is already known to interact with CRY1 protein, was chosen as a positive control. The samples were blotted using an anti-BMAL1 antibody. The specific interaction was detected as shown in Figure 5.12.

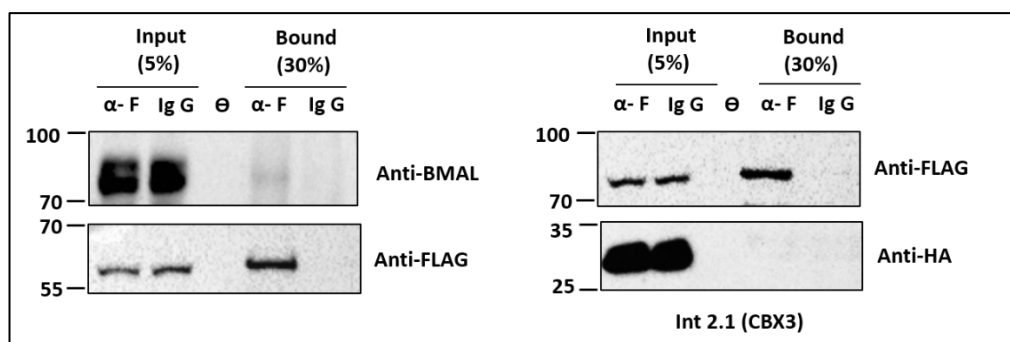


Figure 5.12: Western blot analysis of co-IP sample of BMAL1 and CBX3 proteins. Input indicates the cell lysate (which does not immunoprecipitated), bound represents the immunoprecipitated samples. IgG was used as a control, α-F represents the anti-FLAG antibody. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 8% SDS gel.

6. DISCUSSION

Discovering the protein-protein interaction (PPI) is the predominant approach for an interpretation of molecular pathways. The knowledge of the interaction has made it enable to improve the efficiency of drug therapies by understanding the function and cellular importance of a protein. Until a few years ago, yeast-two hybrid assay and immunoprecipitation coupled to mass-spectrometry (IP-MS) analysis were the two main techniques to discover novel PPIs [Maccarrone et al., 2016]. Actually, with the availability of version of proximity-dependent labeling enzymes with different kinetics such as BioID, BioID2, miniTurbo, TurboID, and APEX2 we now limit the labeling durations from minutes to 24 hours. In this way, we can now narrow PPIs of for example CRY1 to 1 hour with TurboID for example CRY1 is only in nucleus. On the hand, using BioID2 for example would allow to label CRY1 from its synthesis to degradation, which means that this technique will record the history of PPIs of CRY1. In our laboratory, a graduate student discovered CRY1 PPIs in U2OS cell line using miniTurbo based BioID technique [Olfaz, 2020].

In this thesis, we wanted to confirm some of the CRY1 PPIs from that previous study. For this, we selected the interactor candidates that are involved in DNA damage responses. Because, it is known that DNA damage response are controlled by the circadian clock, and CRY protein are human homologs of bacterial photolyase enzymes which repair UV-induced damages using the blue light as energy source. On the other hand, BioID method labels all proteins in the close proximity of the bait protein which does not necessary bait and prey will interact. Therefore, the co-IP would confirm physical interaction between bait and prey which help the interactors to study further.

From several interactors from BioID results, we identified only one strong interaction. When we analyze the presence of BMAL1 in CRY1-3xFLAG precipitated complex and actually observed a weak interaction. CRY1 and BMAL1 interactions are essential for the core circadian clock mechanism, and it is supposed to be a strong interaction. Therefore, considering that the weak BMAL1 signal in CRY1-3xFLAG precipitated complex, we cannot say that other interactors do not interact with CRY1. We can speculate that we can improve co-IP efficiency by applying some modifications. For example, the precipitation of CRY1 from only nucleus would

increase the possibility of the detecting interactors that are located in nucleus. This is also true for co-IP from cytoplasm after cellular fractionation for the interactors which are located in cytoplasm.

More importantly, we analyzed the PPIs of CRY1 under normal conditions, however giving a DNA damage to cells may specifically cause the real interaction between CRY1 and candidate interactor. This may explain why we failed to detect most of the PPIs.

In summary, an optimization in the co-IP conditions such as cellular fractionation before co-IP or activating DNA damage response pathway would increase the possibility of detecting direct interactions using co-IP technique.

In case, we have to keep in mind that these interactions can be very weak and transient and therefore could not be confirmed at all with co-IP method. In this case, functional assay would clarify the question of why those interactors were in close proximity of CRY1. These functional assays include that stability or activities of the interactors in the presence or absence of CRY1 or vice versa.

This thesis was a model on how to discover the novel interactors of CRY1. At the end we learnt that classical co-IP conditions should be optimize and may be more importantly functional assay should be considered if no physical interaction is detected with co-IP method. With the advances in BioID and optimization of co-IP it looks like that we will be able to discover more PPIs which were not possible so far due to technical difficulties.

The discovery of novel PPIs will provide novel target for intervention using different agents such as small molecular weight chemicals or gene-editing or RNA-interference. In future, these interventions could open new therapeutic strategy for either circadian clock or DNA damage response to exploit in the management of diseases such as cancer.

All these associations of DNA-damage repair component and *CRY1* genes suggests that *CRY1* is an important suppressor of abnormal cell neoplasms, and tumor aggressiveness. Validation of the *CRY1* interactions may open a new point of view for the understanding of the relation between the circadian clock and DNA damage. The exploration of the novel *CRY1* interactomes helps the *CRY1* targeted drug therapy and better prognosis in cancer patients.

REFERENCES

- Akashi M., Tsuchiya, Y. Yoshino, T. Nishida E., (2002), “Control of intracellular dynamics of mammalian period proteins by casein kinase I ϵ (CKI ϵ) and CKI δ in cultured cells”, *Molecular and Cellular Biology*, 22(6), 1693–1703.
- Akashi M., Takumi T., (2005), “The orphan nuclear receptor ROR α regulates circadian transcription of the mammalian core-clock Bmal1”, *Nature Structural and Molecular Biology*, 12(5), 441–448.
- Allen A. E., (2019), “Circadian rhythms in the blind, *Current Opinion in Behavioral Sciences*”, *Current Opinion in Behavioral Sciences*, 30, 73–79.
- Angelousi A., Kassi E. Ansari-Nasiri N., Randeva H., Kaltsas G., Chrousos G., (2019), “Clock genes and cancer development in particular in endocrine tissues”, *Endocrine-Related Cancer*, 26(6), 305–317.
- Baker A., Saltik M., Killisch I., Mautner V., Lamm G., Christofori G., Cotton 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, 773-782
- Baldi L., Muller N., Picasso S., Jacquet R., Girard P., Thanh H. P., Derow E., Wurm F., M., (2008), “Transient gene expression in suspension HEK-293 cells: application to large-scale protein production”, *Biotechnology Progress*, 21(1), 148-153.
- Brown S., Kowalska E., Dallmann, R., (2012), “(Re)inventing the circadian feedback loop”, *Developmental Cell*, 22(3), 477–487.
- Chaves I., Yagita K., Barnhoorn S., Okamura H., van der Horst G. T. J., 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.
- Duez H., Staels, B., (2009), “Rev-erb- α : an integrator of circadian rhythms and metabolism”, *Journal of Applied Physiology*, 107(6), 1972–1980.
- Everett L. J., Lazar M. A., (2014), “Nuclear receptor Rev-erb α : up, down, and all around”, *Trends in Endocrinology and Metabolism*, 25(11), 586–592.
- Foley L. E., Gegear R. J., Reppert S. M., (2011), “Human cryptochrome exhibits light-dependent magnetosensitivity”, *Nature Communications*, 2(1).
- Gaddameedhi S., Selby C. P., Kaufmann W. K., Smart R. C., Sancar, A., (2011), “Control of skin cancer by the circadian rhythm”, *Proceedings of the National Academy of Sciences*, 108(46), 18790–18795.

Gallego M., Virshup D. M., (2007), “Post-translational modifications regulate the ticking of the circadian clock”, *Nature Reviews Molecular Cell Biology*, 8(2), 139–148.

Gao P., Yoo S. H., Lee K. J., Rosensweig C., Takahashi J. S., Chen B. P., Green C. B., (2013), “Phosphorylation of the Cryptochrome 1 C-terminal tail regulates circadian period length”, *Journal of Biological Chemistry*, 288(49), 35277–35286.

Go C.D., Knight J.D.R., Rajasekharan A. *et al.*, (2021), “A proximity-dependent biotinylation map of a human cell”, *Nature*, 595, 120–124.

Godinho S. I. H., Maywood E. S., Shaw L., Tucci V., Barnard A. R., Busino L., Pagano M., Kendall R., Quwailid M. M., Romero M. R., O’Neill J., Chesham J. E., Brooker D., Lallanne Z., Hastings M. H., Nolan P. M., (2007), “The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period”, *Science*, 316(5826), 897–900.

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

Jia M., Su B., Mo L., Qiu W., Ying J., Lin P., Yang B., Li D., Wang D., Xu L., Li H., Zhou Z., Li X., Li J., (2020), “Circadian clock protein CRY1 prevents paclitaxel-induced senescence of bladder cancer cells by promoting p53 degradation”, *Oncology Reports*, 45(3), 1033–1043.

Kang T. H., Lindsey-Boltz L. A., Reardon, J. T., Sancar, A., (2010), “Circadian control of XPA and excision repair of cisplatin-DNA damage by cryptochrome and HERC2 ubiquitin ligase”, *Proceedings of the National Academy of Sciences*, 107(11), 4890–4895.

Kang T. H., Leem, S. H., (2014), “Modulation of ATR-mediated DNA damage checkpoint response by cryptochrome 1, *Nucleic Acids Research*”, 42(7), 4427–4434.

Lee J. H., Gaddameedhi S., Ozturk N., Ye R., Sancar A., (2012), “DNA damage-specific control of cell death by cryptochrome in p53-mutant Ras-transformed cells”, *Cancer Research*, 73(2), 785–791.

Lee T. H., Park J. M., Leem S. H., Kang, T. H., (2014), “Coordinated regulation of XPA stability by ATR and HERC2 during nucleotide excision repair”, *Oncogene*, 33(1), 19–25.

Lockley S. W., Skene D. J., Arendt J., Tabandeh H., Bird A. C., DeFrance, R., (1997), “Relationship between melatonin rhythms and visual loss in the blind”, *The Journal of Clinical Endocrinology and Metabolism*, 82(11).

Maccarrone G., Bonfiglio J. J., Silberstei S., Turck C. W., Martins-de-Souza D., (2016), “Characterization of a protein interactome by co-immunoprecipitation and shotgun mass spectrometry”, *Multiplex Biomarker Techniques*, 223–234.

Mouritsen H., Ritz, T., (2005), “Magnetoreception and its use in bird navigation, *Current Opinion in Neurobiology*”, 15(4), 406–414.

Niida H., Nakanishi M., (2005), “DNA damage checkpoints in mammals”, *Mutagenesis*, 21(1), 3–9.

Olfaz S., (2020), “Exploring the interactome of recombinant CRY1 protein by proximity labeling” [Unpublished Master of Science Thesis], Gebze Technical University.

Ozturk N., (2017), “Phylogenetic and functional classification of the photolyase/cryptochrome family”, *Photochemistry and Photobiology*, 93(1), 104–111.

Ozturk N., Lee J. H., Gaddameedhi S., Sancar A., (2009), “Loss of cryptochrome reduces cancer risk in p53 mutant mice”, *Proceedings of the National Academy of Sciences*, 106(8), 2841–2846.

Partch C. L., Green C. B., Takahashi J. S., (2014), “Molecular architecture of the mammalian circadian clock”, *Trends in cell biology*, 24(2), 90–99.

Reppert S. M., Weaver D. R., (2002), “Coordination of circadian timing in mammals”, *Nature*, 418(6901), 935–941.

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

Sancar A., Lindsey-Boltz L. A., Kang T. H., Reardon J. T., Lee J. H., Ozturk N., (2010), “Circadian clock control of the cellular response to DNA damage”, *FEBS Letters*, 584(12), 2618–2625.

Shafi A. A., McNair C. M., McCann J. J., Alshalalfa M., Shostak A., Severson T. M., Zhu Y., Bergman A., Gordon N., Mandigo A. C., Chand S. N., Gallagher P., Dylgjeri E., Laufer T. S., Vasilevskaya I. A., Schiewer M. J., Brunner M., Feng F. Y., Zwart W., Knudsen K. E., (2021), “The circadian cryptochrome, CRY1, is a pro-tumorigenic factor that rhythmically modulates DNA repair”, *Nature Communications*, 12(1).

Swamy M., Siegers G., Minguet S., Wollscheid B., Schamel W. W. A., (2006), “Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the identification and analysis of multiprotein complexes”, *Science Signalling*, 345, pl14.

Takahashi J., (2016), “Transcriptional architecture of the mammalian circadian clock”, *Nature Reviews Genetics*, 18, 164–179.

Teo G., Liu G., Zhang J., Nesvizhskii A., I., Gingras A., C., Choi, H., (2014), “SAINTexpress improvements and additional features in significance analysis of INTeractome software”, *Journal Proteomics*, 100, 37–43.

Vitaterna M. H., Selby C. P., Todo T., Niwa H., Thompson C., Fruechte E. M., Hitomi K., Thresher R. J., Ishikawa T., Miyazaki J., Takahashi J. S., 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.

Wan G., Hayden A.N., Iiams S.E., Merlin, C., (2021), “Cryptochrome 1 mediates light-dependent inclination magnetosensing in monarch butterflies”, *Nature Communications*, 12(1).

Wang H., Liu R., (2011), “Advantages of mRNA display selections over other selection techniques for investigation of protein–protein interactions”, *Expert Review of Proteomics*, 8, 335–346.

Yoo S. H., Mohawk J., Slepka S., Shan Y., Huh S., Hong H. K., Kornblum I., Kumar V., Koike N., Xu M., Nussbaum J., Liu X., Chen Z., Chen Z., Green C., Takahashi J., (2013), “Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm”, *Cell*, 152(5), 1091–1105.

Zhao H., Piwnicka-Worms H., (2001), “ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1”, *Molecular and Cellular Biology*, 21(13), 4129–4139.

Zhao X., Li G., Liang S., (2013), “Several affinity tags commonly used in chromatographic purification”, *Journal of Analytical Methods in Chemistry*, 1-8.

Zhou L., Yu Y., Sun S., Zhang T., Wang M., (2018), “Cry 1 regulates the clock gene network and promotes proliferation and migration via the Akt/P53/P21 pathway in human osteosarcoma cells”, *Journal of Cancer*, 9(14), 2480–2491.

BIOGRAPHY

Saba SELVİ was received her B. Sc. Degree in Molecular Biology and Genetics Department at Gebze Technical University in 2019. She then joined the MSc. Program in Gebze Technical University, Graduate School of Natural and Applied Science, Molecular Biology and Genetics Department and after that she graduated with MSc degree in 2021. She worked for 2 years under the supervision of Prof. Dr. Nuri OZTURK.

APPENDICES

Appendix A: Protein and DNA Markers

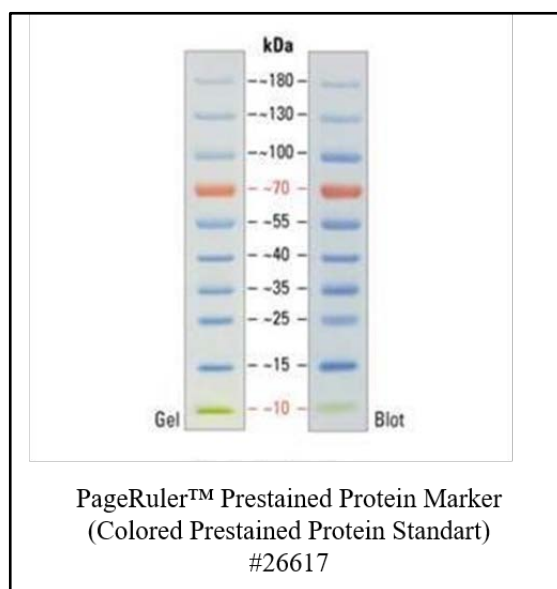


Figure A1.1: Protein Ladder.

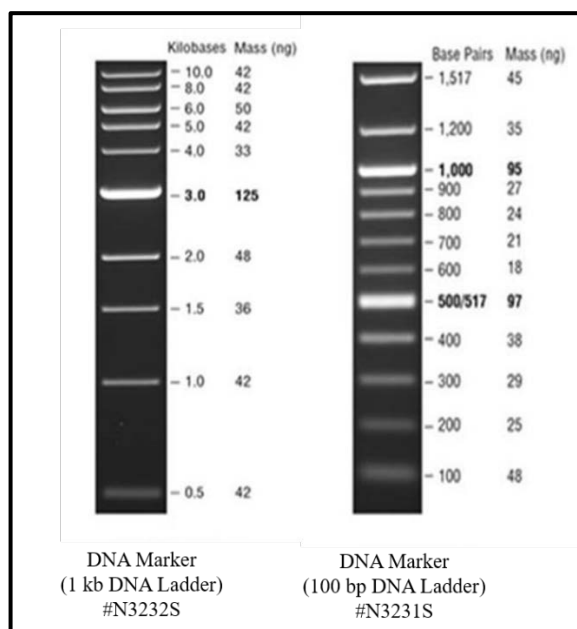


Figure A1.2: DNA Ladder.

Appendix B: List of CRY1 interactors generated by Saintexpress Analysis

Table A1.1: A list of CRY1 interactors associated with DNA damage and response pathways was created using SAINTexpress software (saint score of 0.98 and above).

Uniprot Accession Number	Saint Score
Q13185	1.0
Q9GZR7	1.0
O75533	1.0
Q8TAD8	1.0
Q43684	0.99
Q53HL2	0.99
Q9NQS7	0.99
Q03188	0.98
P49736	0.98
P33981	0.98