

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

EXPLORING NOVEL BMAL1 INTERACTIONS

KEVSER ÇİLENK
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

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BELİRLENMESİ

KEVSER ÇİLENK
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

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2022



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SUMMARY

The circadian clock is the mechanism found in almost all organisms that regulates metabolic, physiological, and behavioral processes in approximately 24-hour rhythms. These rhythms are generated by the transcription-translation feedback loop (TTFL) mechanism. In the positive arm of the TTFL, CLOCK and BMAL1 transcription factors transactivate the expression of circadian clock-regulated genes (CCGs). Two of these CCGs, *CRY* (*CRYPTOCHROME*) and *PER* (*PERIOD*) function in the negative arm of the TTFL, and their products translocate into the nucleus to inhibit CLOCK-BMAL1 activity. This inhibition and activation between positive and negative arms generate the oscillations in the CCGs which are also tuned by consolidator loops and post-translational modifications. Disturbances in the circadian clock are associated with various pathological conditions such as cancer, sleep disorders, and metabolic syndrome.

In this study, we aimed to confirm the novel BMAL1 protein-protein interactions which were discovered in our laboratory using the proximity-dependent biotinylation identification (BioID) technique. These interactions were confirmed by a co-immunoprecipitation method. For this purpose, HA-tagged interactors were expressed in HEK293T cells expressing 3xFLAG-BMAL1. The presence of interactors in anti-FLAG precipitated complexes were analyzed with anti-HA immunoblotting.

We confirmed some of these interactions, which suggests that BMAL1 has some undiscovered functions and functional studies of these interactions may reveal novel reaction mechanisms.

Key Words: BMAL1, Circadian Clock, Protein-Protein Interactions (PPI), Interactor, co-Immunoprecipitation (co-IP).

ÖZET

Sirkadiyen saat, metabolik, fizyolojik ve davranışsal süreçleri yaklaşık 24 saatlik ritimlerde düzenleyen hemen hemen tüm organizmalarda bulunan mekanizmadır. Bu ritimler, transkripsiyon-translasyon geri besleme döngüsü (TTFL) mekanizması tarafından üretilir. TTFL'nin pozitif kolunda, CLOCK ve BMAL1 transkripsiyon faktörleri, sirkadiyen saat tarafından düzenlenen genlerin (CCG) ekspresyonunu trans-aktif eder. Bu CCG'lerden ikisi, *CRY* (*CRYPTOCHROME*) ve *PER* (*PERIOD*), TTFL'nin negatif kolunda işlev görür ve ürünleri, CLOCK-BMAL1 aktivitesini inhibe etmek için sitoplazmadan çekirdeğe göç ederler. Pozitif ve negatif kollar arasındaki bu inhibisyon ve aktivasyon, aynı zamanda konsolidatör (yardımcı) döngüler ve translasyon sonrası modifikasyonlar tarafından da ayarlanarak CCG'lerdeki salınımları üretir. Sirkadiyen saatteki bozukluklar kanser, uyku bozuklukları ve metabolik sendrom gibi çeşitli patolojik durumlarla ilişkilidir.

Bu çalışmada, daha önce laboratuvarımızda yakınlığa bağlı biyotininilasyon tanımlama (BioID) tekniği kullanılarak keşfedilmiş olan yeni BMAL1 protein-protein etkileşimlerini doğrulamayı amaçladık. Bu etkileşimler, birlikte immünopresipitasyon yöntemiyle doğrulandı. Bu amaçla, HA etiketli interaktörlerin, 3xFLAG-BMAL1'i eksprese eden HEK293T hücrelerinde anlatımı sağlandı. Anti-FLAG antikorlarıyla çökeltilmiş komplekslerde interaktörlerin varlığı, anti-HA immüno blotlama ile analiz edildi.

Bu çalışmada BMAL1'in keşfedilmemiş etkileşimleri doğrulanırken, bu çalışma bu etkileşimlerin özgün işlevleri olduğunu ve bu etkileşimlerin işlevsel çalışmalarının yeni reaksiyon mekanizmalarını ortaya çıkarabileceğine işaret etmektedir.

Anahtar Kelimeler: BMAL1, Sirkadiyen Saat, Protein-Protein Etkileşimleri, İnteraktör, Ko-İmmünopresipitasyon.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: (degree) Celsius
µg	: Microgram
µl	: Microliter
APS	: Ammonium persulfate
ARNT	: Aryl hydrocarbon receptor nuclear translocator-like protein
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle ARNT-like 1
bp	: Base pair
CCG	: Circadian clock-regulated gene
CK2 α	: Casein kinase II subunit alpha
co-IP	: co-Immunoprecipitation
CRY	: Cryptochrome gene or protein
ddH ₂ O	: Double-distilled water
dNTPs	: Deoxynucleotide triphosphate
DMEM	: Dulbecco's Modified Eagle's Medium
<i>E. coli</i>	: <i>Escherichia coli</i>
EDTA	: Ethylenediaminetetraacetic acid
FBXL3	: F-box/leucine-rich repeat protein 3
FBS	: Fetal Bovine Serum
GSK3B	: Glycogen synthase kinase-3 beta
HEK293T	: Human embryonic kidney 293T cells
HRP	: Horseradish peroxidase
kb	: Kilobase pair
LB	: Luria Bertani
MAPK	: Mitogen-activated protein kinase
NEAA	: Non-essential amino acids
nm	: nanometer
	:

PCR	: Polymerase chain reaction
PER	: PERIOD gene or protein
PEI	: Poly-ethylenimine
PI	: Protease inhibitor
PPI	: Protein-protein interaction
RE	: Restriction enzyme
RER	: RORE-RevRE
REV-ERB	: Retinoic acid orphan nuclear receptors
RRE	: Rev response element
ROR	: Retinoic acid-related orphan receptor
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAP	: Tandem affinity purification
TBE	: Tris/Boric Acid/EDTA
TBS	: Tris Buffered Saline
TEMED	: Tetramethylethylenediamine
TTFL	: Transcription Translation Feedback Loop
Y2H	: Yeast 2 hybrid

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1. INTRODUCTION

This thesis includes validation of novel BMAL1 interactions using the co-immunoprecipitation method. These interactions were previously discovered in our laboratory using the proximity-dependent biotinylation (BioID) technique followed by mass spectrometry. Before moving on from these interactions to functional studies, another method is required to confirm the identity of these interactions because the BioID method may give false positive results. Therefore, we have analyzed and confirmed the presence of some of the novel BMAL1 interactors in the purified BMAL1 complex. We can speculate about their functions based on our findings in this thesis, but functional studies are still needed to understand their biological relevance/meaning.

With the further confirmation of more novel BMAL1 interactions and functional studies of them, new connections between the circadian clock and other signaling pathways are expected to be discovered.

1.1. The Purpose, Contribution, and Content of Thesis

In this study, we aim to validate some of the circadian clock protein's interactions with the co-immunoprecipitation (co-IP) technique. These possible interactions were previously discovered in our laboratory using the proximity dependent biotin labelling and identification (BioID) method. In this thesis, the candidates from mass spectrometry analysis were subjected to SAINT Express Analysis to determine statistically significant ones and eliminate false positives. To confirm the physical interaction between BMAL1 and novel interactors, we first stably expressed 3xFLAG-tagged BMAL1 in HEK293T human embryonic kidney cells (HEK293T-BMAL1.3xFLAG). Then individual interactors, which were cloned from U2OS human osteosarcoma cells line's cDNA, were transfected into HEK293T-BMAL1.3xFLAG cells and BMAL1 complex was immunoprecipitated using anti-FLAG antibody-magnetic beads. The presence of the interactions in the BMAL1 complex was analyzed using anti-HA antibody immunoblotting because interactors had 3xHA tagged in a frame. This strategy confirmed some the novel interactions. The presence of known interactors such as CLOCK and CRY1 in

BMAL1 complexes suggested that our system was enough functional to detect significant physical interactions. The specificity of the system was controlled by normal IgG control to immunoprecipitate and the absence of the candidates in control beads confirmed these interactions are real.

With this study, we confirmed some novel BMAL1 interactions which can be further studied by functional assay to understand their biological relevance to the circadian clock or other signaling pathways.

Chapter 2 presents a literature review on the circadian clock, a molecular transcription-translation feedback loop (TTFL), BMAL1 protein, BioID technique and Co-Immunoprecipitation technique. Section 3 lists the materials and equipment used in the experiments. All experimental protocols are described and detailed in Chapter 4. In Chapter 5, the results of the experiment are explained with figures. Finally, the thesis is concluded by discussing the results in Chapter 6.

2. LITERATURE REVIEW

2.1. Circadian Clock

All organisms on Earth are under the influence of daily and seasonal changes due to the planet's rotation and orbit around the sun. To adapt to these changes, organisms need an endogenous circadian clock system that synchronizes their biological functions with the environment [Albrecht and Eichele, 2003]. The circadian clock is a mechanism that regulates physiological, biochemical, and behavioral events in approximately 24-hour cycles to maintain organism homeostasis. Many biological activities such as blood pressure, hormone release, sleep-wake, and liver metabolism occur under the control of the circadian clock [Doruk et al., 2020]. Therefore, circadian (derived from the word circa-dies, meaning “about one day”) rhythms generated by the molecular clock are found in nearly all living organisms and its disturbances are associated with various diseases such as cancer, diabetes, sleep disorders [Yamada and Forger, 2010].

The endogenous master clock, which is regularly reset or resynchronized daily by sun light early in the morning, is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain [Çevik and Aslan, 2015]. The light-dark cycle resetting signal is received from the retina through to the retinohypothalamic pathway [Sinturel et al., 2020]. The SCN communicates with other organs or tissues by neuronal and humoral signals to synchronize the peripheral clocks in organs. Thus, the SCN coordinates subcellular oscillators in the brain and peripheral organs with each other and with external time [Pilorz et al., 2020].

2.2. Molecular Transcription-Translation Feedback Loop (TTFL)

In mammals, the circadian clock is generated by the transcription-translation feedback loop (TTFL) mechanism. This TTFL mechanism consists of a positive and a negative arm [Albrecht, 2012]. BMAL1 and CLOCK proteins, the two members of the bHLH-PAS (Basic-helix-loop-helix - PER-ARNT-SIM) transcription activators, are the transcription factors that make up the positive arm [Okamura et al., 2010].

BMAL1/CLOCK forms a heterodimer structure and activates the gene expression by binding to E-box elements (CACGTG) in the promoters of negative arm genes (*PERIOD* and *CRYPTOCHROME*) and clock-controlled genes (CCGs) [Krishnaiah et al., 2017]. The PER 1-3 (*PERIOD*) and CRY 1-2 (*CRYPTOCHROME*), which act as negative regulators, oligomerize after expression [Grimaldi et al., 2009]. The PER/CRY complex is phosphorylated by protein kinases (CK1 ϵ and CK1 δ) and translocates to the cell nucleus. In the nucleus, PER and CRY heterodimers inhibit transcription of the CLOCK/BMAL1 complex. Therefore, they also indirectly inhibit the expression of CCGs along with the expression of themselves [Preußner and Heyd, 2016].

The stability of PER/CRY proteins in the cytoplasm and nucleus is regulated by ubiquitination-mediated degradation. In addition, casein kinases provide the determination of the internal period length of the clock by controlling the rate of entry of the PER/CRY complex into the nucleus. After the degradation of the PER and CRY, the inhibition of the CLOCK/BMAL1 complex is relieved, which is the re-start of the circadian cycles [Partch et al., 2014]. In addition to this core loop, there is a secondary (consolidator) feedback loop. The Rev response element (RRE) in this regulatory loop regulates the BMAL1 promoter by interacting with both RAR-related orphan receptors (RoR α , RoR β , and RoR γ) and Retinoic acid orphan nuclear receptors (Rev-eRB α and Rev-eRB β) [Zhang and Kay, 2010]. The expression of these receptors is regulated by the BMAL1/CLOCK complex. Transcription of *Bmal1* is activated by ROR and suppressed by REV-ERB [Chen et al., 2020]. An additional level of regulation is provided by the expression of circadian clock-regulated genes undergoing various post-translational modifications such as phosphorylation, sumoylation, acetylation, and ubiquitination [Chaudhari et al., 2017].

Phosphorylation is one of the most important post-translational modifications that regulate various cellular events in eukaryotic and prokaryotic cells. Therefore, phosphorylation can alter the activity and stability, binding capacity, localization, and function of a protein in the molecular clock mechanism. Phosphorylation is very important in mammalian circadian clocks. But other post-translation modifications are also needed to regulate circadian oscillations [Sinturel et al., 2020]. To establish a circadian rhythm, the oscillations must occur in approximately 24-hour periods. Biochemical events take place in seconds or minutes instead of hours. Therefore, a

delay must occur in the negative feedback mechanism. The role of post-translational modifications is crucial for this delay to occur. Phosphorylation of many molecular clock genes such as CRY, BMAL1, and PER is performed rhythmically. Phosphorylation of these genes is achieved by the balanced function of kinases and phosphatases [Reischl and Kramer, 2011].

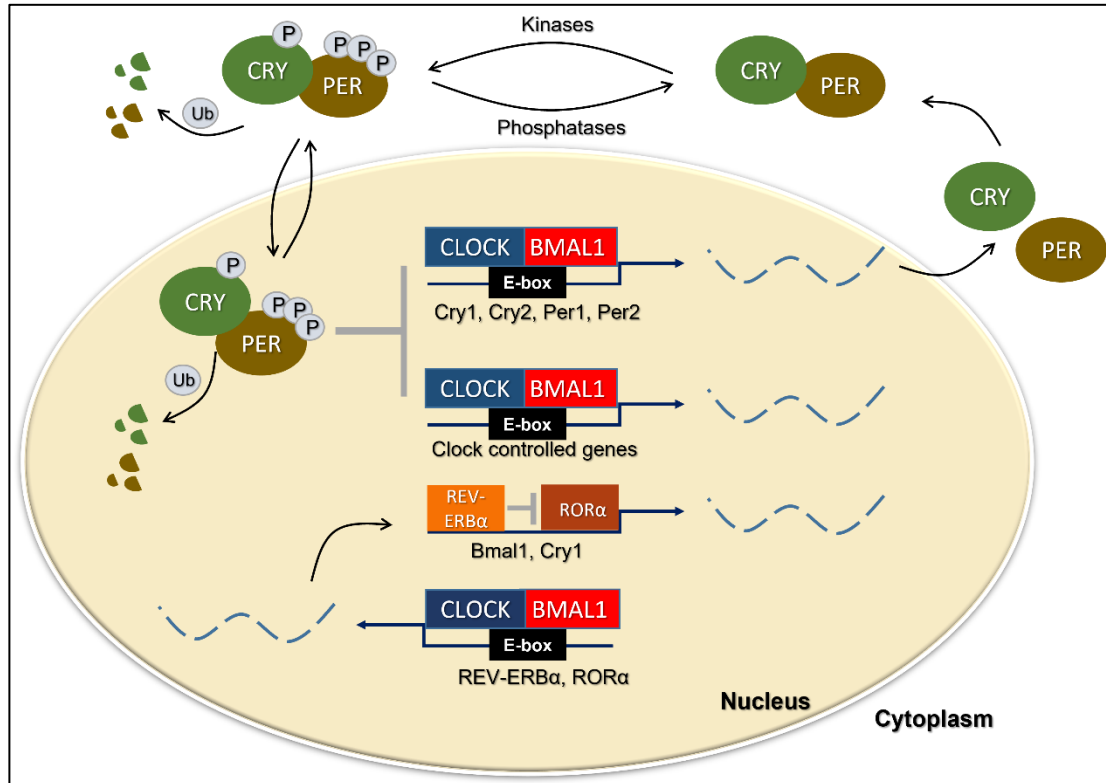


Figure 2.1: A general model of the circadian molecular transcription-translation feedback loop.

2.3. BMAL1 (Brain and Muscle ARNT-Like 1) Protein

In humans, the BMAL1 protein is encoded by the *Bmal1* gene. The *Bmal1* gene is also called ARNTL, MOP3, BMAL, BMAL1C, JAP3 etc. BMAL1 is a transcription factor consisting of a basic helix-loop-helix (bHLH) and two PAS domains. The BMAL1 protein plays a role in the positive arm of the transcription-translation feedback loop (TTFL), which establishes the molecular circadian clock [Reppert and Weaver, 2002]. Deletion of the transcription factor *Bmal1* gene prevents the formation of 24-hour rhythms in the circadian clock [Ray et al., n.d.]. As a result,

it was determined that the circadian clock was disrupted when the *Bmal1* gene is absent in humans and this is associated with many pathological conditions such as disruptions in the sleep-wake cycle, and abnormal retinal function [Reppert and Weaver, 2002]. Mutations in BMAL1 have been associated with diseases such as diabetes, hypertension, sleep disorders, infertility, and gluconeogenesis [Richards et al., 2014]. The deacetylation activity of the SIRT1 protein inhibits the transcription of the CLOCK/BMAL1 complex and regulates the degradation of the Per protein [Asher et al., 2008]. Degradation of the Per protein release the inhibition on the activity of the CLOCK/BMAL1 heterodimer. In addition, the FBXL3 (F-box/LRR-repeat protein 3) protein provides polyubiquitination of CRY proteins and targets them for proteasomal degradation [Ko and Takahashi, 2006].

The CLOCK/BMAL1 complex also regulates the transcription of target genes by binding to the E-box element in the promoters of the ROR α / β and Rev-Erba genes. The REV-ERBa and ROR proteins regulate the expression of the *Bmal1* gene through a secondary feedback loop. In addition, these proteins compete for binding to Rev-Erb/ROR response elements located in the promoter of *Bmal1*. Thus, *Bmal1* expression is activated by ROR proteins and suppressed by REV-ERBa proteins [Guillaumond et al., 2005]. Post-translational modifications of BMAL1 regulate the timing of the transcription-translation feedback loops of the CLOCK/BMAL1 heterodimer.

Phosphorylation of BMAL1 makes it a target for other modifications (ubiquitination, degradation, deubiquitination, and stabilization). Acetylation of BMAL1 stimulates CRY1 to inhibit transcriptional activation of the CLOCK/BMAL1 complex [Hirayama et al., 2007]. Phosphorylation of BMAL1 by kinases is important for both its stability and activity. Phosphorylation of the CLOCK/BMAL1 heterodimer is activated by casein kinase 1 ϵ and inhibited by MAPK (Mitogen-activated Protein Kinase) [Eide et al., 2002]. The intracellular localization of BMAL1 is regulated by CK2 α (Casein kinase II subunit alpha) phosphorylation, while the stability of BMAL1 is controlled by GSK3B (Glycogen synthase kinase-3 beta) phosphorylation. Thus, the BMAL1 protein is prepared for ubiquitination [Tamaru et al., 2009, Sahar et al., 2010]. The BMAL1 protein structurally contains four regions. These are a bHLH domain, two PAS domains (PAS-A and PAS-B), and a transactivating domain. The CLOCK/BMAL1 heterodimer has strong interactions within these domains [Huang et al., 2012].

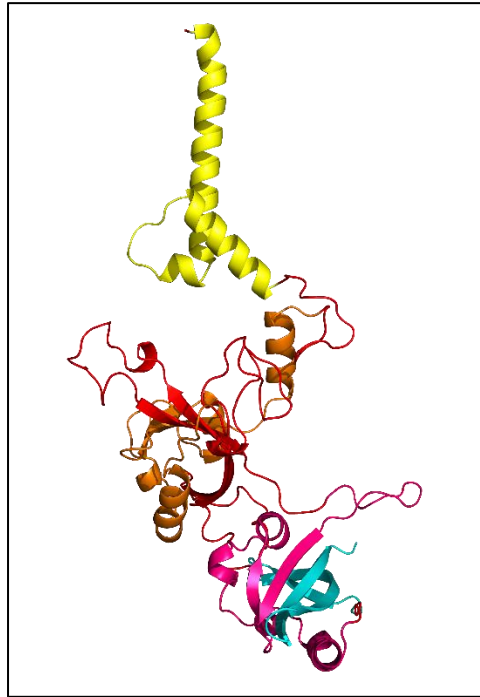


Figure 2.2: 3-D Crystal structure of the BMAL1(it was created as a PDB ID: 4F3L template by Swissmodel protein as a cartoon. The images were created with PyMOL(Open-Source).

2.4. BioID Technique

Protein-protein interactions (PPI) play a role in important biological processes such as cell communication, cell growth and division, immune response, and metabolism. The proteins involved in these processes perform their functions by acting in conjunction with each other. It is necessary to understand the functions of proteins and their behavior in interaction. Therefore, various methods such as proximity-dependent labeling methods have been developed to detect and identify candidate protein-protein interactions (PPI) [Béganton et al., 2019].

The traditional approaches to identify protein-protein interactions are affinity purification, Y2H (Yeast 2 hybrid), and TAP (Tandem affinity purification). However, these traditional scanning methods have limitations such as cost, time, etc [Rao et al., 2014].

Also, not all protein-protein interactions exhibit strong affinity binding. For this reason, BioID (Proximity-dependent biotin identification), APEX (Ascorbate

peroxidase-based), or their variants, which have been developed in recent years, are used to identify weak and transient protein interactions in living cells. There are two groups of protein labeling enzymes that are mostly used in affinity labeling methods. These are biotin ligases and peroxidases.

The characteristics of the enzymes used for proximity labeling are very important. APEX is widely used because of its fast kinetics and high activity. However, biotin ligase-based methods are preferred because of the difficulty of delivering peroxidase substrates to target tissues [Cho et al., 2020].

The BioID technique ensures that the physiological states of proteins are maintained, and interactors are identified with high sensitivity. In the BioID method, the biotin ligase enzyme is fused to the target protein and performs the biotinylation of other proteins in the immediate vicinity of the target fusion protein. In this way, biotinylated proteins are purified with streptavidin-coated beads and identified by mass spectrometry [University et al., 2018].

The use of the BioID method is limited due to its long labeling times and low activity. TurboID and MiniTurbo have shorter labeling kinetics and efficiency than other biotin ligase-based methods. TurboID is widely preferred because it has higher activity than the BioID method. Although MiniTurbo's activity is lower than TurboID, it has much shorter labeling times than BioID [Cho et al., 2020].

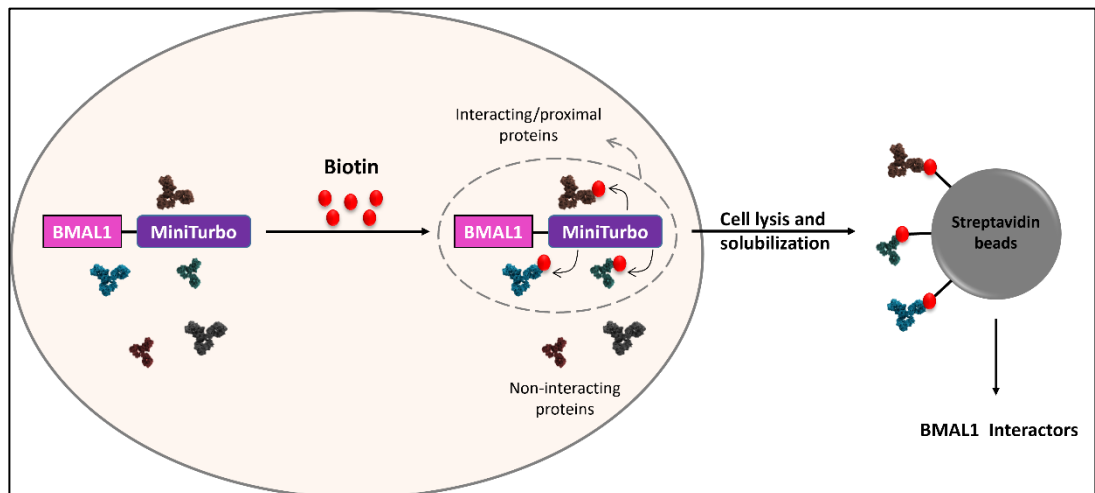


Figure 2.3: Stages of the BioID technique. MiniTurbo biotin ligase enzyme is fused to BMAL1 protein. Endogenous proteins in the immediate environment of BMAL1 are labeled with biotin. After the biotinylation process is finished, the cells are lysed and solubilized. Then biotinylated proteins are precipitated with Streptavidin beads. Subsequently, BMAL1 interactors are identified by mass spectrometry.

In this thesis, we used the affinity labeling technique with the MiniTurbo biotin ligase enzyme. The precipitated interactors were identified by mass spectrometry. We used the co-IP (co-immunoprecipitation) method to confirm the interaction of these interactors with BMAL1.

2.5. Co-Immunoprecipitation Technique

Protein-protein interactions (PPI) are an integral part of nearly all cellular processes (such as metabolism, signal transduction, protein localization, and enzyme activation). These processes involve proteins acting together in complex and interrelated systems [Béganton et al., 2019].

Although various methods are available to identify protein-protein interactions, co-immunoprecipitation (co-IP) is a powerful technique that is widely used to discover new protein interactions or to identify indirect/direct physical relationships between the target protein and other proteins [Tang and Takahashi, 2018]. This method exploits the specificity of an antibody to bind to the protein of interest. If a specific antibody for the target protein is not available, the labeled version of the protein can be used. Here, the antibody is selected specifically for the tagged protein [Isono and Schwechheimer, 2010]. Basically, a tag is attached to the target protein (such as HA, FLAG, Myc, EGFP) and precipitation is provided by incubation with a commercial antibody specific to this tag [Tang and Takahashi, 2018].

Here, the antibody is immobilized by binding to Protein A/G-linked matrices (such as agarose, sepharose, or magnetic beads). The cell lysate containing the targeted protein is then incubated with a mixture of magnetic beads and antibodies. In this way, the protein of interest and its interactomes bind to the antibody and are precipitated. Non-precipitating and non-specific interactions on the beads are removed by washing. The precipitated protein complexes are separated by SDS-PAGE and then analyzed by immunoblotting.

In this thesis, a large number of possible protein-protein interactions (PPI) were identified using the BioID technique with U2OS-MiniTurbo-BMAL1 cells in our laboratory. The co-IP technique was used to confirm the presence of possible PPIs obtained by the BioID technique and identified by mass spectrometry.

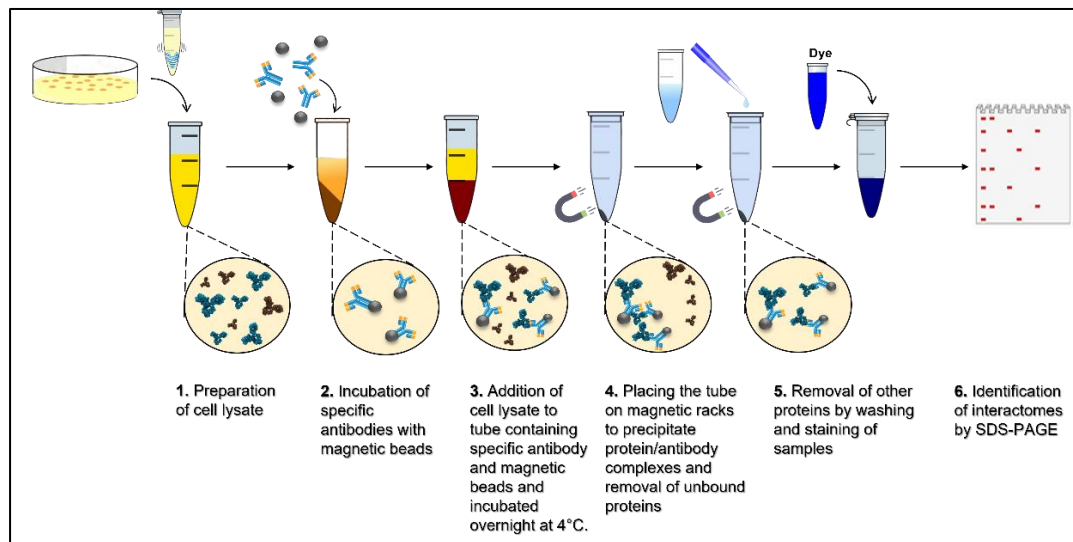


Figure 2.4: Stages of the co-immunoprecipitation method. 1) Cells expressing the protein of interest are prepared. Cells are scraped to obtain pellets, and washed once after centrifugation. Lysis buffer supplemented with protease inhibitor is then added and vortexed every 5 minutes for 15 minutes on ice. In this way, cell pellets are lysed. These cell lysates are centrifuged for 15 minutes, and the supernatants are transferred to new tubes. 2) Protein G-linked magnetic beads with specific antibodies (anti-FLAG) are incubated for 1 hour with rotation in the microcentrifuge tube (to bind the anti-Flag antibody to the magnetic beads). 3) The cell lysate is added to the tube containing the magnetic bead and specific antibody targeting the protein of interest and incubated overnight at 4°C with rotation. 4) These tubes are placed on magnetic racks to precipitate protein/antibody complexes. 5) The magnetic bead-antibody-protein complexes are then washed multiple times (3-4) with lysis buffer to remove unbound proteins. After removing the excess liquid, this complex is suspended in the Laemmli-SDS loading buffer. 6) After the samples are separated by SDS-PAGE, immunoblotting analysis is performed to detect proteins and protein complexes of interest.

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1: List of kits and reagents.

NucleoSpin Plasmid Kit	740588.50 [Macherey-Nagel (MN)/Germany]
Gel Extraction and PCR Clean up Kit	740609.50 [Macherey-Nagel (MN)/Germany]
PureLink Genomic DNA Mini Kit	K1820_02 [Invitrogen/USA]
Protease Inhibitor Tablets	S8830_2TAB [Sigma-Aldrich/ USA]
Protein Molecular Weight (MW) Marker	26612 [ThermoFisher Scientific/USA]
Q5® High-Fidelity DNA Polymerase	M0491S [New England Biolabs (NEB)/USA]
T4 DNA Ligase	15274017 [Invitrogen/USA]
Chemiluminescent ECL-HRP Substrate	K-12043D10 [Advansta/USA]
1kb DNA Ladder Marker	N3232S [New England Biolabs (NEB)/USA]
100bp DNA Ladder Marker	N3231S [New England Biolabs (NEB)/USA]
DNA Gel Loading Dye (6X)	B7021S [New England Biolabs (NEB)/USA]
Dynabeads™ Protein G Beads	10004D [Invitrogen]
Puromycin	P8833 [Sigma-Aldrich/ USA]

3.2. Buffers and Solutions

Table 3.2: List of Buffers and Solutions.

10X SDS-PAGE Running Buffer	250mM Tris-Base (pH:8.3), 95mM Glycine 1% SDS
1X Tris-Buffered Saline (TBS)	20mM Tris-HCl (pH:7.6), 150mM 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 powder in 1x TBS-T
10X Tris/Borate/EDTA (TBE) Buffer	10.8g Tris-Base, 5.5g Boric Acid, 40 ml 0.5M EDTA (pH:8.0)
100 ml 4x Protein-Loading Dye (Laemmli Buffer)	277.8 mM Tris-HCl (pH:6.8), 4.4% 8g SDS, 40% 44.4 ml Glycerol, 20% β -mercaptoethanol, 0.02% 20 mg Bromophenol-Blue
NP-40 Lysis Buffer (co-IP)	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	41965-039 [Gibco Invitrogen/USA]
Fetal Bovine Serum(FBS)	10270-076 [Gibco Invitrogen/USA]
100X Penicillin--Streptomycin	15140-122 [Gibco Invitrogen/USA]
LB Agar (Luria-Bertani Medium)	LBP04 [Caisson-Lab/USA]
Restriction Endonucleases	#ER0681; #FD0644; #ER0971; #ER0693; #ER0271; #IVGN0366 [ThermoFisher Scientific/USA-New England Biolabs (NEB)/USA]
Restriction Endonuclease Buffers	ThermoFisher Scientific/USA

Table 3.3: List of basic SDS-PAGE recipes.

Separating Gel (6%)	5 ml	10 ml
Autoclaved ddH ₂ O	2.9 ml	5.8 ml
Acrylamide/Bisacrylamide (40%)	0.75 ml	1.5 ml
1.5M Tris/HCl, (pH:8.8)	1.25 ml	2.5 ml
10% SDS	0.05 ml	0.1 ml
10% APS	0.05 ml	0.1 ml
TEMED	0.004 ml	0.008 ml
Stacking Gel (5%)	4 ml	8 ml
Autoclaved ddH ₂ O	2.92 ml	5.84 ml
Acrylamide/Bisacrylamide (40%)	0.5 ml	1 ml
1.5M Tris/HCl, (pH:6.8)	0.5 ml	1 ml
10% SDS	0.04 ml	0.08 ml
10% APS	0.04 ml	0.08 ml
TEMED	0.004 ml	0.008 ml

3.3. Antibodies

Table 3.4: List of antibodies.

Name	Dilution rates	Source/Catalog No
Anti-BMAL1	1:1000	Cell Signalling Technology [#14020]
Anti-FLAG	1:3000	Sigma-Aldrich [#F1804]
Anti-HA	1:3000	Cell Signalling Technology [#C29F4]
Anti-Rabbit_HRP	1:5000	Thermo Fisher Scientific [#31460]
Anti-Mouse_HRP	1:6000	Cell Signalling Technology [#7076S]

3.4. Equipment

Table 3.5: List of equipment.

Centrifuges	521-1647 Refrigerated [Micro-Star 17R-VWR/USA] 521-1646 [Micro-Star 17R-VWR/USA] 521-2844 [Mini-Star VWR/USA] 02-1827 Nuve (NF400)
Agarose Gel Electrophoresis Materials	1704406 [Bio-Rad/USA]
Electrophoresis System	ThermoFisher Scientific/USA
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, Bio-Rad, USA
Incubator [CO2]	1658004 [Mini-PROTEAN®_Bio-Rad/USA]
Inverted Microscope	Nikon-Eclipse Series-E100
Laminar Airflow Cabinet	Class-II Cabinet [ThermoFisher Scientific/USA]
Power Supply	Bio-Rad/USA
PCR Thermal Cycler	Bio-Rad/USA (41798)
Transblot Transfer System	1704155 [Bio-Rad/USA]
Nanodrop	206-26300-48 [Shimadzu-Biotech]
Shaker	Standard Analog Shaker [VWR/USA]
Waterbath	Wisd/Germany
Vortex	Scientific Industries/USA
Magnetic Rack	DynaMag™.2/Invitrogen

3.5. Oligonucleotide Sequences

Table 3.6: List of PCR primer oligonucleotides.

Oligo Name	Oligo Sequence (5' to 3')
O00567 _Forward	GCAGCTAGCATGGTGCTGTTGCACGTGCTG
O00567 _Reverse	ATGCTCGAGCTAATCTTCCTGGGATGCTTTATG
P11387 _Forward	GCATCTAGAATGAGTGGGGACCACCTCCAC
P11387 _Reverse	ATGGTCGACCTAAAACTCATAGTCTTCATCA
Q9BQG0 _Forward	GCAGCTAGCATGGAGAGCCGGGATCCCCG
Q9BQG0 _Reverse	ATGCTCGAGTTATTTTTTTAGAGCTGCTGATTG
Q9NX58 _Forward	GCAGCTAGCATGGTATTTTTTTACATGCAATGC
Q9NX58 _Reverse	ATGCTCGAGTCATTTTACAAGCTTGACTTTG
P60660 _Forward	GCAGCTAGCATGTGTGACTTCACCGAAGA
P60660 _Reverse	ATGCTCGAGTCACCCCGACAGGATATGCC
P22087 _Forward	GCATCTAGAATGAAGCCAGGATTCAGTCCC
P22087 _Reverse	ATGGTCGACTCAGTTCTTCACCTTGGGGGGTGG
Q8NI92 _Forward	GCAGCTAGCATGATCTTGCAGTGCTCA
Q8NI92 _Reverse	ATGCTCGAGTTATTGACCAAATGCTTTACTG
Q6I9V5 _Forward	GCAGCTAGCATGACGGAACAGGCCATC
Q6I9V5 _Reverse	ATGCTCGAGTTAGATCACCTTCTTGAGCTC
A0A024QZU2 _Forward	GCAGCTAGCATGCGTGAGATCGTGCACATC
A0A024QZU2 _Reverse	ATGCTCGAGCTACGCCTCGTCCTCGCCCT
Q6NZI2 _Forward	GCAGCTAGCATGGAGGACCCACGCTCTA
Q6NZI2 _Reverse	ATGCTCGAGTCAGTCGCTGTCGCTCTTGTC
P23284 _Forward	GCAGCTAGCATGCTGCGCCTCTCCGAACG
P23284 _Reverse	ATGCTCGAGCTACTCCTTGGCGATGGCAAAG
P27635 _Forward	GCAGCTAGCATGGGCCGCGCCCCGCCCCGT
P27635 _Reverse	ATGCTCGAGTCATGAGCAGGGCTGAGTTGTC
Q9NR30 _Forward	GCAGCTAGCATGAATTCTCCTAAATCCA
Q9NR30 _Reverse	ATGCTCGAGTTATTGACCAAATGCTTTACTG
Q03701 _Forward	GCATCTAGAATGGCCGCAGTCAAGGAGCCTTTG
Q03701 _Reverse	ATGGTCGACTCATTTTCCTTTGTTTTTTAGT

4. METHODS

4.1. Molecular Cloning

4.1.1. PCR Reaction with Cloning Primers

Coding sequences of interactors were amplified by PCR reaction using Q5® High-Fidelity Polymerase (#M0491S, New England Biolabs, USA). Master mix was prepared in a microcentrifuge tube. Reagents added to the PCR reaction mix: 500 ng of U2OS cDNA; 5 µl 5x Q5 Reaction Buffer; 1.25 µl 10 µM Forward and Reverse primers; 0.5 µl of 10 mM dNTPs (deoxynucleotide triphosphate); 0.25 µl of Q5 High-Fidelity Polymerase enzyme and ddH₂O to make up the final volume to 25 µl.

Forward and reverse primers are added to the mixture transferred to separate microcentrifuge tubes for each interactor and mixed with a micropipette. Then the tubes are placed in the PCR device and the process is started.

PCR reaction cycles, in order: pre-denaturation at 98°C for 30 seconds; In 35 cycles, denaturation at 98°C for 10 seconds, annealing at 58°C for 15 seconds, elongation at 72°C for 1 minute; Final elongation at 72°C for 2 minutes. At the end of the process, the tubes are removed from the device and stored at +4°C, or electrophoretic processes are started.

To analyze the accuracy of the PCR reaction, the amplified BMAL1 interactors were run and visualized on a 1% agarose gel electrophoresis to which Redsafe™ Nucleic Acid Staining Solution was added (Preparation of agarose gel will be detailed under the heading of agarose gel electrophoresis).

Table 4.1: PCR reaction contents.

Stock Solutions and Concentrations	Total 26 μ l Reaction (μ l)
5X Q5 Reaction Buffer	5
10 mM dNTPs	0.5
10 μ M Forward and Reverse Primer	1.25
DNA	1 (500 ng)
Q5 DNA Polymerase	0.25
ddH ₂ O	18

Table 4.2: PCR conditions of thermocycler.

Step	Temperature	Time	Cycle
Initial Denaturation	98°C	30 seconds	
Denaturation	98°C	10 seconds	35 cycles
Annealing	58°C	15 seconds	
Extension	72°C	1 minute	
Final Elongation	72°C	2 minutes	

4.1.2. Digestion of Plasmid and Primers with Restriction Enzymes

Appropriate buffers and restriction enzymes were used to insert the amplified interactors' coding sequences into linearized vectors (Enzymes and buffers used are mentioned in Table 3.2 in the material section). For digestion of the plasmid vector and amplified DNA, a reaction mix of 20 μ l was created in a microcentrifuge tube: 2 μ l of 10x appropriate restriction enzyme buffer (final volume, 1x), 1 μ l of restriction enzymes (NheI-XhoI or XbaI-SalI), 2 μ l DNA (200 ng) and ddH₂O to a final volume of 20 μ l. The digestion reaction of interacting DNA and plasmid with restriction enzymes (same) was carried out in separate tubes by incubating at 37°C for 1-2 hours.

Table 4.3: List of restriction enzymes.

Interactors (UniprotID)	Restriction Enzymes
O00567	NheI-XhoI
Q9NX58	
P60660	
Q8NI92	
Q6I9V5	
A0A024QZU2	
Q6NZI2	
P23284	
Q9NR30	
P11387	XbaI-SalI
P22087	
Q03701	

The coding sequence of the interactors was cloned into pcDNA3.1.3xHA of 3xHA-MiniTurbo-NLS_pCDNA3 (from Addgene Plasmid #107172). Ligation reaction of amplified interactor coding sequences into linearized pcDNA3.1.3xHA plasmid was performed using T4 DNA Ligase enzyme.

After cloning, ligation products were transformed into *E. coli* DH5-a strain. Obtained clones were cut with Anza (Eco31I) restriction enzyme and analyzed.

4.1.3. Analysis of DNA by Agarose Gel Electrophoresis

DNAs amplified by PCR or restriction digestion products were run on a 1% agarose gel to which Redsafe™ Nucleic Acid Staining Solution was added. First of all, 0.4 grams of agarose was added to 40 ml of 1X TBE buffer and melted in the microwave oven for the 1% gel. Then 2.3 µl of staining solution was added. The gel was allowed to solidify after pouring it into a horizontal electrophoresis tank. After the gel was solidified, 1X TBE buffer was added to the tank. DNA samples were mixed with 6X Loading Dye to a final volume of 1X and loaded into the wells. 1kb

and 100kb DNA markers were also loaded into the wells to accurately observe the sizes of the bands.

DNA samples were run at 100V for approximately 1 hour. Bands were run on agarose gel and were viewed under black light (366 nm). The bands of interest were cut from the gel with a band cutting apparatus (scalpel) and transferred to a microcentrifuge tube. The weight of the obtained bands was determined. In accordance with the protocol, Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit were used for DNA isolation. DNA samples were eluted into 50 µl of elution buffer by centrifugation. The concentrations of the isolated DNAs were measured in Thermo NanodropLite Spectrophotometer.

After cloning, ligation products were transformed into *E.coli* DH5-a strain. Obtained clones were cut with Anza (Eco31I) restriction enzyme and analyzed on 1% agarose gel. Positive clones observed on agarose gel were transfection into the HEK293T cell line and their expression was observed by Western blot analysis.

4.1.4. Ligation of the Inserts with Plasmid

After the relevant gene region (cDNA) is amplified in PCR with forward and reverse primers, ligation is performed under appropriate conditions. The interactors coding sequences into plasmid pcDNA3.3xHA; coding sequence for BMAL1.3xFLAG into pcDNA4-MycHisA plasmid were ligated using 1X T4 Ligase enzyme (#1522-4017, Invitrogen, USA) at +4°C for approximately 16 hours. Components used for ligation were as follows: 1µl T4 DNA Ligase, 1X T4 Ligation Buffer, 3µl Plasmid vector, 4µl Insert DNA in a total volume of 10 µl (1:3 molar vector/insert ratio was applied).

4.1.5. Transformation into Competent Cells

In this step, the ligation product obtained in the previous step is used to transform into bacteria. *Escherichia coli* (*E. coli*) DH5-α strain was preferred as a competent cell (stored at -80°C). Competent cells were prepared using a published protocol [Baldi et al., 2005].

Competent cells to be used in the experiment were taken from -80°C and left to thaw slowly on ice. Then, 100 µl of competent cells and 5 µl of ligation product were added into a different microcentrifuge tube. This tube was incubated on ice for 20 minutes. The competent cells were incubated for 1 minute at 42°C to heat shock, then the tubes were placed on ice again and incubated for 1 minute. Subsequently, 900 µl of Luria Broth (LB) was added to each tube. Cells were grown in a rotating shaker at 37°C for 50 minutes to express the antibiotic resistance gene ampicillin. Incubated cells were spread 100-120 µl on LB agar plates containing 100 µg/µl ampicillin. Plates were kept at room temperature for 5 minutes since all procedures were performed near the fire. The plates were then inverted and incubated overnight at 37°C for growth.

The selected colonies from plates were transferred to a 3.5 ml liquid LB medium containing 100 µg/µl of ampicillin antibiotic and incubated overnight at 37°C.

4.1.6. Plasmid Isolation

Macherey-Nagel Nucleospin Plasmid Isolation kit was used to isolate the plasmids from *E. coli* strain. First, bacterial colonies grown at 37°C overnight were centrifuged at 4000 rpm for 10 minutes and the supernatant was removed. The pellet was dissolved by adding resuspension buffer, and alkali lysis and isolation of the plasmids were performed by following the manufacturer's instructions. The eluted plasmid DNA concentration was measured using Thermo Nanodrop Lite Spectrophotometer device.

4.1.7. Verification of the Transformation

To determine the accuracy of the transformed candidate clones, the isolated plasmid DNAs were digested with Anza Eco31I restriction enzyme. Mixture was prepared in a microcentrifuge tube for cutting reaction: 14.9 µl water, 2 µl 10x FastDigest Buffer, 0.1 µl EcoRI FastDigest enzyme. The mixture was added to new tubes. Then, 3 µl of DNA was added to each tube and mixed, and the final volume was adjusted to 20 µl. Tubes containing DNA were incubated for at least 1 hour at

37°C. After the incubation was completed, DNA samples were separated on a 1% agarose gel.

The confirmed plasmids were again transformed into *E. coli* and cultured in larger volumes of LB medium containing 100 µg/µl ampicillin to obtain enough plasmids for transfection studies. The plasmids were again isolated as described above using larger volumes of kit buffers.

4.2. Cell Culture

4.2.1. Maintenance of the Cell Line

HEK293T cells were grown in high glucose-Dulbecco's Modified Eagle Medium (DMEM) with 1% Penicillin, 1% Streptomycin, 1% non-essential amino acids (NEAA), and 10% Fetal Bovine Serum (FBS). The cells were cultured at 37°C and 5% CO₂ in a humidified incubator. Cells were passaged when their densities reached 80% and the medium was changed every 3-4 days. During the passage, the medium was discarded and the cells were then washed with 1x PBS. Afterwards, 0.05% Trypsin-EDTA solution was added to the cells and incubated for 5 minutes at 37°C. DMEM (three times the amount of trypsin) was added to the detached cells to inhibit trypsin activity. Cells were diluted at 1:10 and seeded in a new petri dish and incubated with 5% CO₂ at 37°C.

4.2.2. Transfection

Transfection was performed using poly-ethylenimine (PEI) agent. PEI is a cationic transfection agent that stabilizes DNA by binding to anionic cell surfaces. This complex is taken up into the cytoplasm by endocytosing by cells [Sonawane et al., 2003].

The amplified interacting DNA was transfected into a stable cell line (HEK293T) expressing 3xFLAG-BMAL1. Cells at 60% density were seeded into 60 mm dishes and incubated overnight in an incubator providing 5% CO₂ and 37°C. One day later, the densities of the cells were checked. If the densities of the cells were approximately 80%, transfection was performed. 150 µl of transfection buffer

[containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA (pH 8.0)] was mixed with PEI (based on DNA amount) and amplified plasmid DNA in a 3:1 ratio.

Plasmid DNA (1 µg) was used for transfection of each interactor of interest. This mixture was vortexed and spun, then incubated at room temperature for 15 minutes. The old medium was removed from the dishes and 1 ml of fresh medium was added to the dishes. Fresh medium (2 ml) was mixed with transfection buffer solution and pipetted slowly and transferred to 60 mm dishes. Dishes containing transfected cells were incubated in an incubator (under 5% CO₂ and 37°C conditions) for 2 days.

4.2.3. Generation of a Stable Cell Line

HEK293T cells were transfected with the pcDNA4-BMAL1-3xFLAG plasmid using the PEI transfection agent (as specified in the transfection section) to generate a stable HEK293T cell line expressing the 3xFLAG-tagged BMAL1 protein. After 48 hours of the transfection, cells were seeded into 96-well plates by serial dilution. To select stable clones, cells were incubated in an antibiotic (Zeocin)-containing medium. Single-cell clones were seeded into 12-well plates, then expressions of the candidates were screened by immunoblotting using an anti-FLAG tag antibody (#F1804, Sigma Aldrich). The resulting stable HEK293T cell line was used to transiently transfect HA-tagged interactors.

4.3. Co-IP

4.3.1. Cell Collection

HEK293T cells transfected in cell culture were harvested by scraping together with their medium. Then these cells were transferred to a new centrifuge tube and the pellet was obtained by spinning at 5000 rpm for 1 minute. The pellets were washed with cold 1x PBS solution to remove all residue and the supernatant was removed. Cell pellets were placed on ice for lysis.

4.3.2. Cell Lysis and Sample Preparation

Cell pellets were lysed using NP-40 Lysis Buffer (#FNN0021, Thermo Fisher Scientific, USA) supplemented with 1x Protease inhibitor (PI). Cells were incubated on ice for a total of 20 minutes by vortexing every 5 minutes. Cell lysates were centrifuged at 13300 rpm for 20 minutes at 4°C. The obtained supernatants were transferred to new 1.5 ml microcentrifuge tubes. A portion of the supernatant was transferred to new microcentrifuge tubes to use as a positive control (input). These lysates were mixed with 4x Protein loading dye (final volume 1x) and stored at -20°C until analyzed with other samples in Western blot.

4.3.3. Immunoprecipitation of Interactomes with Magnetic Beads

Dynabeads Protein G (10 µl) (#10003D, Thermo Fisher Scientific) were added to tubes placed in a magnetic rack. The beads were washed 4 times using 500 µl of NP-40 lysis buffer. Then, 1 µg of anti-FLAG antibody (#F1804, Sigma Aldrich) or IgG (#7076S, Cell Signaling Technologies) as control was added to each tube. NP-40 Lysis buffer (200 µl) was added to each sample and incubated for 1 hour at 4°C. The cleared cell lysate (500 µl) was transferred to tubes containing Anti-Flag/Dynabeads or Control IgG/Dynabeads complexes and incubated at 4°C overnight with rotation. These tubes were placed on magnetic racks to precipitate protein/antibody complexes. The magnetic bead-antibody-protein complexes were washed 4 times with 500 µl of NP-40 lysis buffer to remove unbound proteins. After removing excess liquid, this complex is contained in 70 µl of 1.5x Laemmli-SDS loading buffer (4X Laemmli-SDS loading buffer contained 250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue, and 10% freshly added 2-mercaptoethanol) was suspended. Samples were placed at -20°C for use. For each interaction analysis, 5% of the inputs and 30% of the precipitated complexes were loaded and separated by SDS-PAGE and then analyzed by immunoblotting.

4.4. Western Blot

4.4.1. SDS-PAGE Gel Preparation

When preparing the SDS-PAGE gel, two glass plates (one long and one short) are used and these glasses are cleaned with 70% ethanol. These two glass plates were joined flat and placed in the gel cassette. First, the separating gel was prepared and poured between glass plates (as described in Table 3.3). Air bubbles formed while pouring the gel are removed by adding isopropanol. The polymerization of the gel takes place thanks to the TEMED and APS (ammonium persulfate) components in the gel. Polymerization begins with APS and this reaction is accelerated by the addition of TEMED, which acts as a catalyst. In addition, acrylamide, which is widely used and provides cross-linking, determines the density, pore size, and brittleness of the gel.

The separating gel was allowed to polymerize for approximately 20-40 minutes. Stacking gel (5%) was prepared after the gel had frozen (Table 3.3.). The isopropanol in the separating gel was removed and washed with dH₂O. Wetness between glass plates was dried with Whatman paper. Afterwards, the prepared stacking gel was added to the separating gel. The 1.5 mm comb was gently inserted over the stacking gel. Stacking gel is also ready for use after polymerization.

4.4.2. Sample Preparation for SDS Polyacrylamide Gel

The precipitated samples were taken from -20°C and allowed to dissolve at room temperature and then placed in a heat block set to 95°C. The samples were boiled for 10 minutes to denature the proteins. In this way, proteins are separated according to their molecular weights by SDS-PAGE.

4.4.3. SDS-PAGE Gel Running

The comb was removed, and the wells were washed with ddH₂O to remove the formed bubbles. The gel was placed in a cassette and then into a tank. The space between the tank and the cassette was filled with 1x Running Buffer (10x Running

buffer diluted with ddH₂O). Samples (equal amount) and PageRuler™ Prestained Protein Ladder (#26617, Thermo Fisher Scientific, USA) were loaded into the wells and run at 80V. After the samples entered the separation gel, the device was increased to 120V. After a few hours, the bromophenol blue dye in the loading dye was run until it came out of the gel. After the dye came out of the gel, the device was stopped, and the glasses were removed from the cassette. A spatula was used to remove the gel between the two glasses. The area of the wells was excised, and the gel was placed in transfer buffer for transfer.

4.4.4. Transfer of Proteins from Gel to Membrane

Trans-Blot Turbo Transfer System (Serial No: 690BR011760, Biorad) was used for the semi-dry transfer of proteins from the gel to the nitrocellulose membrane of the same size. A gel-sized membrane and 4 pieces of Whatman paper were incubated in 1x transfer buffer for 2-3 minutes. A transfer sandwich was formed by arranging them in order on the transfer cassette: 2 Whatman paper, membrane, gel, and 2 Whatman paper. Since the gel placed on the membrane caused bubbles to form between the membrane and the gel, the bubbles were removed using a roller. Then the cassette was locked and placed in the device (cathode (-) electrode at the bottom, anode (+) electrode at the top). The transfer process was carried out at 12V for 1 hour and the transfer of proteins from the gel to the membrane was completed.

4.4.5. Blocking and Immunoblotting

Skim milk powder (5% blocking solution) was prepared: 1g skim milk powder, 20ml 1x TBS-T buffer (10x TBS-T buffer diluted with dH₂O). This mixture was homogenized by vortexing. After the transfer was completed, the membrane was placed in blocking solution to prevent binding of non-specific antibodies. The membrane was blocked in a shaker at room temperature for 1 hour. The membrane was then transferred to the primary antibody-containing solution (5% skim milk powder, 1x TBS-T buffer, and 1% sodium azide, which prevents bacterial contamination) and incubated at 4°C overnight.

After 24 hours, the membrane was removed from the primary antibody and washed 4 times for 30 minutes with 1x TBS-T. In the washing step, unbound antibodies were removed. The membrane was taken into the solution containing secondary antibodies and incubated in the shaker for at least 1 hour. The membrane was incubated with an anti-mouse secondary antibody (1:6000, #7076S, CST) for anti-FLAG and an anti-rabbit secondary antibody for anti-HA (1:5000, #31460, Thermo Fisher Scientific). After 1 hour of incubation, the membrane was washed again with 1xTBS-T buffer 4 times for 30 minutes.

4.4.6. Imaging

After washing, the membrane was placed on imaging paper and dried well. WesternBright Sirius HRP substrate (Advansta Inc., USA) was added onto the membrane and incubated for 3 minutes. For chemiluminescence imaging, the HRP substrate was allowed to be conjugated with the secondary antibody. Thus, a chemical reaction takes place and chemiluminescent signals are produced. After incubation was complete, the excess substrate was discarded. The Chemidoc™ XRS+ System with ImageLab™ Software (#1708265, Biorad) was used to image the chemiluminescent signals in the membrane.

5. RESULTS

In this study, we aim to validate the circadian clock protein BMAL1 interactome by using the co-immunoprecipitation (co-IP) method so that we can further investigate the effect of these interactions on the biological clock. For this aim, we developed an experimental design based on immunoprecipitation to validate the candidate BMAL1 interactome in the cell. The candidate proteins were previously identified by the BioID technique followed by mass spectrometry analysis. For this thesis, the candidates were selected using Saint Express Analysis.

We then generated a stable cell line (HEK293T-BMAL1-3xFLAG) from HEK293T cell that has a 3xFLAG tag (C-terminal) and overexpresses the BMAL1 protein. We used this cell line to immunoprecipitate BMAL1. BMAL1 interacting proteins (interactors) were cloned into pcDNA3.1.3xHA plasmid where the HA tag is expressed at the N-terminus of the interactor. These plasmids were transfected into the stable cell line to transiently express the interactors. After immunoprecipitation of the BMAL1 using anti-FLAG antibody magnetic beads, the presence of the interactors in the BMAL1 complexes was analyzed with anti-HA antibody immunoblotting.

5.1. Generation of Interactor Expression Constructs

5.1.1. Determination of the Candidate Interactors

Candidate proteins as possible interactors of BMAL1 were selected from the mass spectrometry analysis data of BMAL1 described in a Ph.D. student's thesis in our laboratory [Gul, 2022]. In this study, the proteins in the immediate proximity of the BMAL1 protein in vivo were labeled with biotin using the BioID method. Biotinylated proteins were precipitated with streptavidin-magnetic beads and candidates identified by mass spectrometry were grouped according to their function. Candidates identified by Saint Express Analysis (≥ 0.95) were listed as high-confidence BMAL1 protein interactors [Abbasi and Schild-Poulter, 2019].

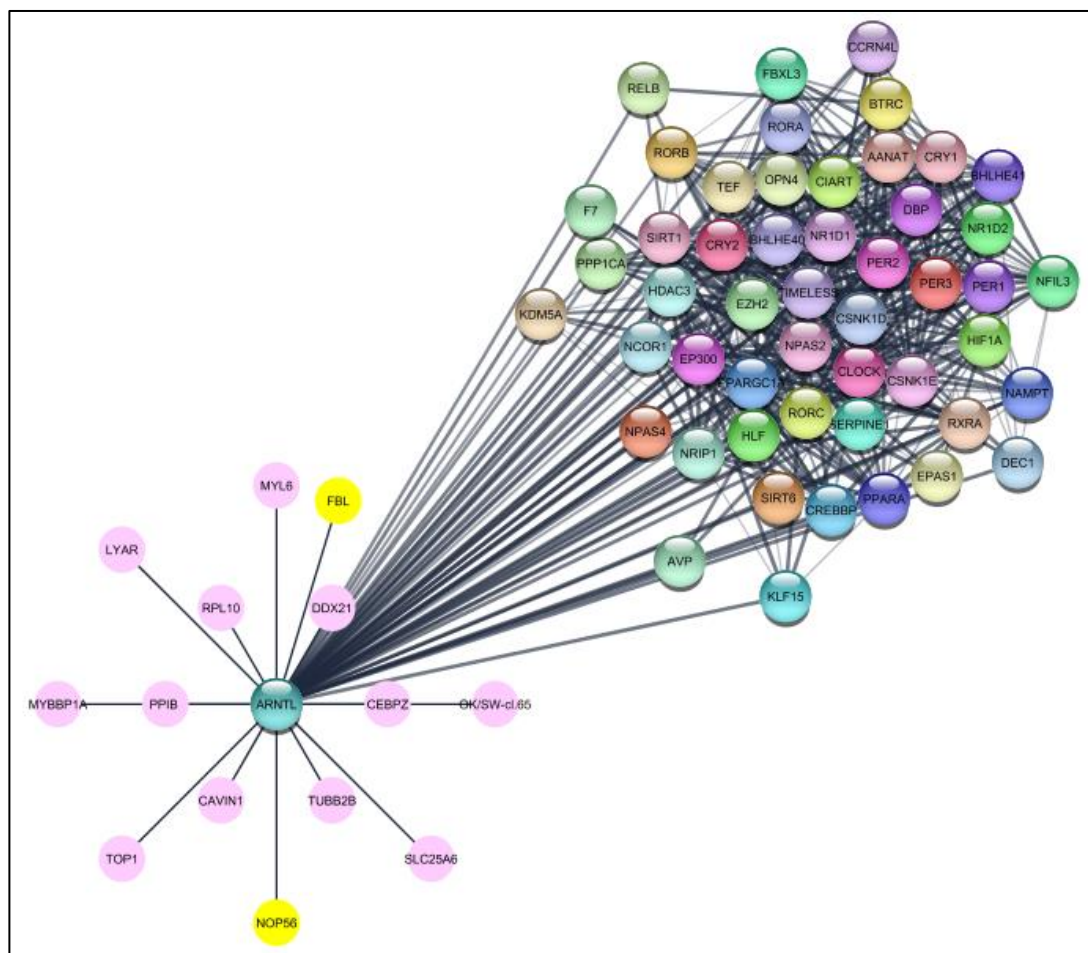


Figure 5.1: Literature and experimental interaction map of BMAL1 protein. The maps were generated using StringApp and Cytoscape(3.7.2) software.

We selected fourteen interactors from candidates identified by mass spectrometry and confirmed the interaction of some with co-IP. We found that two of these interactors interact with BMAL1. These interactors are shown in yellow (Figure 5.1).

5.1.2. Preparation of Plasmids for Cloning

The 3xHA-MiniTurbo-NLS-pcDNA3 (#107172) plasmid used to clone the interactors was purchased from Addgene. Since specific antibodies for the target proteins are not available, the tagged version of the proteins are used to analyze them using an anti-HA antibody against a HA tag. This tag is easily detected by anti-HA antibodies from rabbit and mouse. The HA tag can be added to both the N-terminal and C-terminal regions of the protein to avoid label problems. In addition,

immunoprecipitation of BMAL1 complex was managed by anti-FLAG antibody magnetic beads. The map of the 3xHA-MiniTurbo-NLS-pcDNA3 plasmid which was used to clone interactors was shown in Figure 5.2.

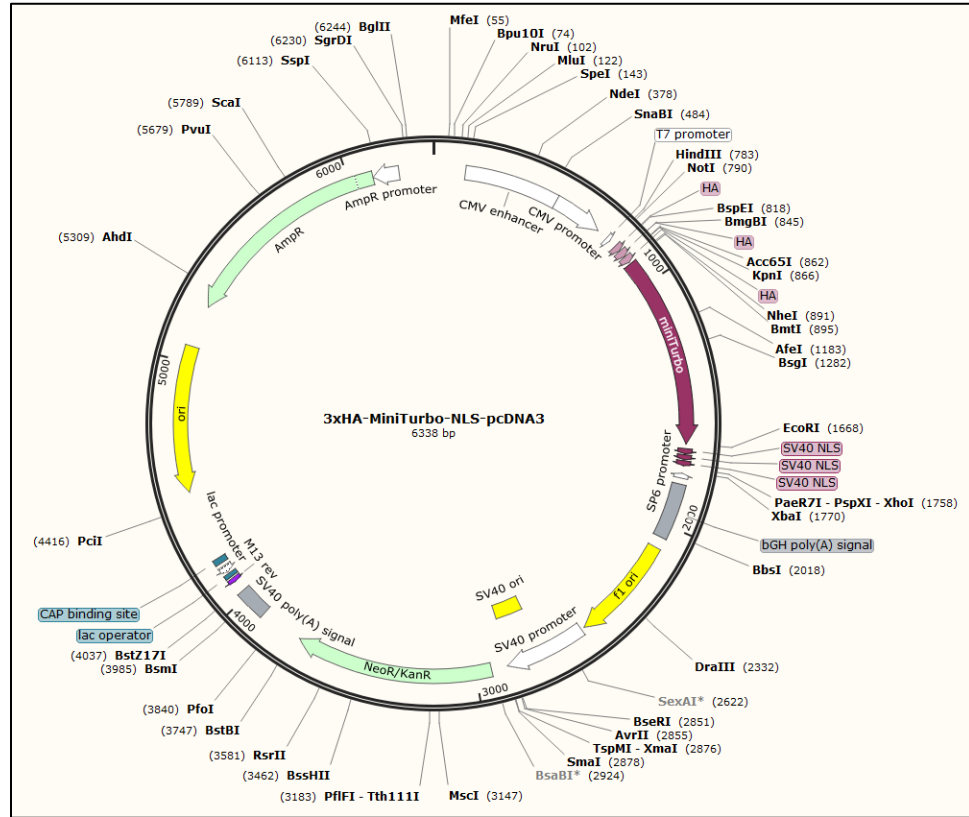


Figure 5.2: Map of the 3xHA-MiniTurbo-NLS-pcDNA3 [#107172] plasmid. This map was created from the SnapGene-Viewer Program.

The cloning process for the interactors is explained below: The selected interactomes were first amplified by a polymerase chain reaction from the cDNA of the U2OS cell, using specific primers containing restriction enzyme recognition sites close to their 5' ends. The amplified interactors were isolated from agarose gel and cut with appropriate restriction enzymes. At the same time, the 3xHA-MiniTurbo-NLS-pcDNA3 plasmid was digested with the same restriction enzymes. Subsequently, the remaining plasmid fragment was isolated from agarose gel by leaving out the MiniTurbo coding sequence to insert interactors into the pcDNA3.1.3xHA part. These interactors were then inserted into the 3xHA-NLS-pcDNA3 plasmid. The protrusions formed by the selected restriction enzymes

specific to each interactor were compatible with the cloning process. This strategy is explained in Figure 5.3.

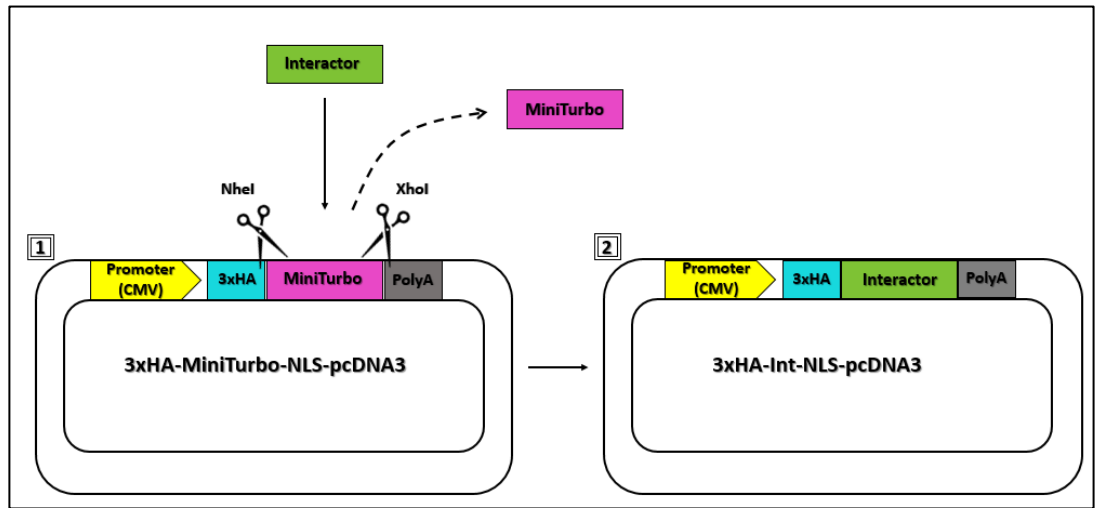


Figure 5.3: Design of the cloning method of the 3xHA-MiniTurbo-NLS-pcDNA3 plasmid. 1) Design, 2) The plasmid containing the protein of interest is expressed.

In order to insert the 3xFlag encoding sequence into the pcDNA4 vector, the 3xFLAG sequence was cut from the EcoRI-NheI cut sites of the inducible Caspex expression plasmid (Addgene #97421) and transferred into the pcDNA4 plasmid. The BMAL1 region was amplified from the U2OS cDNA using the BMAL1-3fl-F (ATCGAATTCGCCACCATGATTAATATAGAAAGC) and BMAL1-3fl-R (ATCTCTAGACAGCGGCCATGGCAAGTC) oligos. The amplified BMAL1 was inserted into the pcDNA4-3xFLAG-His plasmid from the EcoRI-XbaI site. This construct was then used to construct the stable HEK293T-BMAL1-3xFLAG.

We preferred the 3x version of FLAG and HA tags to increase efficiency.

5.1.3. Amplification of the Interactors

The DNA sequences of the interactors were amplified using U2OS (Human Osteosarcoma Cell Line) cDNA using compatible oligos (purchased from Sentebiolab, Turkey), Q5 High Fidelity Polymerase enzyme, and other reagents. Appropriate enzyme recognition sites were added to the 5' ends of the primers for the recognition and binding of DNA by restriction enzymes. PCR reaction steps are given in section 4.1.1. After PCR, whether the gene was expressed or not was checked by

1% agarose gel electrophoresis. The results are shown in Figure 5.4. Bands expected for interactors (Int1-14), respectively: 1785, 2298, 3999, 1140, 456, 966, 438, 897, 1338, 1173, 651, 651, 3724, 3165 bp (base pairs).

These interactors were identified according to the 1 kb size marker [N3232S New England Biolabs (NEB)/UK] DNA ladders.

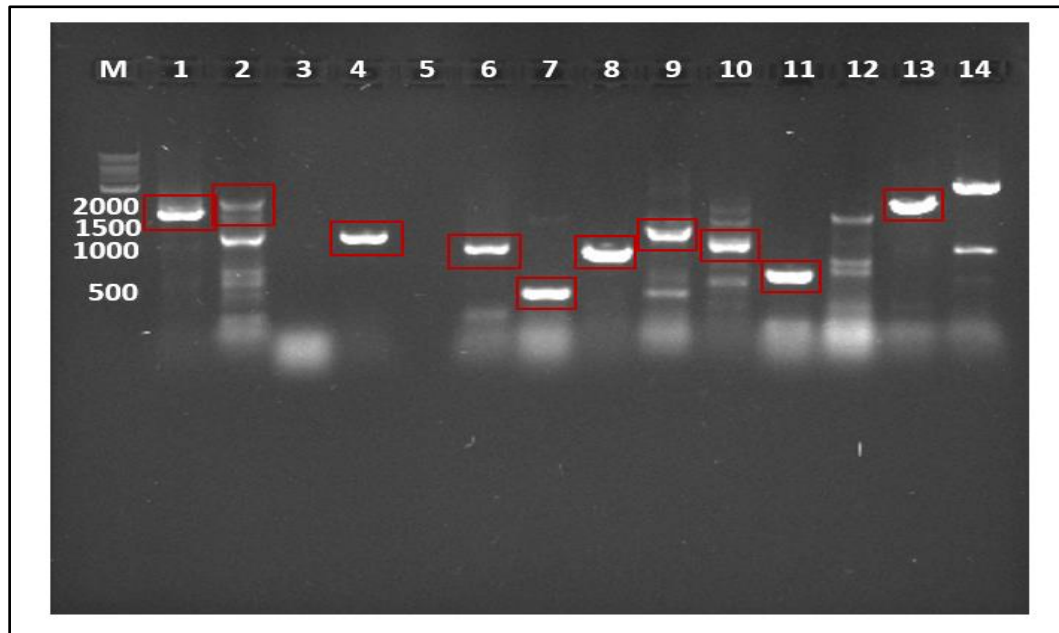


Figure 5.4: Confirmation of the PCR reaction of target interactors. Lane numbers represent candidate interactors. M: 1kb DNA ladder (#N3232S-NEB). Samples were run on 1% agarose gel. Red rectangles show expected bands of interactors.

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

To perform the ligation of the interactors amplified by the PCR reaction with the 3xHA-NLS-pcDNA3 plasmid, a sticky end was formed with the restriction enzymes NheI and XhoI or XbaI and SalI. In order to verify the efficiency of the digestion process, the digestion products were loaded into 1% agarose gel and visualized. These DNAs were observed under 366 nm black light and extracted from the gel. Then, Macherey-Nagel Nucleospin Gel and PCR Clean-up kit were used to purify DNA from the agarose gel. T4 DNA ligase was used to clone interactors in place of the MiniTurbo sequence removed from the 3xHA-NLS-pcDNA3 plasmid.

The ligation products were transformed into *Escherichia coli* DH5-a strain. Colonies were picked up the next day and grown in 3.5 mL LB medium. After overnight incubation, plasmid DNAs were purified from bacterial pellets (obtained by precipitation) using the Macherey-Nagel Nucleospin Plasmid Kit. To confirm that the interactors were cloned appropriately, the isolated plasmid DNAs were cut with Anza 36 Eco31I (Thermo Fisher) enzyme and loaded on the agarose gel. In Figure 5.5, the interactors containing the expected bands were identified for use in the next experiments.

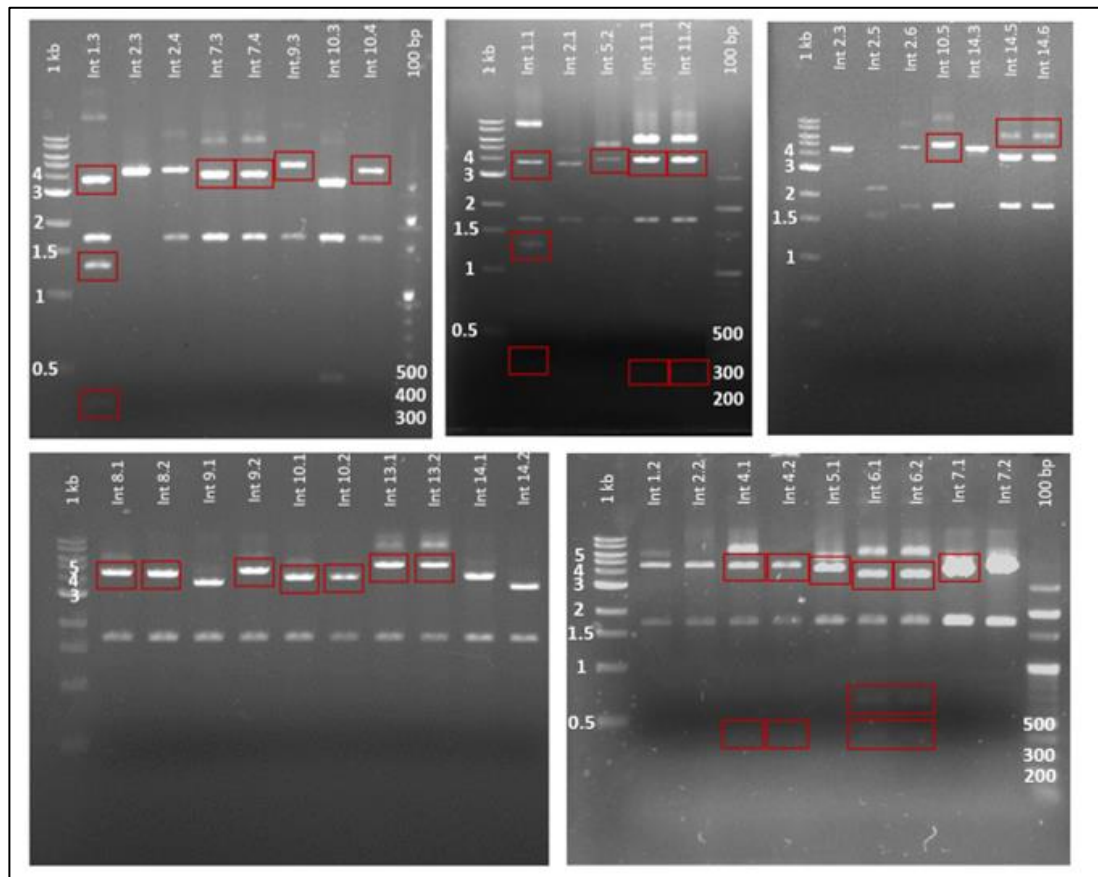


Figure 5.5: Confirmation of cloning of candidate interactors into the 3xHA-NLS-pcDNA3 vector. The vector was cut with the Anza (Eco31I) enzyme. Lane numbers represent interactors. 1kb: DNA ladder. Samples were run on a 1% agarose gel. The expected bands of the interactors are marked with red rectangles.

5.2. Generation and Conformation of 3xFLAG-BMAL1-HEK293T Stable Cell Line

The FLAG tag is used to purify and identify protein complexes. This label should not interfere with the biological function and folding of the proteins to be labeled. For this reason, it is a good choice to use the FLAG tag, which is a small peptide [Li, 2010]. The FLAG tag not only provides better purification efficiency, but also exhibits a high degree of specificity for proteins in the same complex [Chang, 2006].

Due to these advantages, immunoprecipitation of our target protein BMAL1 was performed using the FLAG tag. We chose to use a 3xFLAG tag to immunoprecipitate the BMAL1 protein with anti-FLAG antibody coupled with magnetic beads. The map of the plasmid is shown in Figure 5.6.

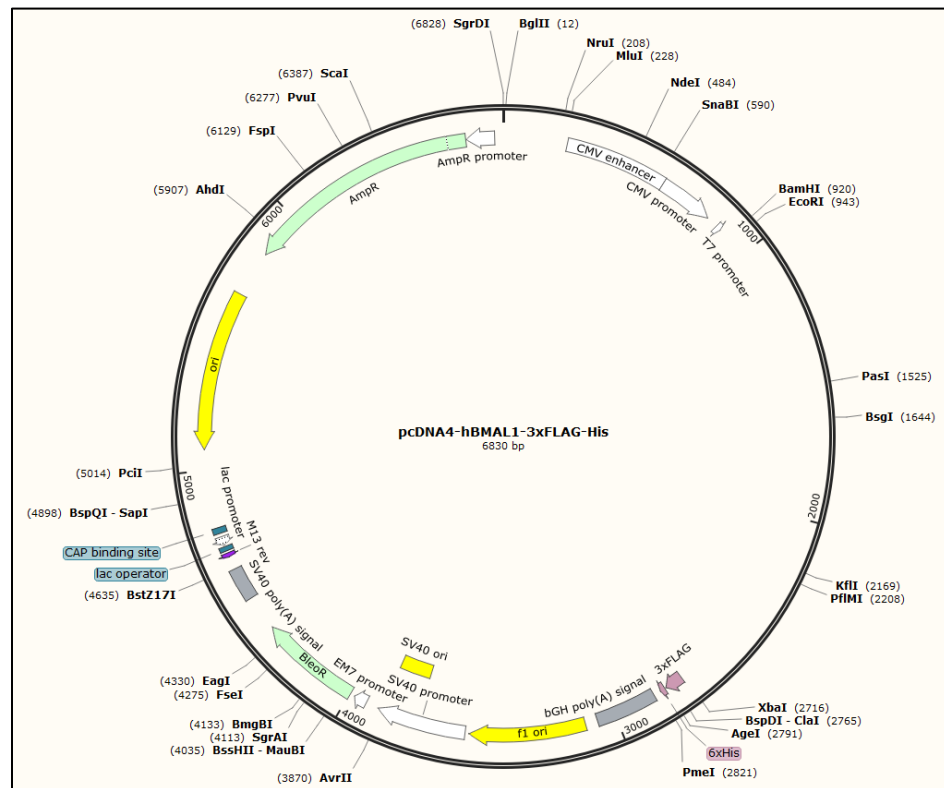


Figure 5.6: Map of the pcDNA4-hBMAL1-3xFLAG-His1 plasmid. This map was made using the SnapGene Viewer Program.

As shown in Figure 5.7, the pcDNA4.BMAL1.3xFLAG plasmid was digested with the appropriate restriction enzyme (Hind III) to validate.

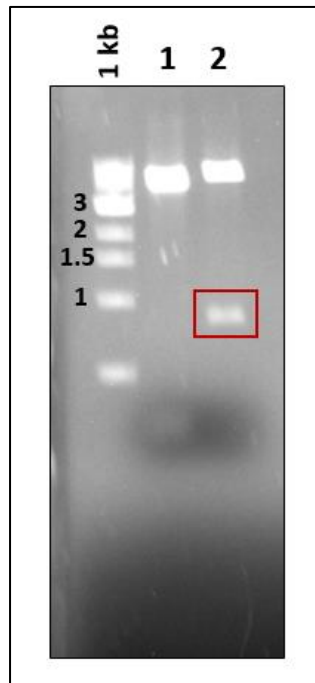


Figure 5.7: RE analysis of pcDNA4-BMAL1-3xFlag plasmid with Hind III digestion. Column 1-2; potential pcDNA4-BMAL1-3xFlag plasmid clones. 1 kb; DNA ladder. Samples were run on a 1% agarose gel. The expected fragment (751 bp) is indicated by the red rectangle.

pcDNA4.BMAL1.3xFLAG plasmid was transfected into HEK293T cells to generate a stable cell line (expressing BMAL1.3xFLAG). As shown in Figure 5.8, subclones were generated from the HEK293T cell line, which was confirmed to express the transfected plasmid. Immunoblotting screening was performed using anti-FLAG antibody to observe the presence of BMAL1.3xFLAG in stable clones (Figure 5.9).

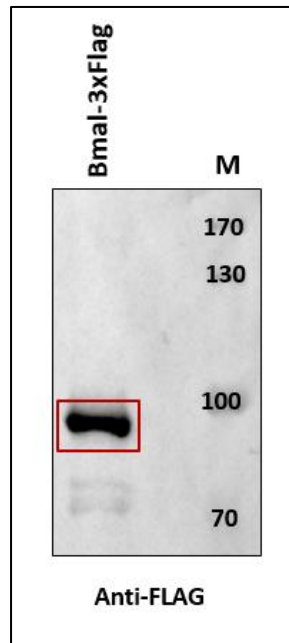


Figure 5.8: Validation of stably expressed 3xFlag-BMAL1 in the HEK293T cell line. The molecular weight of the endogenously expressed BMAL1 gene is 78 kDa. The weight of 3xFlag-BMAL1 is approximately 80 kDa. PageRuler™ Prestained Protein Ladder (#26617, Thermo Fisher Scientific, USA) was used as a marker (M). The sample was run on 8% SDS polyacrylamide gel. The expected band is shown with the red rectangle.

In order to obtain more efficient and stable expression results, the subclones were generated by a second round of positive clones (Figure 5.9). As a result of this analysis, B1 and B5 subclones with observed expression were selected and analyzed with co-IP as shown in Figure 5.10.

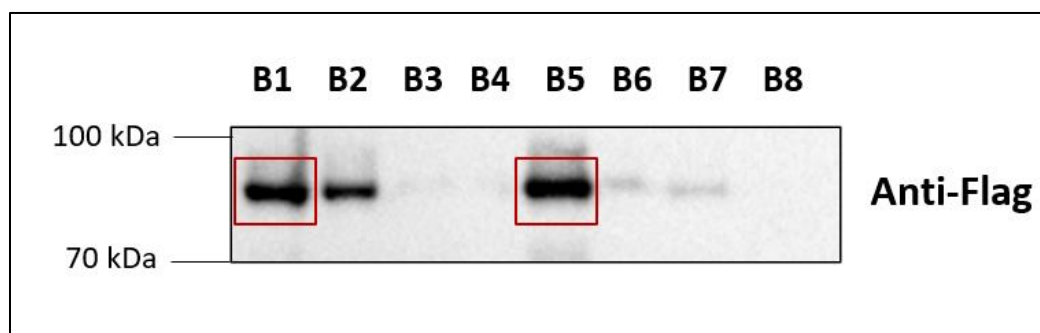


Figure 5.9: Screening of BMAL1-3xFLAG subclones in HEK293T cells. Anti-FLAG antibody was used to detect the presence of subclones of BMAL1-3xFLAG. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 6% SDS polyacrylamide gel. The expected bands are shown with red rectangles.

Immunoblot analysis of BMAL1 protein using anti-FLAG antibody was performed and an expression was detected. As expected, an expression may not be detected in the IgG (control) lane. In addition, anti-Clock and anti-CRY1 antibodies were used to detect the presence of CLOCK and hCRY1 proteins interacting with BMAL1. As a result, expression was detected in both clones, and experiments were continued with B1 with better expression (Figure 5.10).

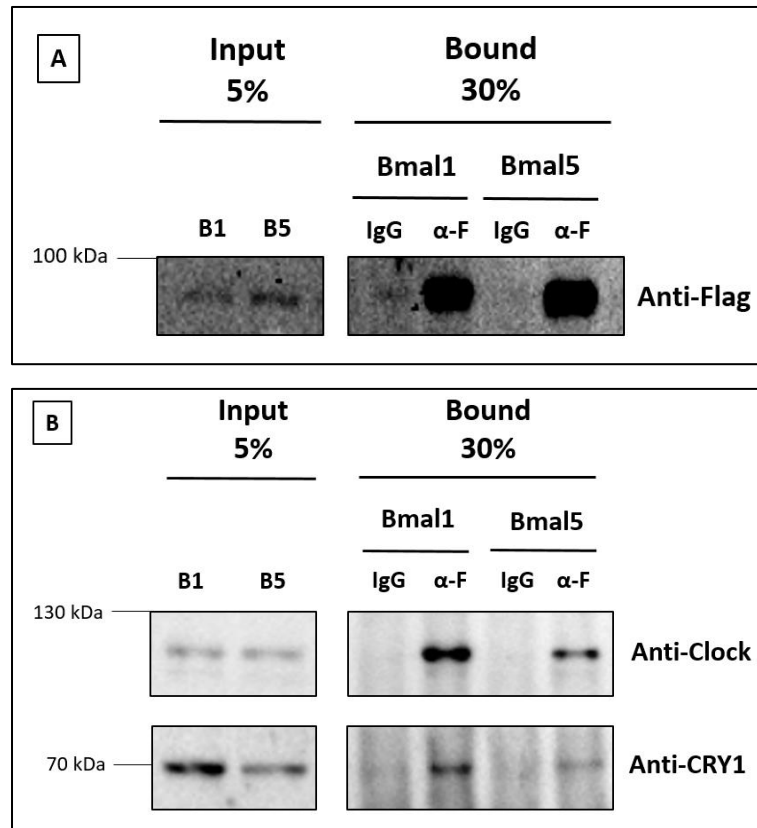


Figure 5.10: Confirmation by co-IP of the presence of 3xFLAG-BMAL1 expressed in the stable HEK293T cell line. The molecular weight of the endogenously expressed BMAL1 gene is 78 kDa. After fusion of the 3xFLAG tag to BMAL1, an 80 kDa band is expected in the SDS polyacrylamide gel. Input indicates cell lysate (not immunoprecipitated). Bound indicates immunoprecipitated samples. IgG was used as a control. α-F represents anti-FLAG antibody. PageRuler™ Prestained Protein Ladder was used as a marker. A) Anti-Flag antibody was used to detect the expression of 3xFlag-BMAL1 in subclones B1 and B5. B) Anti-Clock and anti-CRY1 antibodies were used to detect the presence of Clock and CRY1 proteins interacting with BMAL1. Samples were run on 8% gel.

The resulting stable HEK293T cell line was used to transiently transfect HA-tagged interactors.

5.3. Expression of the Interactors in the Stable Cell Line

In Figure 5.12, the expression observed interactors were selected and transiently transfected into the stable HEK293T cell line. Afterwards, the interactions between BMAL1 and interaction were tested using the co-IP method. Firstly, in Figure 5.5, amplified positive clones were selected and transfected into the HEK293T-BMAL1-3xFLAG cell line to ensure expression is possible in the stable

cell line. Immunoblot analysis was performed using the anti-HA antibody to confirm the expression of the interactors. Molecular weights of bands expected for positive interactors, respectively: 66, 33, 49, 43, 107, 42, 16, 35, 16, 24 kDa (Figure 5.11).

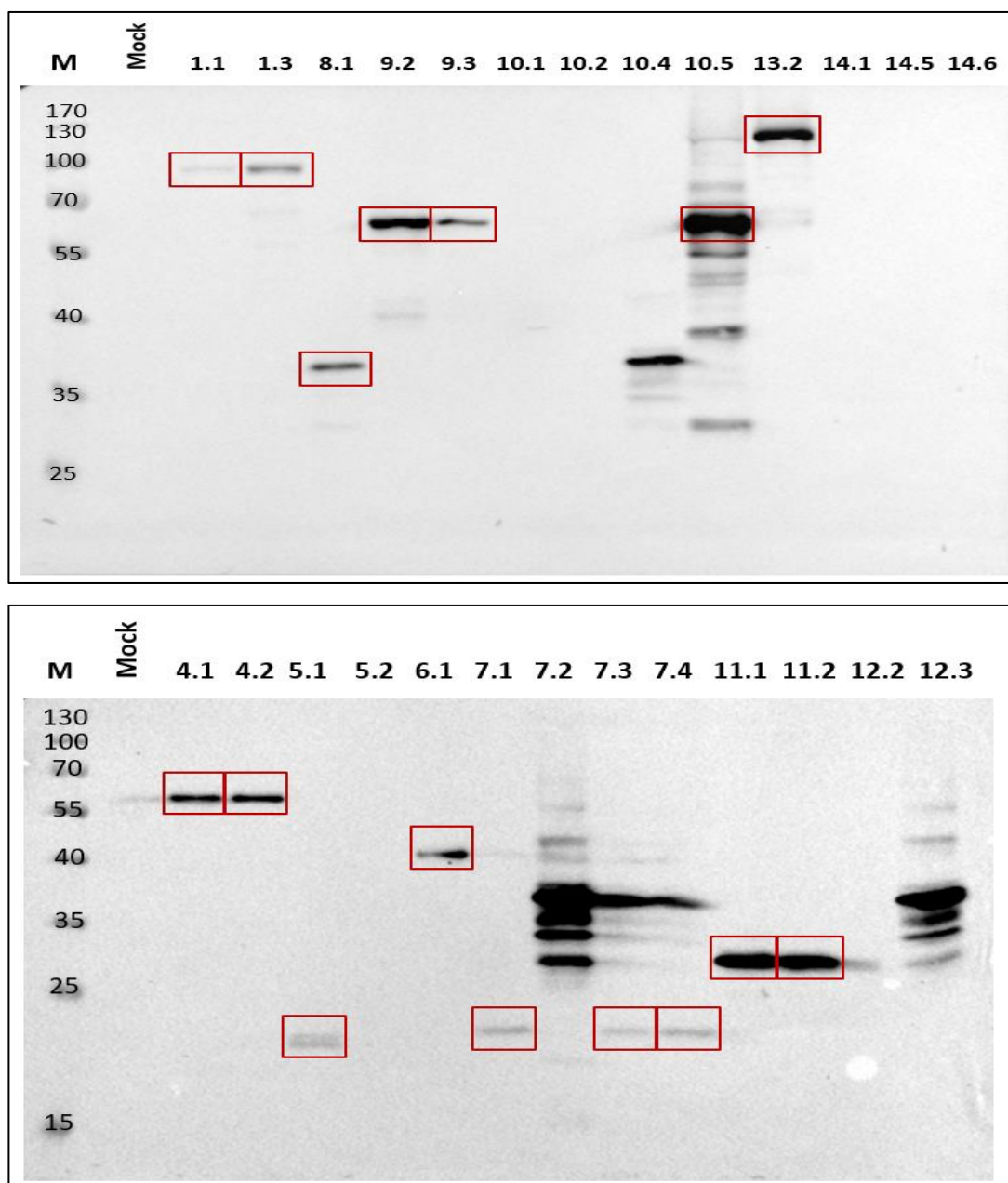


Figure 5.11: Confirmation of the expression of interactors in the stable 3xFlag-BMAL1-HEK293T cell line. Mock (control) contains cell lysate (not transfected). PageRuler™ Prestained Protein Ladder was used as a marker. The samples were run on the 8% gel prepared. Bands observed at expected molecular weights are indicated by red rectangles.

Presence of unexpected bands as well as expected bands in the immunoblot image may indicate protein modifications.

5.4. Co-IP of Interactors from HEK293T-BMAL1-3xFLAG Cell Line

Co-immunoprecipitation (co-IP) technique was used to confirm the physical interactions between BMAL1 protein and its candidate interactors. Using an anti-FLAG antibody, BMAL1-3xFLAG protein was immunoprecipitated. Interactors are also expected to co-immunoprecipitate with the BMAL1-3xFLAG protein. Non-interacting proteins of BMAL1 in cell lysates were removed by washing steps. Immunoblotting analysis was performed using anti-HA antibody to analyze the presence of the BMAL1 interactions. Anti-HA antibody was used because the interactors were HA-tagged. BMAL1-3xFLAG protein was analyzed with immunoblotting using anti-FLAG antibody.

Input contains only cell lysate (not immunoprecipitated). FLAG and HA bands are expected to be seen in anti-FLAG lanes. In contrast, a band should not be observed in the IgG strips due to the absence of a specific antibody-protein interaction.

To validate the BMAL1 protein, the human CRY1 protein known to interact with BMAL1 was used as a positive control, and the interaction was examined. In the HEK293T-BMAL1-3xFLAG cell line, co-IP samples were blotted with both anti-FLAG and anti-hCRY1 antibodies and interaction with endogenous CRY1 was detected. In the hCRY1-HEK293T cell line (which was generated in our laboratory by Prof. Dr. Nuri Öztürk), the same procedures were applied for the hCRY1 protein and blotted with anti-FLAG and anti-BMAL1 antibodies. As seen in Figure 5.12, a specific interaction with endogenous BMAL1 was observed.

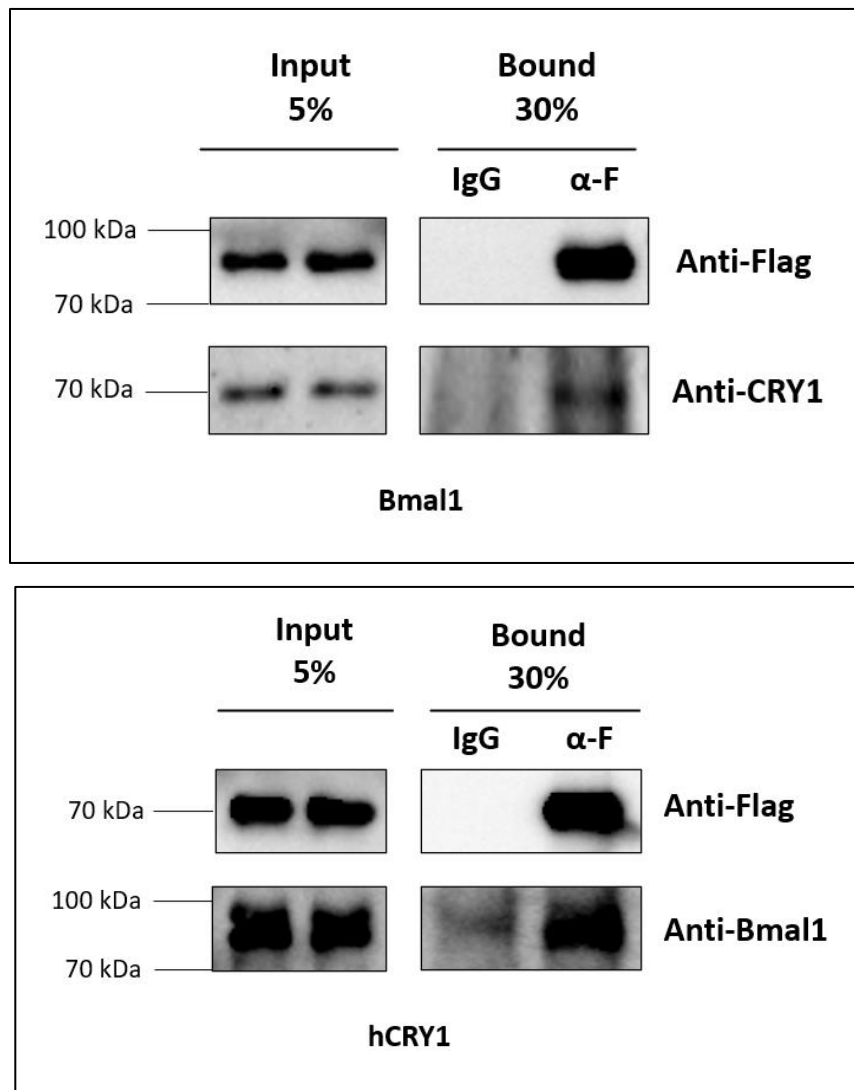


Figure 5.12: Western blot analysis of co-IP sample of endogenous BMAL1 and hCRY1 proteins at HEK293T stable cell line. Input shows cell lysate (not immunoprecipitated). Bound indicates immunoprecipitated samples. IgG is used as a control. α-F represents anti-FLAG antibody. PageRuler™ Prestained Protein Ladder was used as a marker. Samples were run on 8% SDS gel.

Interactor NOP56 was transfected into the HEK293T.BMAL1.3xFLAG cell line. After immunoprecipitation with co-IP, immunoblot analysis was performed using anti-FLAG and anti-HA antibodies. As a result of the analysis, the expected interaction in the anti-FLAG lane was observed. Besides, NOP56 was transfected into a stable cell line expressing 3xFlag-hCRY1, and Western blot analysis was performed using the same antibodies. NOP56 and CRY1 interaction was not observed in the anti-FLAG lane (Figure 5.13). The NOP56 protein was found to interact only with the BMAL1 protein.

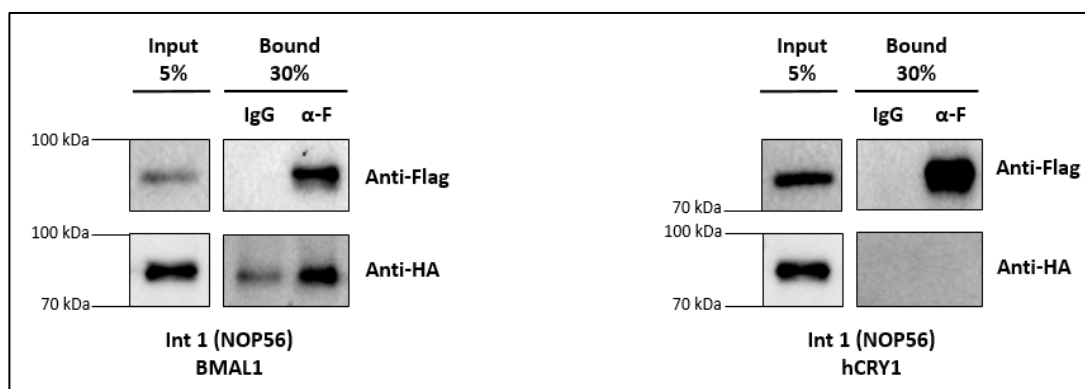


Figure 5.13: Western blot analysis of co-IP of NOP56 protein transfected into cell line expressing both BMAL1 (pcDNA4-BMAL1-3xFLAG) and hCRY1 (pcDNA4-hCRY1-3xFLAG). Input (non-immunoprecipitated sample) indicates cell lysate and bound indicates immunoprecipitated samples. IgG is used as a control, α -FLAG indicates the anti-Flag antibody. PageRuler™ Prestained Protein Ladder was used as a marker. The samples were run on 8% gel by molecular weight.

Interactor FBL was transfected into the HEK293T-BMAL1-3xFLAG expressing cell line. Western blot analysis was performed by blotting with anti-FLAG and anti-HA antibodies. The expected interaction as a result of the analysis was observed in the anti-FLAG lane. Besides, interactor FBL was transfected into a stable cell line expressing 3xFLAG-hCRY1 and Western blot analysis was performed using the same antibodies. As shown in Figure 5.14, the interaction was observed in the anti-FLAG lane. The FBL protein interacts with both BMAL1 and hCRY1.

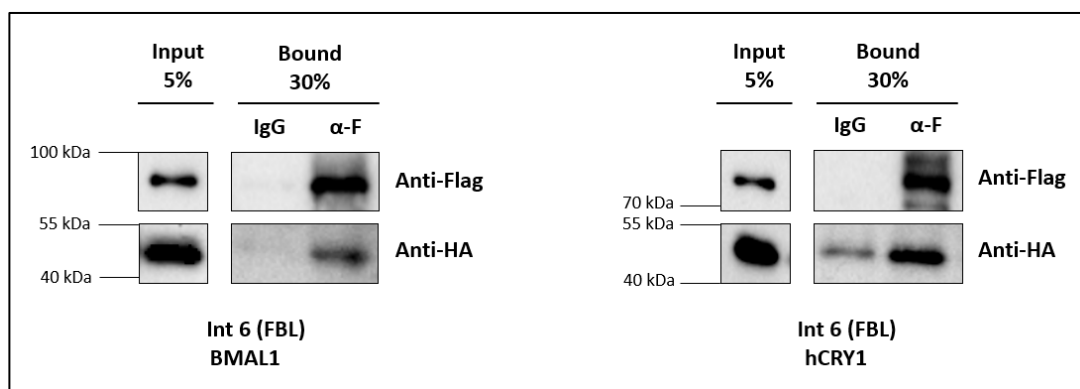


Figure 5.14: Western blot analysis of co-IP of FBL protein transfected into cell line expressing BMAL1 (pcDNA4-BMAL1-3xFLAG) and hCRY1 (pcDNA4-hCRY1-3xFLAG). Input (non-immunoprecipitated sample) indicates cell lysate and bound indicates immunoprecipitated samples. IgG is used as a control, while α -FLAG indicates anti-FLAG antibody. The samples were run on 8% gel by molecular weight.

Interactor PPIB was transfected into the HEK293T-BMAL1-3xFLAG cell line. Western blot analysis was then performed using anti-FLAG and anti-HA antibodies. The expected interaction was not observed in the Anti-FLAG lane. Additionally, the PPIB interactor was transfected into the 3xFLAG-hCRY1 expressing cell line and Western blot analysis was performed using the same antibodies. In Figure 5.15, PPIB and hCRY1 interaction was not observed in the anti-FLAG lane. The PPIB protein may not be in interaction with both BMAL1 and hCRY1 proteins.

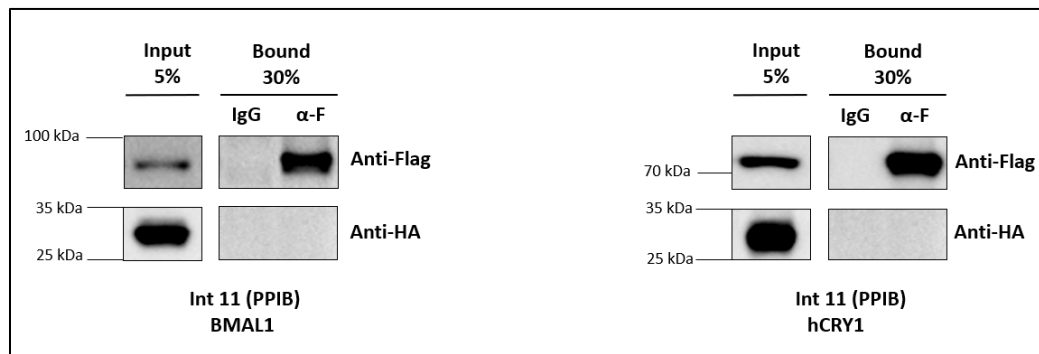


Figure 5.15: Western blot analysis of co-IP of PPIB protein transfected into cell lines expressing BMAL1 (HEK293T-BMAL1-3xFLAG) and hCRY1 (HEK293T-hCRY1-3xFLAG). Input (unprecipitated sample) indicates cell lysate and bound indicates immunoprecipitated samples. IgG is used as a control. α-FLAG indicates the anti-FLAG antibody. The samples were run on 10% gel according to their molecular weight.

6. DISCUSSION

Signaling proteins mostly perform their biochemical functions by protein-protein interactions (PPIs) in the complexes. Therefore, identifying PPIs and understanding their functions can provide insight into the mechanisms and treatments of the related diseases [Li et al., 2017].

Due to the limitations of traditional methods used to describe PPIs, two affinity labeling methods, BioID and APEX, have been developed in recent years. These methods allow the fusion of the protein of interest with the BirA biotin ligase enzyme in the BioID technique and with the ascorbate peroxidase enzyme in the APEX method. When these enzymes are activated, they covalently label all proteins in the proximity of the protein. After biotin labeling, interactors are purified by streptavidin-beads and identified by mass spectrometry [Béganton et al., 2019]. Because this method labels all proteins in close proximity even the ones that do not actually interact with the protein of interest, other methods are required to confirm the possible interactions identified by the BioID method.

Co-IP is one of the common methods used to confirm PPIs. In this method, other proteins that interact with the protein of interest are detected after the complexes of the protein of interest are purified. In the co-IP technique, the target protein (BMAL1 in this thesis) is precipitated by using a specific antibody conjugated to the beads. However, in this method, certain protein interactions cannot be detected if the protein interactions are weak or very short-lived. In addition, the BioID method is limited in use due to the long labeling times and low activity. Although MiniTurbo activity is lower than TurboID, MiniTurbo has much shorter labeling times than BioID [Cho et al., 2020].

In this thesis, we used the MiniTurbo-based BioID method and wanted to confirm the protein-protein interactions (PPI) of BMAL1 identified by this method. Therefore, we selected candidate interactors that interact with the BMAL1 protein. In our BioID approach, the MiniTurbo biotin ligase enzyme was fused to the protein of interest (BMAL1-3xHA-MiniTurbo) and endogenous proteins in the immediate vicinity of BMAL1 were labeled with biotin. In this way, we identified the interactors of the protein of interest (BMAL1). We then confirmed the existence of these protein interactions using the co-IP technique.

Co-IP of some of the highly expressed ones selected from the list of interactors created by BioID and mass spectrometry analysis was performed.

BMAL1 and hCRY1 proteins are known to interact in the circadian clock mechanism. Therefore, when we analyzed the presence of endogenous CRY1 in the BMAL1-3xFLAG-precipitated complex, we observed some of the interactions. Likewise, when we analyzed the presence of endogenous BMAL1 in the 3xFLAG-CRY1 precipitated complex and observed an interaction between CRY1 and BMAL1. With this experiment, we confirmed this interaction and showed that some interactors of BMAL1 can also interact with hCRY1 (Figure 5.12).

When we analyzed the presence of the NOP56 interactor in the BMAL1-3xFLAG-precipitated complex, we observed a strong interaction. In the same way, when we analyzed the presence of the NOP56 interactor in the 3xFLAG-hCRY1 precipitated complex, we did not observe an interaction. With this experiment, we confirmed this strong interaction of BMAL1 and the candidate interactor (NOP56). We also found that this interactor only interacted with the BMAL1 protein, but not with CRY1 (Figure 5.13).

Moreover, when we analyzed the presence of the FBL interactor in the BMAL1-3xFLAG-precipitated complex, we detected an interaction. Likewise, we detected a strong interaction in the analysis of the presence of the FBL interactor in the 3xFlag-hCRY1 precipitated complex. With this experimental result, we confirmed that the FBL protein interacts with both BMAL1 and hCRY1 proteins (Figure 5.14).

During rRNA maturation, nucleolar proteins (such as NOP56, NOP58, and FBL) and snoRNAs (small nucleolar RNA) form snoRNP (small nucleolar ribonucleoproteins) complexes, which are involved in biological processes such as methylation and post-transcriptional modifications [Boisvert et al., 2007].

Nop56 (Nucleolar protein 56) is a nucleolar protein in complex with Nop58 (Nucleolar protein 58). Nop56 is a protein required for the assembly of the 60S ribosomal subunit. In addition, this protein is involved in pre-rRNA processing. The protein encoded by this gene is located in the nucleolus. NOP58 is a box C/D small nucleolar ribonucleoprotein that forms snoRNP complexes that have an important role in pre-rRNA processing in the nucleolus [Lykke-Andersen et al., 2018]. The FBL (Fibrillarin) protein is a member of the snoRNP complexes involved in the processing of pre-ribosomal RNA. The FBL protein associated with Box C/D

snoRNAs is an important methyltransferase that performs rRNA methylation [Wang et al., 2000].

Studies have shown that BMAL1 is required to maintain snoRNP levels and plays a role in pre-rRNA processing. By mass spectrometric analysis, it was determined that BMAL1 interacts with nucleolar proteins (NOP56, NOP58, and FBL) and this relationship takes place in the nucleoli of cells. In addition to being a transcriptional regulator, BMAL1, which is involved in the circadian core clock mechanism, has also been found to be a component of the nucleolus. That is, BMAL1 is in the same complex as RNA polymerase II-related transcription factors with nucleolar localization. This function of BMAL1 also appears to be independent of the circadian [Cervantes et al., 2020].

With this thesis, we determined that BMAL1 can interact with NOP56 and FBL proteins, considering the relationship of NOP56 with NOP58, and we confirmed this interaction with co-IP.

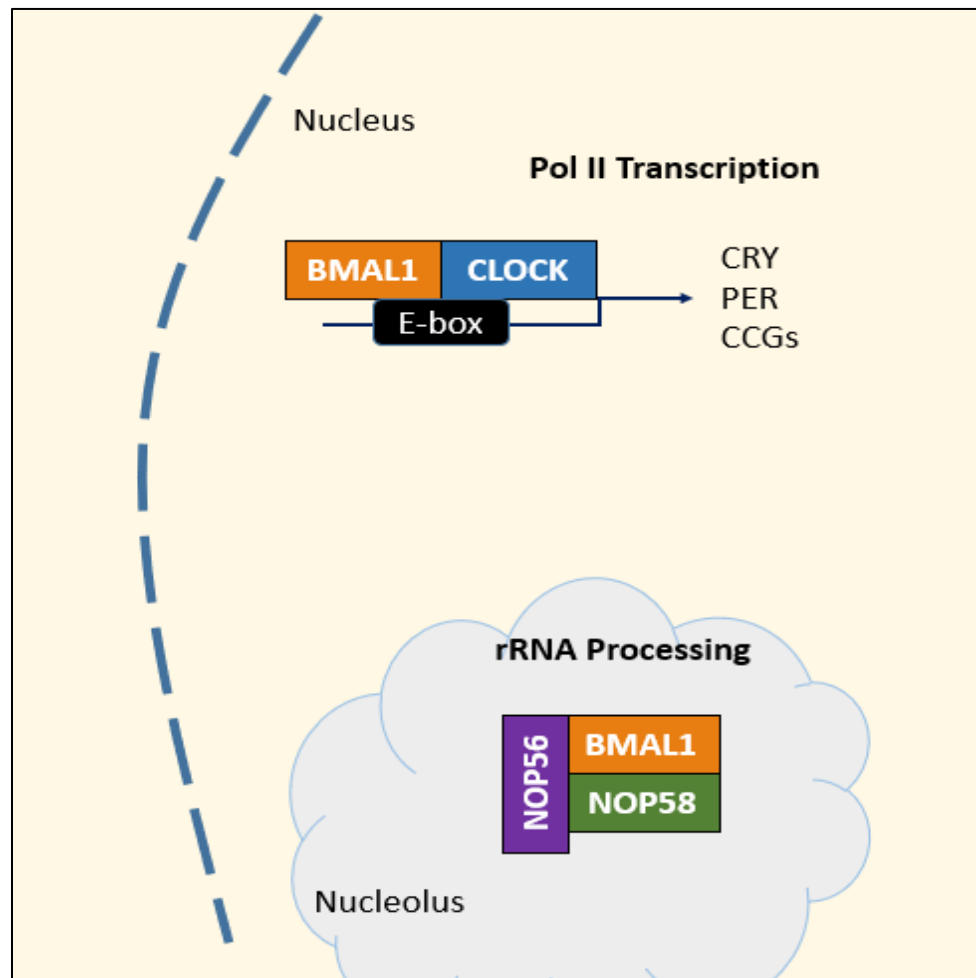


Figure 6.1: Interaction of BMAL1 with nucleolar proteins NOP58, NOP56, and FBL.

In addition to these proteins, we analyzed the presence of the PPIB interactor in the BMAL1-3xFLAG-precipitated complex. We observed that Interactor PPIB did not interact with the BMAL1 protein. We also analyzed the presence of PPIB in the hCRY1-3xFLAG precipitated complex and observed that this protein does not interact with hCRY1. However, we cannot definitively say that this protein does not interact with BMAL1 and hCRY1 (Figure 5.15).

In the presence of weak or transient interactions, an adjustment or modification of the test stages is required. Expression can be enhanced by making some changes in the co-IP process of interactors with weak expression. Solutions and active chemicals used to increase binding and activity should be controlled. For example, in the co-IP process, the activity of the antibody, the lysis buffer, and the washing steps are very important.

Transient or weak protein interactions are usually removed from the complex during capture and washing. The wash buffer should keep the interactions with the protein of interest intact and reduce non-specific binding. Therefore, adjustments can be made to the composition of the wash buffer to optimize the wash condition. Also, the higher concentration of denaturing buffers can be used to reduce non-specific binding. IgGs may mask proteins of interest due to low protein content, transient or low affinity of target interactors. The use of antibodies generated in the same type as the antibodies used in the co-IP procedure in immunoblotting increases non-specific binding. Therefore, antibodies generated from different strains must be used for immunoblotting. In addition, the type and concentration of magnetic beads also affect non-specific binding. However, immunoblot may not be sensitive enough to detect low expression of the target protein. This problem can be solved by using a more specific or active antibody. The activity of bait and target proteins can be increased. In addition, different cell lines can be used for these proteins. At this point, the activity of the plasmid should also be checked.

At the end of this thesis, we showed that new interactors of BMAL1 protein were detected and confirmed by the co-IP technique. As a result, we observed that co-IP conditions should be optimized considering weak or non-existent interactions. With the advancement of technology, both BioID and co-IP techniques are being developed. In this way, in future studies, more interactions will be detected, and functional studies will be carried out to understand the reason for these interactions. In this way, the functional changes that occur in the presence and absence of BMAL1 in the cell and the activity/stability of the interactors will be determined. The results obtained will contribute to the determination of the effects of these proteins and their interactions on diseases.

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BIOGRAPHY

Kevser ÇİLENK was born in Istanbul. She graduated from Atatürk University, Department of Molecular Biology and Genetics in 2019. Then she attended the master's program at Gebze Technical University. After completing her master's program in the Department of Molecular Biology and Genetics at the Institute of Science, she graduated in 2022 with a master's degree. She worked for 2 years under the supervision of Prof. Dr. Nuri ÖZTÜRK.

APPENDICES

Appendix A: Protein and DNA Markers

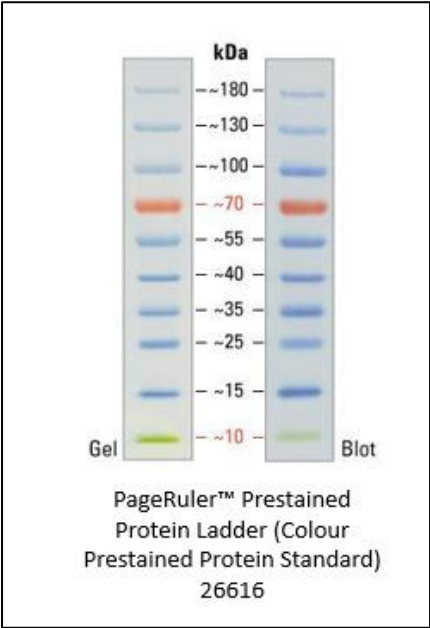


Figure A1.1: Protein Ladder.

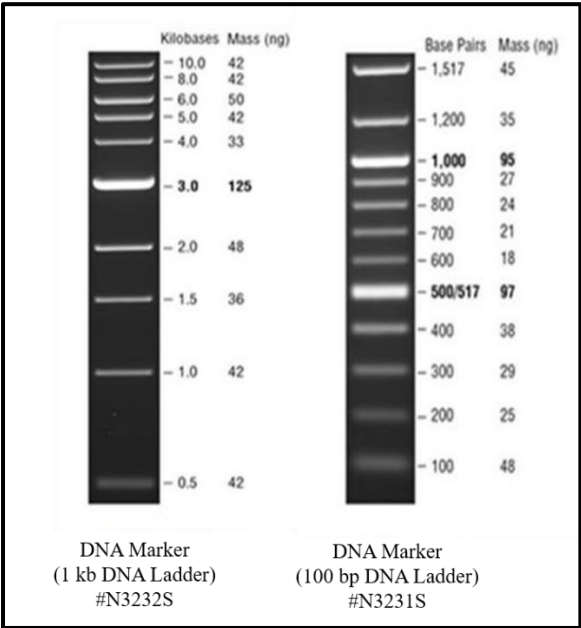


Figure A1.2: DNA Ladder

Appendix B: List of BMAL1 interactors generated by Saint Express Analysis

Table A1.1: A list of candidate interactors of the BMAL1 protein was generated using Saint Express software (Saint score 0.95 and above).

Uniprot Accesion Number	Saint Score
O00567	0.99
P11387	1.0
Q9BQG0	1.0
Q9NX58	1.0
P60660	0.99
P22087	0.99
Q8NI92	0.99
Q6I9V5	0.99
A0A024QZU2	0.98
Q6NZI2	0.98
P23284	0.98
P27635	0.98
Q9NR30	0.97
Q03701	0.99