

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

EXPLORING PERIOD2 INTERACTOME

ŞEYMA YAZAN
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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PROF. DR. NURİ ÖZTÜRK

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

PERİOD2 İNTERAKTOMUNUN KEŞFİ

ŞEYMA YAZAN
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

GEBZE
2023



YÜKSEK LİSANS JÜRİ ONAY FORMU

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SUMMARY

The circadian clock is synchronized by light-dark cycles to control metabolism and physiology in a 24-hour cyclic manner. The endogenous circadian clock is generated by a transcription-translation feedback loop (TTFL) mechanism, and it is adjusted to 24-hour geological clock by sunlight. In the positive arm of the TTFL, BMAL1 and CLOCK transcription factors activate transcription of negative arm members PERIOD (PER) and CRYPTOCHROME (CRY) as well as other circadian clock-regulated genes. After translation, CRY and PER proteins inhibit BMAL1-CLOCK activity by protein-protein interactions (PPIs).

Among the three PER proteins in humans, the PER2 protein, which has the highest oscillation, is found in the cytoplasm and nucleus in a circadian manner. Therefore, it is important to investigate proteins that interact with PER2. Our laboratory discovered new PPIs of PER2 using BioID methods. The aim of this study is to confirm some of those PPIs of PERs using co-immunoprecipitation technique. To this end, we transiently expressed HA-tagged interactors in a stable HEK293T-FLAG-PER2 cell line. The proteins that interact with FLAG-PER2 were pulled down on anti-FLAG antibody-coated magnetic beads and non-specific binders were washed away. The presence of the interactors in anti-FLAG antibody-immunoprecipitation complex was analyzed by immunoblotting the samples with an anti-HA antibody. We confirmed several PPIs of PER2 using this method.

In conclusion, we conducted a study in which interactions of PER2 can be demonstrated by the co-immunoprecipitation technique. Identifying new molecular partners of PER2 will provide clues to understand biological processes affected by PER2 directly or indirectly.

Keywords: Period2, Circadian Clock, Protein-Protein Interaction, Interactor, Co-Immunoprecipitation.

ÖZET

Sirkadiyen saat, metabolizmayı ve fizyolojiyi ~24 saatlik döngüsel bir şekilde kontrol etmek için aydınlık-karanlık döngüleriyle senkronize edilir. Endojen sirkadiyen saat, bir transkripsiyon-translasyon geri bildirim döngüsü (TTFL) mekanizması tarafından üretilir ve güneş ışığı ile 24 saatlik jeolojik saate göre ayarlanır. TTFL'nin pozitif kolunda BMAL1 ve CLOCK transkripsiyon faktörleri, negatif kol üyeleri PERIOD (PER) ve CRYPTOCHROME (CRY) ile diğer sirkadiyen saat tarafından düzenlenen genlerin transkripsiyonunu aktive eder. Translasyon sonrası, CRY ve PER proteinleri, protein-protein etkileşimleri (PPI'lar) ile BMAL1-CLOCK aktivitesini inhibe eder.

İnsanlardaki üç PER proteini arasında en yüksek salınımı gösteren PER2 proteini sitoplazmada ve çekirdekte sirkadiyen bir şekilde bulunur. Bu nedenle, proteinlerin PER2 ile etkileşimini araştırmak önemlidir. Laboratuvarımız, BioID yöntemlerini kullanarak PER2'nin yeni PPI'larını keşfetti. Bu çalışmanın amacı, ko-immünopresipitasyon tekniğini kullanarak PER2'nin PPI'larından bazılarını doğrulamaktır. Bu amaçla, FLAG-PER2'yi kararlı bir şekilde ifade eden HEK293T hücrelerinde HA etiketli interaktörleri geçici olarak eksprese ettik. FLAG-PER2 ile etkileşime giren proteinler, anti-FLAG antikoru kaplı manyetik boncuklar üzerine çekildi ve spesifik olmayan şekilde bağlananlar yıkama ile uzaklaştırıldı. Anti-FLAG antikor imüno-çökeltilmiş komplekste interaktörlerin varlığı, örneklerin bir anti-HA antikoru ile immüno-blotlanmasıyla analiz edildi. Bu yöntemi kullanarak PER2'nin birkaç PPI'sını doğruladık.

Sonuç olarak PER2'nin interaktörlerinin ko-immünopresipitasyon tekniği ile gösterilebileceği bir çalışma yürüttük. PER2'nin yeni moleküler ortaklarının belirlenmesi PER2'nin doğrudan veya dolaylı olarak etkileyebileceği biyolojik süreçlerin anlaşılmasına olanak sağlayacaktır.

Anahtar Kelimeler: Period2, Sirkadiyen Saat, Protein-Protein Etkileşimi, İnteraktör, Ko-İmmünopresipitasyon.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
α MSH	: α -melanocyte-stimulating hormone
β -TrCP	: β -transducin repeat-containing protein
$^{\circ}\text{C}$	: Celsius degree
μg	: Microgram
μl	: Microliter
AMPA	: α -amino-3-(hydroxy-5-methyl-4-isoxazole) propionic acid
AMPK	: AMP-activated protein kinase
AP	: Affinity purification
APS	: Ammonium persulfate
ARNTL	: Aryl hydrocarbon receptor nuclear translocator-like protein
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle ARNT-like 1
CaMK	: Calmodulin-dependent kinase
CBD	: CRY binding domain
CCG	: Clock-controlled genes
CKI ϵ	: Casein kinase I-epsilon
CK2- α	: Casein kinase 2 alpha
CLD	: Cytoplasm localization domain
CLOCK	: Circadian locomotor output cycles kaput
co-IP	: co-immunoprecipitation
CREB	: cAMP response element binding protein
CRE	: cAMP response element
DBP	: Albumin D-site- Binding Protein
DMSO	: Dimethyl sulfoxide
E4BP4	: Adenovirus E4 promoter Binding Protein 4
FBXL3	: F-box and leucine-rich repeat protein 3

FBXL21	: F-box and leucine-rich repeat protein 21
FASPS	: Familial Advanced Sleep Phase Syndrome
F-PER2-F	: FLAG-PER2-FLAG
FBS	: Fetal Bovine Serum
HEK293T	: Human embryonic kidney 293T cells
HLF	: Hepatic Leukemia Factor
hPER2	: Human Period2
ipRGCs	: Intrinsically photosensitive retinal ganglion cells
LXXLL	: Leucine-rich motif
LB	: Luria Broth (Luria-Bertani medium)
MAPK	: Mitogen-activated protein kinase
NR	: Nuclear receptor
NES	: Nuclear export signal
NMDA	: N-methyl-d-aspartate
NREs	: Nuclear receptor elements
OPN4	: Melanopsin
PACAP	: Pituitary adenylate cyclase-activating peptide
PAS	: PER-ARNT-SIM
PEI	: Polyethyleneimine
PER	: Period
PKA	: Protein kinase A
PTMs	: Post-translational modifications
RHT	: Retinohypothalamic tract
ROREs	: Retinoic acid-related orphan receptor response elements
SCN	: Suprachiasmatic nucleus
SUMO	: Small ubiquitin-like modifier
TBS-T	: Tris-buffered saline + 0.1% Tween 20
TEF	: Thyrotroph Embryonic Factor
TTFL	: Transcription-translation feedback loop
U2OS	: Human bone osteosarcoma epithelial (U2OS) cells

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1. INTRODUCTION

This study mainly focuses on the validation of novel interactions of PERIOD2 (PER2) protein. Our laboratory previously identified proteins that interact with PER2 by using proximity-dependent biotin labeling (BioID) technique combined with mass spectrometry analysis. Interactors identified based on SAINTExpress analysis of mass spectrometry results were then tested by immunoblotting after co-precipitation (co-IP) to demonstrate the physical interaction between PER2 and its interactors. The exploration and discovery of new PER2 interaction partners contribute to the knowledge in the literature about biological processes and pathways involving PER2 and the circadian rhythm.

1.1. The Purpose, Contribution and Content of Thesis

The main aim of this study is to confirm the possible interactions of PER2 which were identified by proximity-dependent biotin labeling combined with mass spectrometry technique (BioID) using co-immunoprecipitation technique. While the the BioID approach was used to label proteins in proximity (10 nm in diameter) of PER2 in our laboratory, this labeling does not confirm that PER2 directly interacts with them [Gul et al., 2022]. Therefore, the candidate interactors were first scored based on a statistical tool called SAINTExpress analysis. Four interactors were selected with a score of 1 analysed with SAINTExpress. In this thesis, we validated possible PER2 interactions using co-immunoprecipitation assay. Human embryonic kidney HEK293T stably expressing the pENTER-FLAG-PER2-FLAG-His cell line was generated. HA-tagged interactors were then transiently transfected into the HEK293T-FLAG-PER2-FLAG cell line. PER2 complexes were immunoprecipitated using anti-FLAG antibody-coated magnetic beads 48 hours after transfection. The presence of HA-tagged interactors in the anti-FLAG antibody-immunoprecipitated PER2 complex was analyzed by immunoblot analysis using anti-HA tag antibody. At the end of this thesis, we validated the possible interacting proteins selected from the PER2 interactome list by the co-immunoprecipitation method.

Part 2 reviews detailed literature on the circadian clock, the components of the mammalian circadian clock, the core clock gene PER2, protein-protein interaction sites of PER2, and co-immunoprecipitation. Parts 3 and 4 describe materials and all experimental procedures. Part 5 provides a detailed description of all the experimental results obtained in this thesis. Finally, this thesis is concluded by giving conclusions and discussion in Part 6.

2. LITERATURE REVIEW

2.1. Circadian Clock

Almost all organisms, from bacteria to humans, are constantly under the influence of daily environmental cycles that occur because of the Earth's axial movement. Most living organisms have an internal biological timer called the circadian clock to predict these repetitive daily environmental changes and adapt to the changes at the maximum level [Takahashi et al., 2008]. Circadian clocks keep the internal timing of physiological, metabolic, and behavioral processes in phase with the geological (solar) clock with a period of 24 hours [Dunlap, 1999]. Thus, it provides time-dependent coordination of the internal events with external environment. The circadian clock governs cellular metabolism besides physiological functions such as the sleep-wake cycle, temperature regulation, heartbeat, and hormone secretion [Hastings et al., 2003]. It also provides separation of different biological functions such as sleep and nutrition. Disruption of the circadian clock system has been associated with the development of a wide variety of diseases or dysfunctions, including sleep disorders, depression, diabetes, metabolic syndrome, and cancer [Scheer et al., 2009].

The word “circadian” is derived from the Latin phrase “circa dies” meaning “about a day”. Circadian clock produces self-sustaining and cell-autonomous rhythms with periods of approximately 24 hours. Therefore, circadian rhythms are endogenous and continue to oscillate under constant conditions (in the absence of light-dark cycles or time cues). There are inter-species differences in the length of endogenous rhythms. However, these free-running rhythms are synchronized by external time cues (zeitgeber, “time-giver” in German) such as light, temperature fluctuations. This process of synchronizing or resetting is also referred to as “entrainment” of the clock [King and Takahashi, 2000]. Another characteristic feature of circadian rhythms is the ability to maintain periodicity over wide temperature changes [Pittendrigh, 1954]. The basis for the properties of circadian oscillators was laid by the research of Pittendrigh and Aschoff [Bass, 2012].

2.1.1. History

The first scientific observation of circadian rhythm was made by the French astronomer Jean Jacques d'Ortous de Mairan in 1729. De Marian placed the *Mimosa pudica* plant (known as also sensitive plant, sleepy plant, touch-me-not, or shameplant) in a light-insulated box and observed that the leaves of the plant open and close rhythmically regardless of daylight. This observation suggested the existence of internal rhythms in plants [Roenneberg and Merrow, 2005]. In 1832, Augustin Pyramus de Candolle discovered that the free-running period of *Mimosa pudica* was 22.5 hours. In 1962, a French speleologist named Michel Siffre lived in a cave alone for six months to investigate the sleep-wake cycle and what it would be like without any external environmental cues. At the end of the experiment, he showed that the sleep-wake cycle was about 24 hours and 30 minutes, not 24 hours like humans on the surface. The findings proved the existence of an internal clock independent of the day/night cycle [Reppert and Weaver, 2002]. In 1965, Jürgen Aschoff, another pioneer in the study of biological rhythms, also conducted more than 150 isolation experiments in an underground cavern. Aschoff coined the term 'zeitgeber' for the first time as a result of his findings [Aschoff, 1965].

2.1.2. Discovery of Clock Components

In the 1970s, Seymour Benzer and his student Ronald Konopka used *Drosophila melanogaster* (fruit fly) to find the genes responsible for regulating the circadian rhythm. Konopka randomly mutated flies and found three mutant fly phenotypes that exhibited abnormal circadian behavior: long-period mutant, short-period mutant, and arrhythmic mutant [Konopka and Benzer, 1971]. These mutants were found to affect the same locus on the X chromosome, and the gene was described as *Period (Per)* gene. In 1994, US neurobiologist Joseph S. Takahashi and his colleagues, using molecular and genetic approaches, identified a mutation in mice that lengthens the circadian period and causes a loss of persistence in rhythms. These researchers mapped the first mammalian circadian rhythm gene *CLOCK* (Circadian locomoter output cycles protein kaput) [Vitaterna et al., 1994]. Shortly after the discovery of the *CLOCK* gene, two cryptochrome proteins named CRY1 and CRY2, which have high sequence

similarity to the blue light photoreceptor in plants were discovered. Human hCRY1 and hCRY2 proteins were found to be responsible for regulating the clock mechanism [Todo et al., 1996]. In a study on mice, it was shown that mice lacking both mCRY1 and mCRY2 protein exhibit arrhythmic behavior under constant darkness conditions [Van der horst et al., 1999]. ARNTL (Aryl hydrocarbon receptor nuclear translocator-like protein 1), also known as BMAL1 and MOP3, encoded by the Bmal1 gene, another component of the circadian clock, was discovered by Hogenesch and his colleagues [Hogenesch et al., 1998]. Following these discoveries, many other clock components in the circadian oscillator were elucidated.

2.2. The Central Circadian Oscillator

The mammalian circadian clockwork consists of three main interrelated physiological components: the inputs that transmit external cues to the central oscillator, a self-sustaining circadian oscillator (rhythm generator), and output signals (which generates overt rhythms). In mammals, the master circadian oscillator is generated by about 20,000 neurons in the suprachiasmatic nucleus (SCN) of the anterior part of the hypothalamus [Mohawk and Takahashi, 2011]. The master clock in the SCN regulates and synchronizes the peripheral clocks, which are found in almost every tissue of the body, through neural and humoral cues [Ko and Takahashi, 2006]. The surgical damage in the SCN disrupts the circadian clock which shows that the master clock is located the SCN [Ralph and Menaker, 1988].

While rod and cone photoreceptors located in the retinal layers detect light as a visual input for image forming [Morin and Allen, 2006], a subset of retinal ganglion cells, which express the photopigment melanopsin rendering them intrinsically photosensitive (ipRGCs), transmits light signals to SCN neurons for the entrainment the circadian clock [Panda et al., 2002]. The light signals transmitted to the terminal synapse via the retinohypothalamic tract (RHT), which consists of the axon bundle of ipRGCs, are converted into an electrochemical signal with neurotransmitters, releasing neurotransmitters such as glutamate and pituitary adenylate cyclase-activating peptide (PACAP) in SCN neurons [Hannibal et al., 2004]. Glutamate is the main excitatory depolarizing neurotransmitter of central nerve cells [Ebling, 1996]. Glutamate provides Ca^{++} permeability by opening voltage-dependent Ca^{++} channels of AMPA (α -

amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), NMDA (N-methyl-d-aspartate), and kainate receptors located at the nerve end [Meijer and Schwartz, 2003]. Increased Ca^{++} in the cytoplasm activates calmodulin-dependent kinase (CaMK), then CaMK phosphorylates protein kinase A (PKA). PKA then phosphorylates cytoplasmic cAMP response element-binding protein (CREB) [Carlezon et al., 2005]. Thus, activated CREB binds to the cAMP response element (CRE) of the front of *PER1* and *PER2* genes in the SCN and induces transcriptions of target genes [Travnickova-Bendova et al., 2002], [Shearman et al., 1997].

2.3. Molecular Mechanism of the Mammalian Circadian Clock

Molecular and genetic studies have shown that circadian clock modulates approximately 10% of gene expression and 30-40% of second-order genes and post-translational modifications (PTMs) [Zhang et al., 2014]. The molecular architecture of the mammalian circadian clock is formed by a cell-autonomous transcription-translation feedback loop (TTFL) that integrates environmental and metabolic stimuli to generate internal circadian oscillation [Ko and Takahashi, 2006]. The core TTFL model applies across species, with some variation in the components [Reppert and Weaver, 2002].

In the positive arm of the mammalian TTFL, transcription factors CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1) transactivate the expression of the clock-controlled genes (CCGs) [Gekakis et al., 1998]. Two of the CCGs called *CRYPTOCHROME* and *PERIOD* form the negative arm of the TTFL and the translation products of them inhibit the CLOCK:BMAL1 activity by protein-protein interactions to close the loop [Reppert and Weaver, 2002], [Kume et al., 1999].

CLOCK and BMAL1 proteins, both containing bHLH (basic Helix-Loop-Helix) and PAS (Per-Arnt-Single-minded) domains, heterodimerize to initiate the transcription of CCGs [Bunger et al., 2000]. The BMAL1/CLOCK heterodimeric complex binds to E-box elements (CACGTG) in the promoter or enhancer region of *CRY*, *PER*, and other CCGs to activate transcription; thereby activating downstream genes [Yu et al., 2002]. The translated proteins of CCGs gradually accumulate outside the nucleus in the afternoon and reach their highest protein level in the evening. As

being the products of two of CCGs, the PER and CRY proteins then form heterotypic complexes in the cytoplasm. Upon interaction of the casein kinase I-epsilon (CKIε) with the PER/CRY complex, the CKIε-PER-CRY ternary complex moves to the nucleus with appropriate circadian timing [Young and Kay, 2001]. The PER/CRY repressor complex then blocks the transcriptional function of the BMAL1/CLOCK heterodimer to inhibit transcription of its own promoters and promoters of other CCGs (“Blocking-type” repression) [Ye et al., 2014], [Sato et al., 2006], [Yu et al., 2002].

In this repression mechanism, although both CRY and PER bind to the BMAL/CLOCK complex, only CRY interacts directly with the CLOCK/BMAL1/E-Box ternary complex. Biochemical studies have shown that the CRY protein can also suppress BMAL-CLOCK-dependent transcription even in the absence of the PER protein [Yang et al., 2021]. PER mediates removal of the BMAL1-CLOCK heterodimers from the E-Box sequence in the presence of CRY (“displacement-type” repression) [Ye et al., 2014].

During the night period, proteasome-mediated degradation of PER and CRY proteins terminates the negative feedback loop which reinitiates BMAL-CLOCK-mediated transcription. As a result, the transcriptional feedback loop driven by the negative and positive limbs produces a repetitive oscillation pattern every 24 hours [King and Takahashi, 2000].

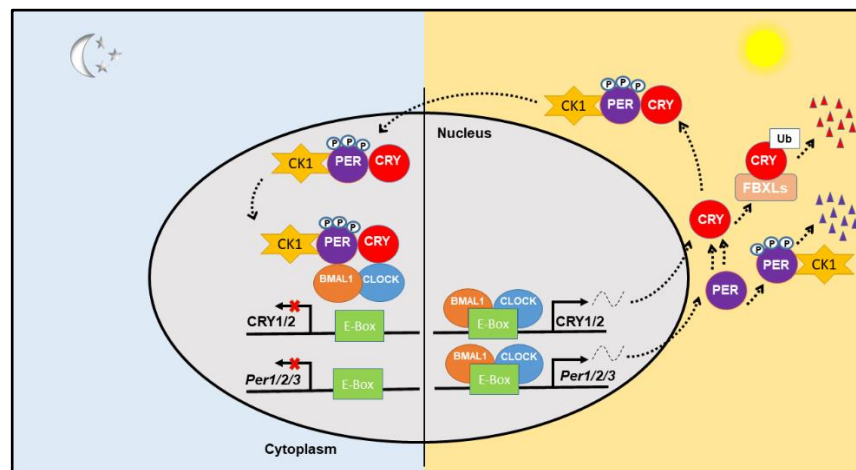


Figure 2.1: Transcription/translation feedback loop (TTFL) model of the molecular clock in mammals. CLOCK-BMAL1 is the positive regulator that regulates the transcriptions of negative regulators of the clock and clock-controlled genes (ccg).

A secondary loop intertwined with the primary TTFL, also called the stabilizing loop, contains Nuclear Receptor SubFamily 1 group D Members 1 and 2 (Rev-erb homologs α and β) and Nuclear Receptor SubFamily 1 group F Members 1, 2, and 3 (ROR homologs α , β and γ) [Takahashi, 2017]. The BMAL1/CLOCK heterodimeric complex binds to nuclear receptor elements (NREs), REV-ERBs and RORs, which contain E-box sequences in their promoters and stimulates their gene expression. The protein products, REV-ERBs and RORs, translocate to the nucleus and regulate the expression of the *BMAL1* gene, which have retinoic acid-related orphan receptor response elements (ROREs/RRE) in its promoter [Preitner et al., 2002]. In the secondary loop, REV-ERBs with negative regulation on the transcription of BMAL1 suppress the rhythmic gene expression of BMAL1, whereas RORs promote expression [Adelmant et al., 1996]. Between RORs and REV-ERBs, the antagonist effect on the BMAL1 promoter helps to fine-tune the oscillation [Guillaumond et al., 2005], [Preitner et al., 2002]. Although REV-ERB α and β do not show tissue-specific circadian oscillation, ROR α , β , and γ are specific in some tissues. BMAL1 up-regulates the expression of REV-ERB α , while another regulator, PER2, down-regulates it [Sato et al., 2004]. In a study in *Per2* knockout mice, expression of REV-Erb α was high and non-rhythmic [Preitner et al., 2002].

The tertiary feedback loop mediated by additional D-Box sequences to other TTFLs includes the PAR (proline and acidic amino acid-rich)-bZip (basic region leucine zipper) transcription factor family [Cowell, 2002]. The BMAL1/CLOCK heterodimer complex binds the E-Box sequences within the promoter regions of the PAR-bZip factors DBP (Albumin D-site- Binding Protein), TEF (Thyrotroph Embryonic Factor), and HLF (Hepatic Leukemia Factor) and upregulates their expressions. Gene products recognize and bind D-Box elements [Mitsui et al., 2001]. Another PAR-bZip factor, E4BP4 (Adenovirus E4 promoter Binding Protein 4, also called NFIL3), contains RORE in its promoter region, and the REV-ERB and ROR proteins compete to bind to this region. E4BP4 and DBP are proteins with opposite functions and compete to bind the D-Box elements of target genes; E4BP4 represses transcription, while DBP activates it. The winner of the competition binds to the D-Box sequences in the promoter regions of REV-ERB and ROR, which control the expression of E4BP4 [Cowell, 2002].

In addition to interlocking feedback loops, proper tuning of circadian oscillations requires post-translational modifications (PTMs) of core clock proteins [Harms et al.,

2004]. PTMs (phosphorylation, ubiquitination, histone acetylation, methylations, and SUMOylation) on core clock proteins affect the function, speed, and precision of the circadian clock [Hirano et al., 2016].

Phosphorylation is required for the PER/CRY complex to enter the nucleus and perform its inhibitory activity. Degradation of phosphorylated proteins for termination of repression activity occurs via the ubiquitin-proteasome system. PER is phosphorylated by two members of the casein kinase I family, CKI- ϵ and CKI- δ . PER phosphorylated by CKIs is ubiquitinated by an E3 ubiquitin ligase, β -transducin repeat-containing protein (β -TrCP). PER is then targeted and degraded by the 26S proteasome [Eide et al., 2005]. The other protein of the negative limb, CRY, is phosphorylated by AMP-activated protein kinase (AMPK) [Lamina et al., 2009]. After CRY proteins are phosphorylated, they are ubiquitinated by members of the F-box protein family, FBXL3 (F-box and leucine-rich repeat protein 3) and FBXL21 (F-box and leucine-rich repeat protein 21), which mediate their degradation by the 26S proteasome [Xing et al., 2013]. FBXL3 functions only in the nucleus, while FBXL21, which works as an antagonist, functions both in the nucleus and in the cytoplasm. Thus, the stability and degradation of CRY is regulated [Yoo et al., 2013].

CLOCK has intrinsic histone acetyltransferase (HAT) activity and BMAL1, the heterodimerization partner of CLOCK, enhances HAT function [Doi et al., 2006]. This acetylation of BMAL1 by CLOCK facilitates recruitment of CRY1 to CLOCK-BMAL1 and promotes transcriptional repression [Hirayama et al., 2007]. Moreover, the rhythmic acetylation of histones H3 and H4 in the chromatin region spanning the promoters of the core clock genes (PER1, PER2, and CRY1) is associated with their circadian transcription [Etchegaray et al., 2003].

Phosphorylation of BMAL1 by casein kinase 2 alpha (CK2- α) is an essential regulator of the mammalian circadian system [Tamaru et al., 2009]. In addition to phosphorylation and acetylation, the activity of BMAL1 is also regulated by small ubiquitin-related modifier (SUMO) called SUMOylation and this plays an important role in BMAL1 circadian expression and clock rhythmicity [Cardone et al., 2005]. Thus, PTMs help maintain circadian oscillation every 24 hours by controlling the stability, entry into the nucleus, subcellular localization, protein-protein interactions, and degradation of clock proteins.

2.4. PERIOD2

The human *Period2* (*hPER2*) gene is located on the long (q) arm of chromosome 2 and contains 23 exons. *PER2*, one of the three members of the period gene family (*PER1–3*) [Reppert, 2000], resembles *Drosophila* Period (dPER) [Hennig et al., 2009], [Shearman et al., 1997]. The core clock protein PER2 plays a role in the negative arm of TTFL and inhibits the activity of the positive arm members by acting with the other negative arm clock protein, the CRY protein, to inhibit their own transcription [Dunlap, 1999], [Jin et al., 1999]. In knockout mouse model, it was found that *Per2* plays a more effective role than *Per1* in the circadian clock, whereas *PER3* plays the least role [Shearman et al., 2000]. *Per2* mRNA expression can be detected in peripheral organs as well as in the superior olivary nucleus and limbic system, which includes the central clock [Albrecht, 2007].

The *Per2* clock gene is essential for the robust maintenance of the circadian rhythm because any disruption in *PER2* expression alters circadian oscillation [Chen et al., 2009]. Its dysfunction is associated with various diseases such as cancer, sleep disorders, eating disorders, metabolism, and neurodegenerative disorders [Zheng et al., 2022], [Chen et al., 2005], [Lavebratt et al., 2010]. Decreased *PER2* expression levels were observed in various cancerous tissues. In addition, tissue culture studies have showed that *PER2* overexpression in cancer cells suppresses proliferation and metastasis and promotes cell apoptosis [Hua et al., 2006]. *PER2* acts as a tumor suppressor by regulating pathways that respond to DNA damage in cancerous tissues [Fu and Lee, 2003]. In a study comparing *mPer2* mutant (*mPer2^{m/m}*) mice with wild-type mice after exposure to gamma radiation, mutant mice showed increased susceptibility to radiation and neoplastic transformation. In addition, the expression of genes that have important roles in tumor suppression and cell cycle, *Mdm-2*, *c-myc*, *Cyclin A*, and *Gadd45 α* , were downregulated in these mice [Fu et al., 2002]. The overexpression of *PER 2* in MCF-7 breast cancer cells induces a significant growth-inhibitory effect. These studies indicate that *PER2* is an important suppressor clock protein in cancer progression and development [Xiang et al., 2008].

Environmental factors such as light, temperature, and food are known to affect the circadian clock. It has been found that the risk of developing ovarian cancer is higher in workers who are exposed to artificial light, compared to individuals who do

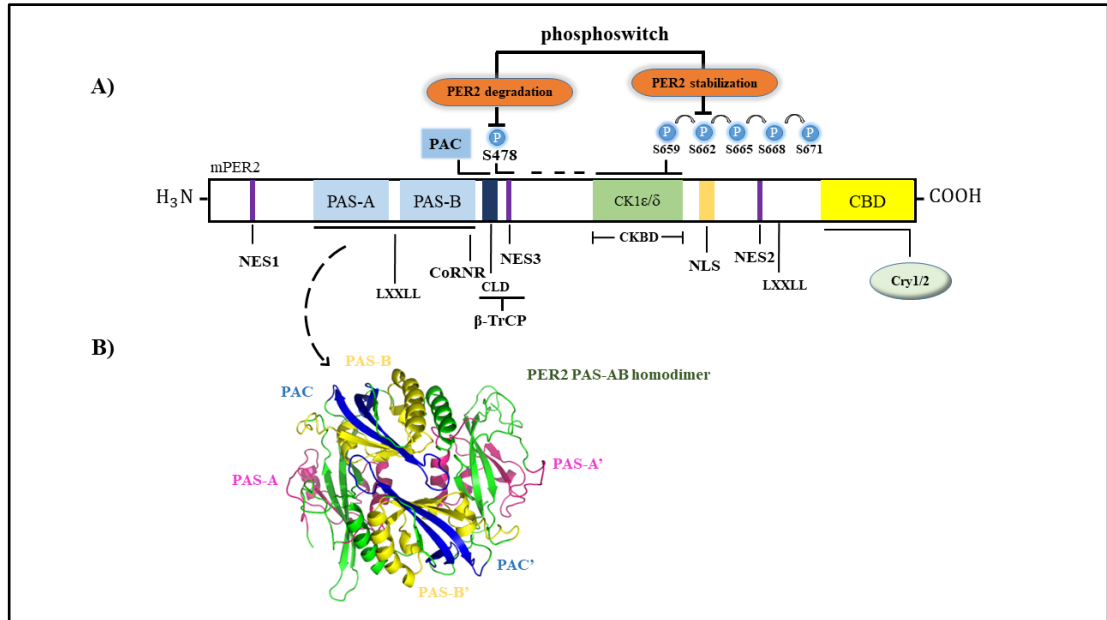
not work the night shift [Wang et al., 2020]. Expression of PER2 is induced in the presence of light [Albrecht et al., 1997], [Shearman et al., 1997]. In zebrafish light-induced PER2 contributes to the expression of the rate-limiting arylalkylamine N-acetyltransferase 2 in melatonin production [Ziv and Gothilf, 2006]. PER2 is closely associated with glucose metabolism and liver function as the deletion of this gene alters the release pattern of the neuroendocrine peptide α MSH (α -melanocyte-stimulating hormone), resulting in night eating syndrome and obesity [Adamovich et al., 2014], [Yang et al., 2009]. Compared with PER2 normal mice, PER2 mutant mice had a higher hepatic fibrosis score [Tahara and Shibata, 2016]. Studies on liver-specific deletion of PER2 have revealed susceptibility of mammals to nonalcoholic fatty liver disease. It causes non-alcoholic fatty liver disease, hyperlipidemia defined as metabolic syndrome, type II diabetes mellitus, and obesity. It has been shown that β -hydroxybutyrate levels in the blood increase as a result of the development of metabolic syndrome, disruption of fat metabolism and breakdown of fatty acids. Thus, high β -hydroxybutyrate disrupts eating and drinking patterns and eliminates daily food expectations [Chavan et al., 2016]. Diabetic atherosclerosis (vascular occlusion) caused by high glucose causes myocardial damage by causing myocardial infarction. PER2 plays an important role in preventing diabetic myocardial damage [Huang et al., 2022]. PER2 is also closely associated with depression. Deletion of the *Per2* gene has been shown to be effective in eliminating the unhappiness that causes insomnia due to sleep disorder [Martini et al., 2021]. However, it has not been shown to have an effect on the relief of anxiety. Insomnia is also known to reduce depression [Hampp et al., 2008]. Studies in *Per2* knockout mice are theoretically known to reduce depression. In mice with thyroid gland removal, decreased expression of PER2 was observed in the absence of thyroid hormone. In these mice that the circadian rhythm was also disrupted in the ganglionic cells other than the SCN in the central nervous system, and the same result was detected in the gonadal cells [Chen et al., 2021].

2.4.1. Protein-Protein Interaction Sites of PER2

The PER protein has two sequential PER-ARNT-SIM (PAS) domains (PAS-A and PAS-B), as well as three nuclear export sites (NES), a nuclear localization site (NLS), and a cytoplasm localization domain (CLD) [Hennig et al., 2009] (Figure 2.2).

Of the PAS-A and PAS-B domains located in the N-terminal region of PER2, especially PAS-B mediates interaction with PER proteins and other proteins [Hayasaka et al., 2011]. In the mammalian system, the PAS-B layer surface mediated mPER2-mPER2 homodimer formation, while in the *Drosophila* system, it was confirmed using yeast-two hybrid assays that it mediated dPER-dTIM heterodimerization [Hennig et al., 2009]. The deletion of the PAS-B domain abolishes the circadian rhythm [Zheng et al., 1999]. Another study performed an immunoprecipitation assay, showing that mutation of a single residue (W419E) at the dimer interface in the PAS-B domain disrupts protein-protein interaction [Philpott et al., 2022]. Phosphorylation of PER2 by CK1 δ or CK1 ϵ of the S478 phosphodegron domain located just downstream of the PAS domain core leads to ubiquitination and proteosomal degradation of PER by β -TrCP. The S478 phosphorylation domain of PER2 is controlled by the FASP (also called the casein kinase binding domain) domain in which sequential serines are located [Eide et al., 2005]. Phosphorylation of the first serine S659 (corresponding to human S662) in this region by CKIs leads to rapid phosphorylation of downstream serine residues [Narasimamurthy et al., 2018]. Thus, it is known to stabilize PER2 and cause higher *Per2* levels [Hirano et al., 2016]. A missense mutation (conversion of serine to glycine) in amino acid 662 of hPER2 (S662G) disrupts the phosphorylation of residues downstream, thereby shortening the circadian period, causing Familial Advanced Sleep Phase Syndrome (FASPS) [Toh et al., 2001], [Xu et al., 2005]. The opposite function of the FASP site in the middle region of PER2 and the B-TrCP binding domains in the N-terminal region of PER2 on the stability of the protein creates a phosphoswitch pattern. This model is temperature sensitive. Studies show that the degradation of the PER2 protein is slowed at high temperatures [Zhou et al., 2015]. In a study of the CKI ϵ Tau mutant hamster (mutated R178C), it was observed that the circadian period of the behavioral rhythms of the hamster was shortened compared to the wild-type phenotype, resulting in a loss of temperature compensation [Lowrey et al., 2000]. PER2 contains the CRY binding domain (CBD), which is involved in complexing with CRY proteins in its C-terminal region. This domain allows PER proteins to form heterodimeric complexes and migrate to the nucleus [Miyazaki et al., 2001]. In addition to CRY proteins, the bZIP transcription factor E4BP4 is known to interact with PER2 through the action of an E-box enhancer-promoter element for potent expression of PER2 [Ohno et al., 2007]. PER2 also has LXXLL (leucine-rich motif) and CoRNR motifs that can interact

directly with some nuclear receptors (NR) [Ripperger and Albrecht, 2012]. Therefore, it is thought that the PER2 protein interacts with many proteins other than circadian clock proteins.



2.5. Co-Immunoprecipitation (co-IP)

Many proteins, including clock proteins, interact with each other inside and outside the cell to maintain homeostasis. Investigation of protein-protein interactions is necessary to elucidate the roles of proteins involved in many biological and physiological processes in the cell [Phizicky and Fields, 1995]. Biochemical methods such as affinity purification (AP), co-immunoprecipitation (co-IP) as well as genetic methods such as yeast-two hybrid assay are widely used to detect PPIs. The co-IP assay is a simple and inexpensive technique in which interacting partners are immunoprecipitated using an antibody specific to the protein of interest (antigen) in a cell lysate [Berggård et al., 2007]. If an antibody specific for the protein of interest is

not available, immunoprecipitation can be performed using an antibody specific for tags such as FLAG, HA, c-myc fused to the C- or N-terminus of the protein of interest [Abu-Farha et al., 2008].

In co-IP method, as the first step, an antibody that precipitates the relevant protein is immobilized to bacterial proteins (protein A or G) attached to magnetic beads (resin) via the Fc region. The cell lysate containing the target protein is then incubated with the antibody and the resulting immune complex is captured with the aid of the immobilized matrix. Proteins that do not bind directly or indirectly to the target protein are removed after washing steps in non-denaturing conditions [Lee, 2007], [Masters, 2004]. The appropriate buffer is then used to separate the complex from the resin and the mixture is run on SDS-PAGE (Figure 2.3). Protein-protein interactions are analyzed by immunoblotting, using antibodies specific to the target protein and interacting partners.

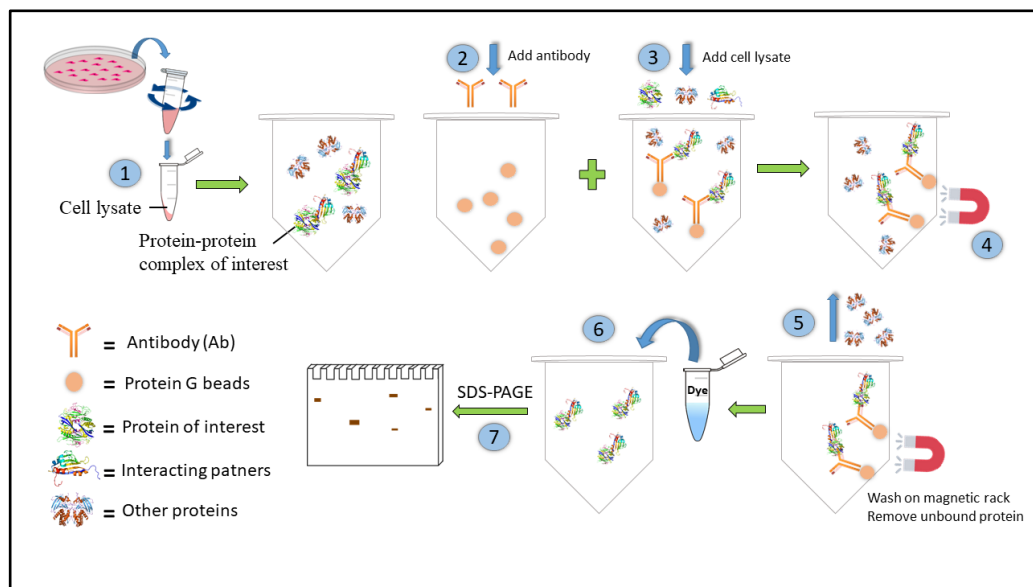


Figure 2.3: Steps of the co-immunoprecipitation method. 1) Cells are lysed with non-ionic detergent; 2) Protein G-coupled magnetic beads and specific antibodies (anti-FLAG antibody in our case) are incubated in a microcentrifuge tube for 1 hour to bind the anti-FLAG antibody to magnetic beads via Protein 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 loaded into gels for SDS-PAGE and then analyzed with immunoblotting to probe the protein of interest and interactors.

3. MATERIALS

3.1. General Kits and Reagent

Table 3. 1: List of kits and reagents.

Nucleospin Plasmid Kit	740588.50, Macherey-Nagel, Germany
PCR Clean-up Gel Extraction Kit	740609.50, Macherey-Nagel, Germany
NucleoBond Xtra Midi kit	740410.10, Macherey-Nagel, Germany
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	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.

10X SDS Running Buffer	250 mM Tris-Base (pH:8.3) 1.90 M Glycine 1% SDS
10X Tris-Buffered-Saline (TBS)	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol
1X TBS-T	1x TBS 0.1%Tween-20
5% Blocking Solution	5% non-fat dry skim milk in 1x TBS-T
10X Tris-Borate-EDTA (TBE)	108g Tris base 55g Boric acid 40 ml 0.5M EDTA (pH 8.0)
4X Protein Loading Dye (Laemmli)	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)
10X PBS	BE17-517Q, Lonza, Belgium
Dulbecco's Modified Eagle Medium (DMEM)	41965-039 (Gibco-Invitrogen, USA)
0.05% Trypsin-EDTA	25300-054 (Gibco-Invitrogen, USA)
Fetal Bovine Serum (FBS)	10270-076 (Gibco-Invitrogen, USA)
100x Penicillin/Streptomycin	15140-122 (Gibco-Invitrogen, USA)
Agar and LB Broth Medium	LBP04, Caisson Labs, USA and LBP03, Caisson Labs, USA
Restriction Enzymes	New England Biolabs, USA
Restriction Enzyme Buffers	New England Biolabs, USA

Table 3. 3: List of SDS-PAGE recipe.

12% Separating Gel	5 ml	10 ml	15 ml
ddH ₂ O	2.15	4.3	6.45
40% Acrylamide: Bisacrylamide	1.5	3	4.5
1.5 M Tris-HCl, pH: 8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006
8% Separating Gel	5 ml	10 ml	15 ml
ddH ₂ O	2.65	5.3	7.95
40% Acrylamide: Bisacrylamide	1	2	3
1.5 M Tris-HCl, pH: 8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.003	0.006	0.009
5% Stacking Gel	2 ml	3 ml	6 ml
ddH ₂ O	1.46	2.19	4.38
40% Acrylamide: Bisacrylamide	0.25	0.375	0.75
1 M Tris-HCl, pH: 6.8	0.25	0.375	0.75
10% SDS	0.02	0.03	0.06
10% APS	0.02	0.03	0.06
TEMED	0.002	0.003	0.006

3.3. Antibodies

Table 3. 4: List of antibodies.

Name	Dilution	Source, Catalog No
Anti-HA	1:1000	Cell Signaling Technology (#C29F4)
Anti-FLAG	1:2000	Sigma-Aldrich (#F1804)
Anti-mouse, HRP	1:2000	Cell Signaling Technology (#7076)
Anti-Rabbit, HRP	1:1000	Thermo Fisher Scientific (#31460)

3.4. Oligonucleotide Sequences

Table 3. 5: List of PCR primer oligonucleotides.

Gene Name	Oligonucleotid Sequence	
	Forward	Reverse
AURKA	5'GCAGCTAGCATGCTCCTGGGGCGCCTGAC3'	5'ATGCTCGAGTCATTTGCCCCGAGGTAGATC3'
PRKCA	5'GCAGCTAGCATGGCTGACGTTTTCCCGGG3'	5'GTCCTCGAGTCATACTGCACTCTGTAAGA3'
H2BC11	5'GCAGCTAGCATGCCAGAGCCAGCGAAGTC3'	5'ATGCTCGAGTTACTTAGCGCTGGTGTACT3'
ILF2	5'GCAGCTAGCATGAGGGGTGACAGAGGCCGT3'	5'ATGCTCGAGTCACTCCTGAGTTCCATGCT3'
MCM3	5'GCAGCTAGCATGGCGGGTACCGTGCTGCTG3'	5'ATGCTCGAGTCAGATGAGGAAGATGATGC3'
HK1	5'GCAGCTAGCATGATCGCCGCGCAGCTCCT3'	5'ATGCTCGAGTTAGCTGCTTGCCTCTGTGC3'
PAICS	5'GCAGCTAGCATGGCGACAGCTGAGGTAC3'	5'ATGCTCGAGTTATAAATTACATTCTCTGAT3'
APMAP	5'GCAGCTAGCATGAGCGAGGCGGACGGGCT3'	5'ATGCTCGAGCTAAACAGCCTGGAGGCTGAG3'
HNRNPA2B1	5'GCAGCTAGCATGGAGAAAACTTAGAAAC3'	5'ATGCTCGAGTCAGTATCGGCTCCTCCAC 3'
CCT2	5'GCAGCTAGCATGGCGTCCCTTTCCCTTGC3'	5'ATGCTCGAGTTAACAGGGGTGGTGATCAGG3'
IMPDH2	5'GCAGCTAGCATGGCCGACTACCTGATTAGT3'	5'ATGCTCGAGTCAGAAAAGCCGCTTCTCAT3'

3.5. Equipment

Table 3. 6: List of equipment.

Agarose Gel Electrophoresis	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
Incubator (CO ₂)	Thermo Fisher Scientific, USA
Electrophoresis System	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	Standart Analog shaker, VWR, USA
Vortex	Scientific Industries, USA
Water Bath	Wisd, Germany
Magnetic Rack	DynaMag™-2 Invitrogen

4. METHODS

4.1. Molecular Cloning

4.1.1. Candidate Interactome PCR (Polymerase Chain Reaction) Amplification

For the amplification of the selected candidate interactors, Q5 High-Fidelity Polymerase (#M0491S, New England Biolabs (NEB) USA) was used. PCR reaction was prepared by adding 500ng U2OS cDNA to a PCR tube containing 5 μ l of 5X Q5 reaction buffer; 0.5 μ l of 10 mM dNTPs; 1.25 μ l of 10 μ M Forward and Reverse Primers; 0.25 μ l of Q5 High-Fidelity Polymerase in nuclease free water to a final volume of 25 μ l.

PCR conditions were as follows: initial denaturation at 98°C for 30 seconds; denaturation at 98°C for 10 seconds, annealing at 58°C for 15 seconds, elongation at 72°C for 30-90 seconds (depending on the size of the interactor) for 35 cycles; and final elongation at 72°C for 2 minutes, respectively.

Table 4. 1: PCR content.

PCR components	Volume in μ l
5X Q5 Reaction Buffer	5 μ l
10 mM dNTPs	0.5 μ l
10 μ M Forward and Reverse Primer	1.25 μ l
Template DNA	0.5 μ l (500 ng)
Q5 High-Fidelity DNA Polymerase	0.25 μ l
Nuclease free water	17.5 μ l
Final volume	25 μ l

Table 4. 2: PCR Conditions.

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

4.1.2. Agarose Gel Electrophoresis

Amplified interactors was run on 1% agarose gel prepared in 1X TBE buffer. For detecting nucleic acid in agarose gels, RedSafe nucleic acid staining solution was added to the agarose gel before the solidification. After solidification of agarose, DNA samples in 1X agarose loading dye was loaded into wells on agarose gel electrophoresis.

4.1.3. Digestion of PCR Products and Gel Purification

Amplified interactor coding DNAs were gel-purified using Nucleospin PCR Clean-up kit following the manufacturer's instructions. Cleaned DNA was eluted in 50µl of nuclease-free water. Interactor coding DNAs were then digested using NheI (#ER0975) and XhoI (#ER0695) or SalI, (#FD0644, all enzymes were purchased from Thermo Fisher Scientific, USA). These restriction sites had been added to the oligos which were used for amplifying interactor coding sequences. For digestion, 2000 ng of PCR product was mixed with 0.5µL of NheI and XhoI or SalI restriction enzymes and 1X digestion buffer. Digestion was performed at 37°C for 4 hours. Digestion samples were loaded onto 1% agarose gel to confirm the integrity.

4.1.4. Digestion of 3xHA-miniTurbo-NLS_pcDNA3 Plasmid and Isolation of the Large Fragment

3xHA-miniTurbo-NLS_pcDNA3 vector was digested with Nhe I and Sal I enzyme in a microcentrifuge tube containing 2µg plasmid DNA, 2µl restriction enzyme buffer (10X concentrate), 0.5µl restriction enzyme (Nhe I and Sal I), and DNase free water to make the volume 20µl. Digestion was incubated at 37°C for 4 hours then combined with 4 µl 6X loading buffer and run on 1% agarose gel.

Digested product was purified using Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit following the manufacturer's instructions. DNA was eluted into 50µl of nuclease-free water.

4.1.5. Ligation of Insert and Vector DNA

T4 DNA Ligase enzyme was used to ligate digested interactors (insert) and 3xHA-NLS_pcDNA3 (vector). The ligation reaction was adjusted at a molar ratio of 3:1 (insert:vector). A total of 10µl of ligation mixture was prepared using 150 ng of insert and vector DNA in 7 µl, 2µl of T4 DNA Ligase Buffer (5X), and 1µl of T4 DNA Ligase. Ligation reaction was incubated overnight at 4°C.

4.1.6. Transformation of Ligation Products into E. coli

For transformation, chemically competent cells of *Escherichia Coli* (DH5α strain) were used. Competent cell aliquots were removed from -80 °C freezer and thawed on ice. Approximately, 80µl of competent cells were mixed with 5µl of ligation product in a microcentrifuge tube. After gentle pipetting, the cells were incubated on ice for 25 minutes. Cells were then removed from ice and incubated in a water bath at 42°C for 1 minute, followed by incubation on ice for 2 minutes.

For cell recovery, 950 µl of warm Luria Broth (LB) was added onto the cells followed by incubation at 37°C in a shaker incubator at 200 rpm for 1 hour. At the end of the incubation, 100 µl of culture was spread on LB agar plates containing an appropriate antibiotic (ampicillin) in which the plasmid carried, and plates were incubated overnight at 37°C. Next day, single colonies were picked up and inoculated

to 3.5mL LB medium containing the 100µg/µl ampicillin in 15 ml conical tubes. Tubes were incubated overnight at 37°C and the bacteria were collected by centrifugation at 4000 rpm for 10 minutes. The supernatant was removed, and the pellet was stored for plasmid DNA isolation.

4.1.7. Plasmid DNA Isolation

Plasmid DNA isolation was performed using the NucleoSpin plasmid kit (Macherey-Nagel, Germany) according to the manufacturer's instruction.

Cell pellets were resuspended in 250 µl of Suspension Buffer (containing RNase), the same amount of Lysis Buffer was added to the tube and inverted gently 3-5 times. The mixture was incubated for 5 minutes followed by addition of 350 µl of Neutralization Buffer. After that, microcentrifuge tubes was centrifuged at 13,300 rpm for 10 minutes. The supernatant was transferred to the NucleoSpin Plasmid Column and centrifuged at 13,300 rpm for 1 minute. The columns were then washed with 600 µl of Wash Buffer by centrifuging at 13,300 rpm for 1 minute. The column was dried by centrifuging at 13,300 rpm for another one minute. DNA was eluted into 50 µl of 1X TE buffer (pH 8.5) by centrifugation at 13,300 rpm for 1 minute. The purity and concentration of the isolated plasmid was analyzed on the NanoDrop Spectrophotometer.

Candidate plasmids were digested with the appropriate restriction enzymes to analyze the presence of the insert. After plasmid validation, plasmids was isolated in large scale for cell culture studies.

4.2. Cell Culture

4.2.1. Maintenance of Cell Line

The human embryonic kidney (HEK) 293T cell lines were grown and maintained in Dulbecco's Modified Eagle medium (DMEM; 4.5g/L D-glucose) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (P/S), and 1% non-essential amino acids (NEAA) incubated at 37°C in a humidified cell culture incubator with 5% CO₂.

4.2.2. Cell Passaging and Cell Stocking

As a routine maintenance procedure, cells were sub-cultured when they reached to 80-90% confluency. For this, the medium was aspirated and cells were washed gently with 1X PBS. After washing with PBS, 0.05% Trypsin-EDTA was added to cover the surface of the adherent cells. The culture plate was incubated at 37°C until all cells were detached from the surface. Warm complete DMEM was added at a ratio of two volumes of trypsin to inhibit trypsin activity. Cells were taken into a 15 ml conical tube and centrifuged at 300 g for 5 minutes. The supernatant was discarded and the pellet was dissolved with complete medium. The cells were counted using hemacytometer. The appropriate amount of cells was then homogeneously seeded into a new 60 mm culture plate containing fresh medium and incubated in a 37°C cell incubator.

Dimethyl sulfoxide (DMSO) was used as a cryoprotectant to freeze the cells. For this purpose, 800 µl of cell suspension, 100 µl of Fetal Bovine Serum (FBS), and 100 µl of DMSO were added into the cryogenic store vials, respectively. After that, vials were incubated at -20 °C for 1-2 hours, at -80 °C for 1-2 days and then transferred to liquid nitrogen for long-term storage.

4.2.3. Generation of Stable Cell Line

For stable cell line generation, cells were seeded in 6 well plates and incubated overnight at 37°C. The following day, transformation of FLAG-PER2-FLAG construct was performed. For this, 1 µg of pENTER-FLAG-hPER2-FLAG was diluted in 150 µl of a transfection buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris.HCl (pH 7.5). Polyethyleneimine (PEI) from 1 mg/ml stock was added to the diluted DNA in 3:1 (PEI: DNA) ratio and incubated for 15 minutes at room temperature. After incubation, the DNA/PEI mixture was mixed with complete DMEM and added onto cells.

After 2-3 days of transfection, cells were selected by puromycin antibiotic treatment. For selection, cells were detached from the culture plate by trypsinization with 0.05% Trypsin-EDTA and washed gently with PBS. After collecting the cells, cells were resuspended in complete medium and seeded into a 96-well plate by serial

dilutions. After that, cells were incubated in medium containing 0.5 µg/ml puromycin to select stable clones. Stable clones expressing FLAG-PER2-FLAG derived from single cells were picked up from the 96 well plate and passaged into larger well plate for screening of expression by western blot using an anti-FLAG antibody (#F1804, Sigma Aldrich). As a result, one of the best expressing clones was selected for further studies.

4.2.4. Transfection of Interactor Plasmids into the HEK293T- FLAG-PER2-FLAG Stable Cell Line

Interactor constructs were transiently transfected to the established cell line expressing FLAG-PER2-FLAG. For transfection, interactor plasmids were mixed with PEI as in the previous section. After DNA:PEI mixture incubation for 15 minutes at room temperature, the DNA/PEI mixture was mixed with complete DMEM and added to onto cells. After transfection, cells were maintained in a cell incubator at 37°C for two days. Then, cells were harvested for co-immunoprecipitation (Co-IP) experiments. Notably, each transfection experiment was performed in 2 replicates.

4.3. Co-Immunoprecipitation

4.3.1. Cell Harvesting

HEK293T-FLAG-PER2-FLAG cells transfected with interactor plasmids were harvested by scraping from the culture plate 48 hours after transfection using a sterile cell scraper. Cells were collected in a microcentrifuge tube prechilled on ice and pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C. The pellet was washed twice with ice-cold 1X PBS buffer and then, the PBS was aspirated. Cell pellets were placed on ice for the lysis step.

4.3.2. Cell Lysis and Sample Preparation

Cell pellets from the previous section were lysed in NP-40 lysis buffer (Thermo Fisher Scientific, cat no: FNN0021) containing 1X protease inhibitor (PI). For cell

lysis, 900-1000 µl of ice-cold non-denaturing NP-40 lysis buffer was added onto the pellets and incubated on ice for 30 minutes. Cell lysates were mixed repeatedly every 5 minutes during this incubation. At the end of the incubation, the lysates were centrifuged at 13,300 rpm for 30 minutes at 4°C. The supernatant containing the cleared proteins was transferred to new 1.5 ml microcentrifuge tubes and placed on ice.

For Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE), 45-50 µl of the supernatant was mixed with 15µl of 4X loading dye (containing 10% β-mercaptoethanol) to reach a final concentration of 1X as input samples. The input samples were kept at -20 °C until SDS-PAGE.

4.3.3. Immunoprecipitation of Cell Lysates with Anti-FLAG Antibody-coated Magnetic Beads

For immunoprecipitation, 1.5 ml microcentrifuge tubes were initially placed on the magnetic rack. Then, 10 µl of MagnaBind Protein G Beads (Thermo Fisher Scientific, cat no: 21349) per sample were added into each tube. The beads were washed three times with 400 µl of NP-40 lysis buffer. After final washing, 200 µl of NP-40 lysis buffer was added to the washed beads. After that, 1µg of anti-FLAG antibody (Sigma Aldrich, cat no: F1804) or control IgG (Cell Signaling Technologies, cat no: 7076S) per sample was added to the beads. Tubes were incubated for 1 hour at 4°C with rotation. At the end of the incubation, 400 µl of cell lysate was added to the tubes and incubated overnight at 4°C with rotation.

The next day, microcentrifuge tubes were placed on the magnetic rack and the NP-40 lysis buffer was removed without disturbing the beads. Then, the beads were washed 4 times with 500-700 µl of NP-40 lysis buffer. After removing the washing buffer, 70 µl of 1.5X Laemmli buffer containing 10% β-mercaptoethanol was added onto the beads. The tubes were removed from the magnetic rack and briefly spun. Input samples and immunoprecipitated complexes were separated using SDS-PAGE and analysed by western blot using suitable antibodies.

4.4. Western Blot

4.4.1. SDS-PAGE Gel Preparation

SDS-PAGE glasses were cleaned with 70% ethanol and placed in a gel-cast frame with thick glass back (1.5 mm) and thin glass plate front. Casted glasses were then clamped to the casting stand. Gels (containing separation and stacking parts) were prepared according to the recipe given in Table 3.2. First, 10 ml of separation gel prepared in a 15 ml conical tube was prepared and then poured between the glasses. Finally, polymerization initiator ammonium persulfate (APS) and TEMED were added to the solution. Sufficient isopropanol was rapidly poured over the gel to protect gels from oxygen and cause a faster polymerization. While waiting for the separation gel to polymerize, a stacking gel (5%) was prepared without adding APS and TEMED. Before pouring the stacking gel, the gel was washed with double-distilled water to remove isopropanol and whatman paper was used to absorb any remaining water in the glasses. APS and TEMED were added to the stacking gel and quickly poured over the separation gel. Immediately afterward, a 15-well comb, previously cleaned with ethanol, was gently inserted. After the gel was polymerized, the comb was removed, and the wells were washed with double-distilled water. To store the gel for a long time (up to 1 week), the gel without removing the comb was wrapped in whatman paper soaked with water and kept at 4°C.

4.4.2. Sample Preparation for SDS-PAGE

Protein lysates were thawed at room temperature and then heated at 95°C for 10 minutes and spun. Alongside with and PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Fisher, Cat. No. 26617) samples were separated using SDS-PAGE.

4.4.3. SDS-PAGE

After slowly removing the comb from the polymerized gel, the wells were washed with double distilled water. The glass plates containing the gel were placed in an electrode assembly and a second gel or plastic buffer dam was placed against it.

The gels were then placed inside the tank. The inner chamber of the tank was filled with 1X running buffer diluted with dH₂O from 10X stock running buffer. Protein samples and ladder treated as in previous section were loaded into wells in sufficient quantities (10 µl from input and 20 µl from precipitated complexes, respectively). The tank was connected to the power supply and ran at 80V until proteins reached the separation gel (approximately 15 minutes). Then the voltage was increased to 120-150 V. The running time of the gel was adjusted according to the size of the protein of interest. The power was turned off after the proteins were separated on the SDS-PAGE.

4.4.4. Western Blot

Electrotransfer of proteins from the polyacrylamide gel to the nitrocellulose membrane was carried out using the Trans-blot Turbo Transfer System (Biorad Transblot Turbo Transfer System, Cat. No. 1704150). The gel was carefully removed from the glass plates with a plastic spatula. The stacking gel was cut away and the remainder of the gel was incubated for several minutes in 1X transfer buffer (containing 20% methanol). Likewise, one nitrocellulose membrane cut to the same size as the gel and 4 blotting papers were incubated in 1X transfer buffer for approximately 10 minutes.

A transfer sandwich was prepared with buffer-soaked components. The filter paper-gel-membrane-filter paper layers are sandwiched between two plates that form an anode (+) and a cathode (-) when an electric field is applied. The membrane should be closest to the anode (+) as the protein with negative charge would move towards the anode. Air bubbles were removed using the roller. The tank was then slightly filled with 1X transfer buffer. Semi-dry blotting was performed at 12 V constant current voltage (0.1 to ~0.4 A) for 60 minutes.

4.4.5. Antibody Incubation

The membrane was removed from the transfer apparatus and washed with 1X TBS-T (Tris-buffered saline plus 0.1% Tween 20). The membrane was then taken up into 20 ml of blocking solution (5% non-fat dry milk in TBS-T) in a container to

prevent binding of non-specific proteins and incubated on an orbital shaker for one hour at room temperature.

At the end of the blocking incubation, the membrane was incubated with the primary antibody overnight at 4°C. The primary antibodies, anti-FLAG antibody (1:2000, Sigma Aldrich, Cat. No. F1804) or anti-HA antibody (1:1000, Cell Signalling Technology (CST), Cat. No. C29F4), were diluted in the blocking solution containing 0.01 % sodium azide to prevent microbial contamination.

Following the primary antibody incubation step, the membrane was washed with 1X TBS-T for 30 minutes (5-15-5-5 minutes, respectively). The membrane was then incubated with the secondary antibody for one hour at room temperature with gentle shaking. An anti-mouse-HRP antibody (1:2000, CST, Cat. No. 7076S) for anti-FLAG antibody and an anti-rabbit-HRP antibody (1:1000, Thermo Fisher Scientific, Cat. No. 31460) for anti-HA antibody were used as the secondary antibody. The membrane was washed again with TBS-T for 30 minutes.

4.4.6. Chemiluminescence Imaging of the Membrane

After the membrane was removed from TBS-T, WesternBright Sirius HRP substrate (Advansta Inc., CA, USA) was applied to the membrane and incubated for 2 minutes. Chemiluminescence was detected and recorded with a Molecular Imager ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were calculated by Image Lab software.

5. RESULTS

The main purpose of this thesis study is to validate the interactions of PER2, which were previously identified in our laboratory by BioID method [Gul et al., 2022]. In BioID method, candidate PER2 interactors were identified by mass spectrometry after biotinylating proteins in proximity of PER2-BioID2 fusion protein.

To perform co-immunoprecipitation (co-IP) studies, a stable HEK293T cell line was established to overexpress FLAG-tagged PER2 protein (FLAG-PER2-FLAG). Interactors, which were cloned into the 3xHA-NLS_pcDNA3 vector for expression with the 3xHA tag, were transiently expressed in the HEK293T-FLAG-hPER2-FLAG stable cell line. After 48 hours following the transfection of interactors, PER2 complexes were immunoprecipitated using anti-FLAG antibody-coated magnetic beads. The presence of HA-tagged interactors in the immunoprecipitated complexes was tested by immunoblotting.

5.1. Establishment of HEK293T-hPER2-FLAG Cell Line

Cloning was performed to generate the pENTER-FLAG-hPER2-FLAG plasmid (shown in Figure 5.2) which expresses the FLAG tags at both N- and C-termini of human PER2 (hPER2) protein. First, the coding sequence for hPER2 was amplified from the human bone osteosarcoma epithelial cell line (U2OS) cDNA using primers Per2cl_1(5'GCAGGTACCGCTAGCGCCACCATGGATTATAAGGATGACGACATAAAAATGGATACGCGGAATTTTC-3') and Per2cl_2 (5'CGTACCGGTGTCGACCTACGTCTGCTCTTCGATCCTGTGA-3'). The PCR-amplified FLAG-hPER2 gene region was then digested with NheI and SalI restriction enzymes and cloned into the NheI and XhoI restriction enzyme sites of the pENTER plasmid (Vigene Biosciences). The strategic description of this cloning is shown in figure 5.1.

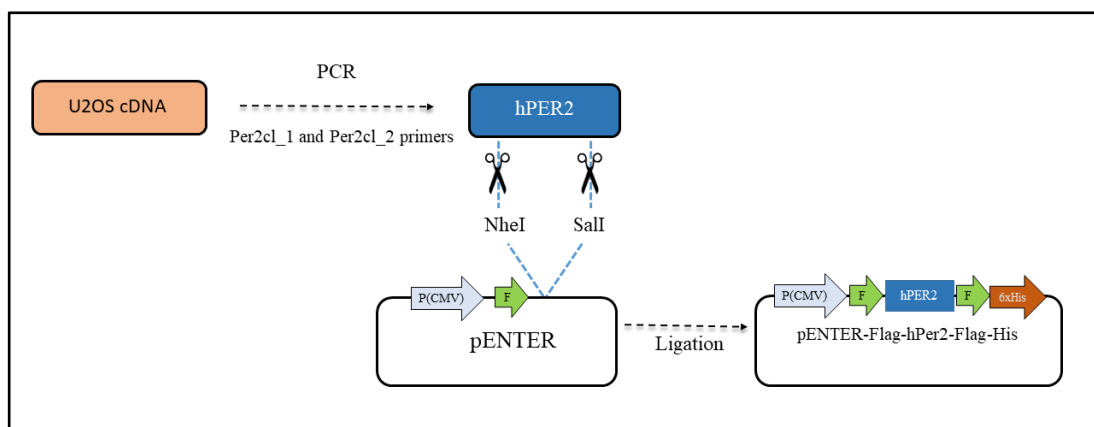


Figure 5.1: Cloning Strategy of pENTER-FLAG-hPER2-FLAG plasmid. PCR-amplified FLAG-hPER2 coding sequence was inserted into pENTER plasmid upstream of FLAG sequence.

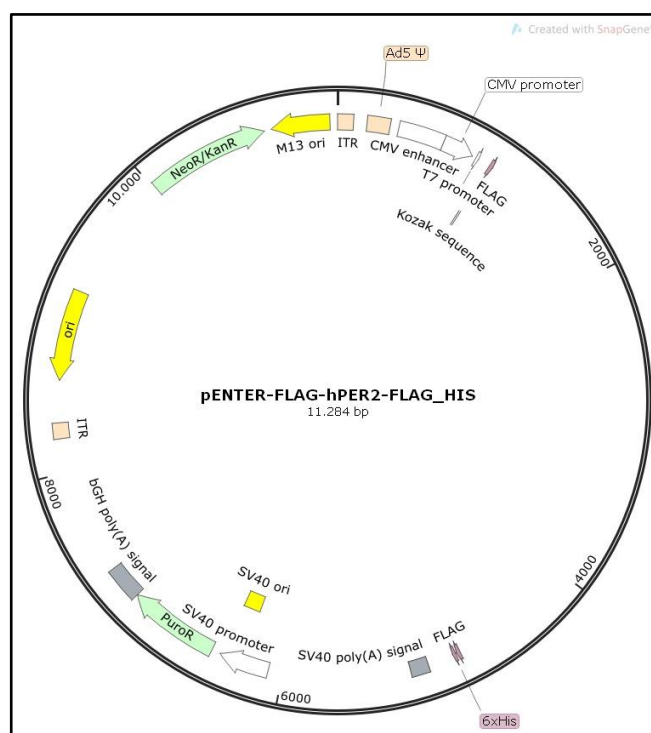


Figure 5.2: The map of the pENTER-FLAG-hPER2-FLAG plasmid. The map was generated using SnapGene Viewer Program.

Ligated plasmids were transformed into competent *E. coli* DH5-alpha cells. Then, plasmid DNA isolation was performed as described in 4.1.7. To screen the candidates, the presence of the right inserts was screened by BamHI restriction enzyme digestion. Digestion products were run on a 2% agarose gel. As shown in Figure 5.3, a 331 bp band was expected.

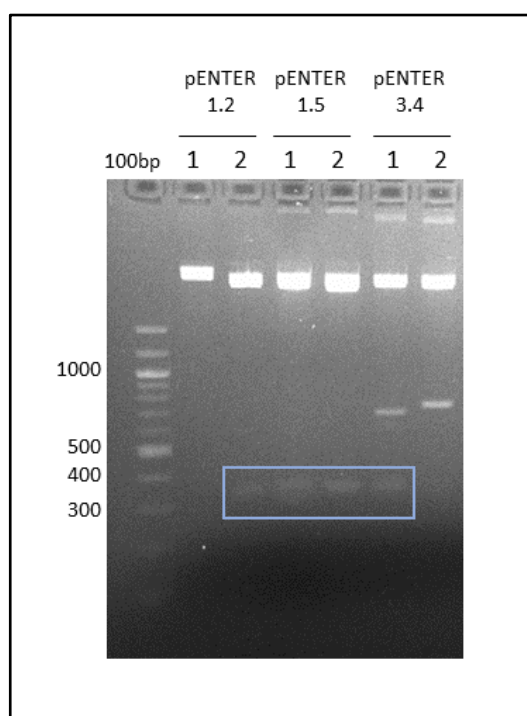


Figure 5.3: BamHI restriction enzyme digestion analysis of candidate pENTER-FLAG-hPER2-FLAG plasmids. The clones encircled with the blue box were selected for transfection to HEK293T cell line. Samples were run on 2% DNA agarose gel. 100 bp was used as a DNA Ladder.

The plasmids with correct inserts were transfected into the HEK293T cell line with PEI transfection reagent. Cells were harvested and analyzed by western blotting using anti-FLAG antibody to see their expression levels. The weight of the FLAG-tagged hPER2 protein was expected to be 170 kDa (Figure 5.4.a). The clone pENTER 1.5 C.1 expressing FLAG-tagged-PER2 (F-PER2-F) was selected for stable expression. Stable clones from transfected HEK293T cells were then obtained by puromycin selection. According to the result of immunoblot, the clone 5 (C5) with the highest expression level was selected among the clones (Figure 5.4.b) and used for co-IP experiments.

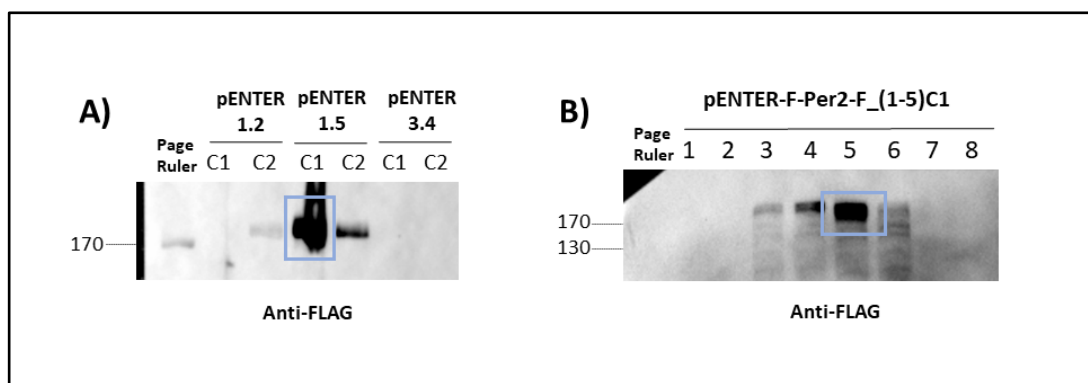


Figure 5.4: Screening of stable FLAG-PER2-FLAG expressing clones. A) The pENTER 1.5 C1 clone giving a strong band of 170 kDa was selected for stable transfection. 6% SDS-PAGE gel was used to run the samples. B) pENTER 1.5 C1 was propagated using the serial dilution method while treating with puromycin to kill untransfected cells. 8% SDS-PAGE gel was used to run the samples. The clone of Lane 5 which showed the highest expression level was selected for further experiments. Samples were analyzed using an anti-FLAG antibody. The marker used was the PageRuler Prestained Protein Ladder.

5.2. Determination of Candidates and Preparation of Interactor Expressing Constructs

5.2.1. Determination of the Candidates

In a previous study conducted in our laboratory, candidate proteins (prey) interacting with the PER2 (bait) protein were identified as PER2 interactome by the BioID proximity-based biotin labeling method combined with mass spectrometry [Gul et al., 2022]. The candidate proteins were then scored with the SAINT (Significance Analysis of INTeractome) calculation tool, which calculates the probability of protein-protein interaction [Teo et al., 2014]. For this thesis, 4 proteins were selected from candidate proteins with a Saint EXPRESS score of 1. Figure 5.5 was generated by the String App and Cytoscape 3.7.2 bioinformatics tool. Candidate proteins with a Saint EXPRESS score of 1 were mapped according to their localization in the cell (nucleus, cytoplasm and extracellular).

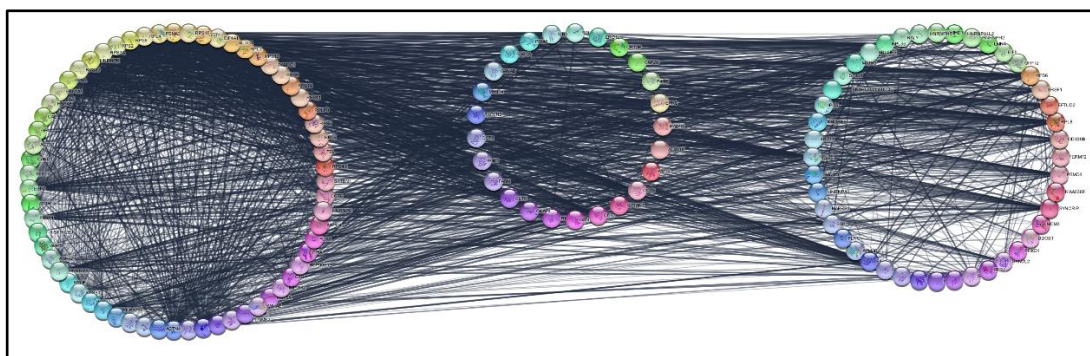


Figure 5.5: Map of PER2-associated candidate proteins with a Saint EXPRESS score of 1. Candidate proteins were separated into nucleus, cytoplasm, and extracellular groups according to their localization sites. The map was created with the String App and the Cytoscape 3.7.2 bioinformatics tool.

5.2.2. Preparation of Plasmids Expressing PER2 Interactor Candidates

First, selected PER2 interactor candidates were amplified from U2OS cDNA using Q5 polymerase (NEB) (Figure 5.8). Candidate interactors were cloned into the 3xHA-NLS miniTurbo_pcDNA3 (#107172) expression plasmid as shown in figure 5.6 to express the interactors with 3xHA tag at the N-terminus. (1) Large fragment of the 3xHA-miniTurbo-NLS-pcDNA3 plasmid was excised from gel after digestion with NheI and XhoI to remove miniTurbo cassette. (2) The amplified interactor sequences were digested with the enzymes NheI and XhoI or SalI. (3) The digested interactor coding sequences were then ligated into NheI-SalI site of 3XHA-NLS_pcDNA3 plasmid. The strategic description of this cloning is shown in Figure 5.7.

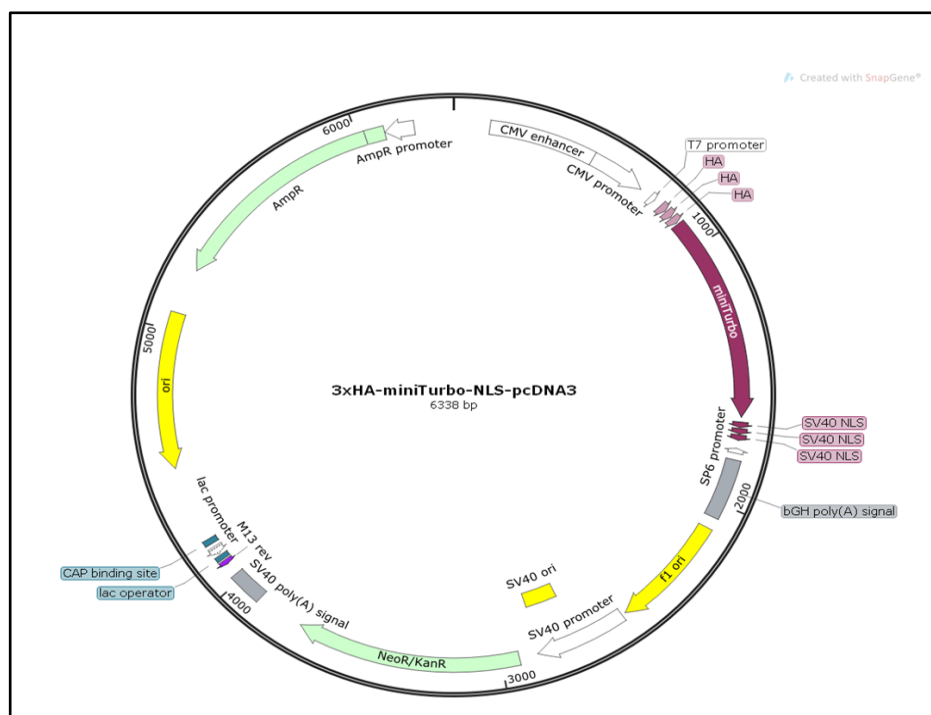


Figure 5.6: Map of the 3xHA-miniTurbo-NLS_pcDNA3 vector used for cloning of interactors. The map was generated using SnapGene Viewer Program.

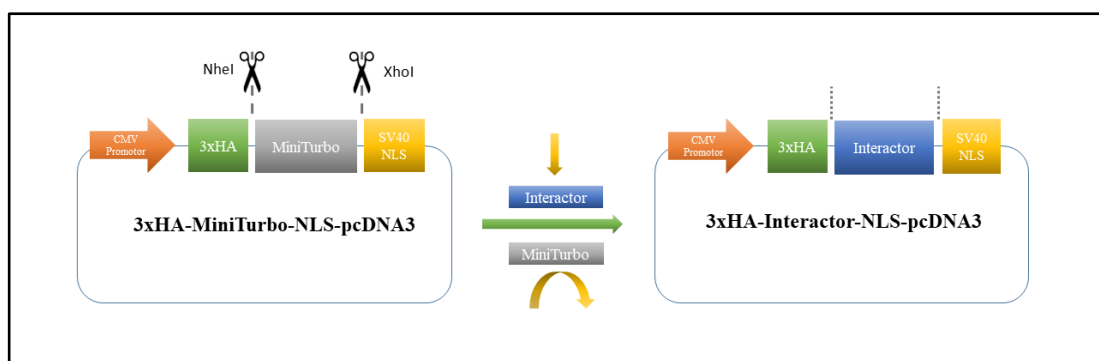


Figure 5.7: Strategy for making 3xHA-Interactor-pcDNA3 plasmids.

Interactor genes were amplified from U2OS cDNA using the primers in Table 3.5. Amplification products were isolated from 1% agarose gel using Nucleospin PCR Clean-up kit (Figure 5.8). Purified PCR products were digested from NheI and XhoI or SalI restriction enzyme sites and the enzymes were heat-inactivated at the end of the restriction digestion. To prepare the vector plasmid, the 3xHA-NLS-miniTurbo_pcDNA3 expression vector was digested with NheI and SalI enzyme restriction sites and run on agarose gel. The large fragment without the miniTurbo expressing cassette was then excised from the gel under 366 nm black light and

purified using the Macherey-Nagel Nucleospin Gel and PCR Clean-up kit. The interactors were mixed with digested 3xHA_pcDNA3 plasmid and ligated using T4 DNA ligase.

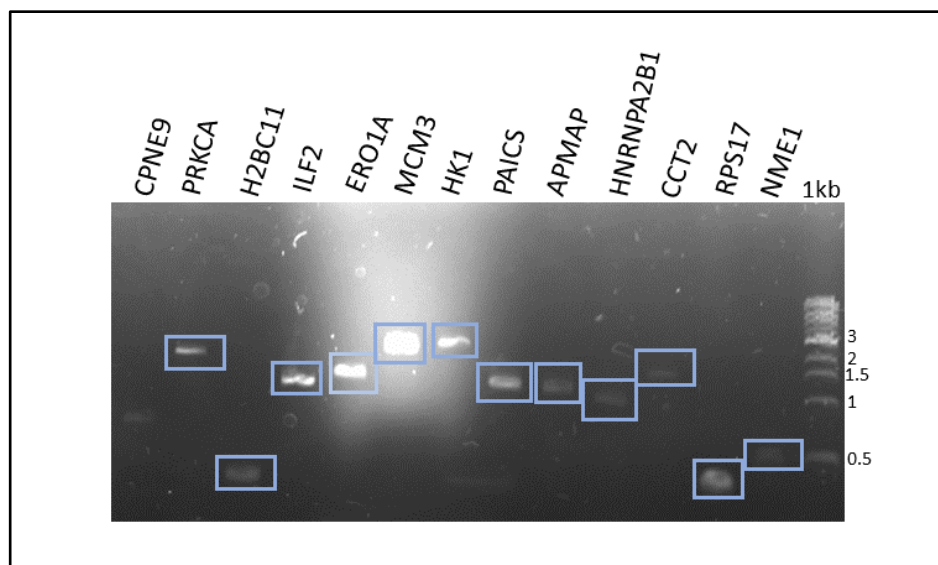


Figure 5.8: Analysis of PCR amplified interactors. Amplicons were run on 1% DNA agarose gel. Expected bands are encircled with a blue box. 1 kb was used as a DNA Ladder.

After ligation, 5 μ l of ligation product was transformed into competent DH5 α strain. Two separate colonies were selected for each interactor and transferred to 3ml LB broth containing ampicillin. Plasmid DNAs were isolated as described in section 4.1.7. Then, plasmid DNAs were digested with SacI enzyme and run on 1% agarose gel to confirm the presence of the interactor coding sequences (Figure 5.9a and b).

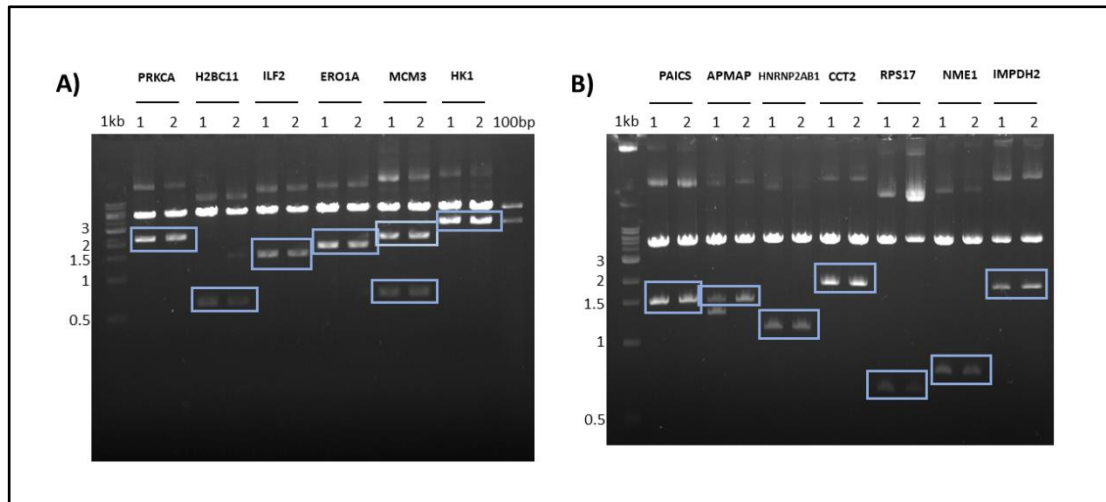


Figure 5.9: SacI restriction enzyme digestion assay to confirm the presence of interactor sequences in plasmids after ligation. A) The expected bands for interactors ranging from PRKCA to HK1 were as follows: 2339 bp for PRKCA, 701 bp for H2BC11, 1493 bp for ILF2, 1542 and 185 bp for ERO1A, 1998 and 749 bp for MCM3, and 2493 bp for HK1. 100bp and 1 kb were used as DNA Ladders. B) The expected bands for candidate interactors from PAICS to IMPDH2 were: 1628 bp for PAICS, 1580 bp for APMAP, 1391 bp for HNRNP2AB1, 1937 bp for CCT2, 737 bp for RPS17, 863 bp for NME1, and 1873 bp for IMPDH2, respectively. 1 kb was used as the DNA Ladder. Samples were run on a 1% DNA agarose gel.

5.3. Expression of Interactors in the Stable HEK293T Cell Line

Each interactor cloned into the 3xHA-pcDNA3 vector for expression was transiently transfected with the PEI transfection reagent into the HEK293T stable cell line expressing FLAG-hPER2-FLAG. Cells were harvested by scraping 48 hours after transfection, and overexpression of interactors was analyzed by western blot. Samples were run on 8% SDS-PAGE gel and analyzed by western blot using anti-HA antibody (Figure 5.10). Interactors whose expression were validated were then used in co-IP experiments.

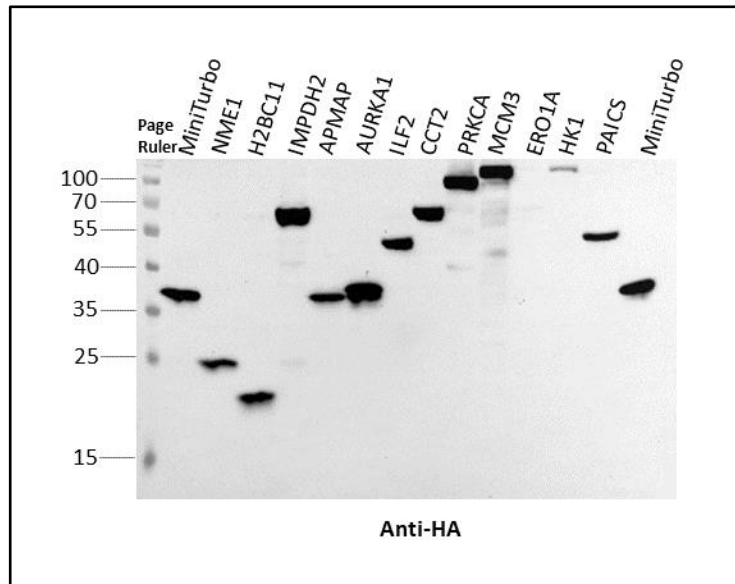


Figure 5.10: Western blot analysis for the expressions of interactors in the HEK293T-FLAG-hPER2-FLAG stable cell line. MiniTurbo expression was used as a control. The expected bands for interactors ranging from MiniTurbo to PAICS are as follows: 30 kDa, 19 kDa, 14 kDa, 57 kDa, 46 kDa, 22 kDa, 43 kDa, 60 kDa, 74 kDa, 90 kDa, 52 kDa, 100 kDa, and 48 kDa. 12% SDS-PAGE gel was used to run the samples. The marker used was the PageRuler Prestained Protein Ladder.

5.4. Validation of Interactions Between PER2 and Candidate Interactors by Co-immunoprecipitation

Co-immunoprecipitation was performed to confirm the interactions between PER2 and candidate interactors identified by mass spectrometry analysis. First, HEK293T-FLAG-hPER2-FLAG was transfected with the plasmids to express HA-tagged interactors, and the cells were then harvested after 48 hours. A portion of the cell lysates was saved to indicate equivalent input. The remaining lysate was incubated overnight with a mixture of anti-FLAG antibody or non-specific control IgG antibody on protein G-magnetic beads for immunoprecipitation procedure. The next day, proteins that did not interact with PER2 were washed away 4 times with lysis buffer using a magnetic stand. After final wash, the beads were resuspended in 1.5X Laemlli buffer. To analyze the immunoprecipitated samples, 5% of the input sample and 30% of the immunoprecipitated complexes (co-IP samples) were run on the SDS gel. To confirm FLAG-hPER2-FLAG precipitation, it was immunoblotted with anti-FLAG antibody. The presence of HA-tagged interactors in the FLAG-PER2-FLAG/anti-

FLAG antibody/magnetic bead complex was analyzed by western blot using anti-HA antibody.

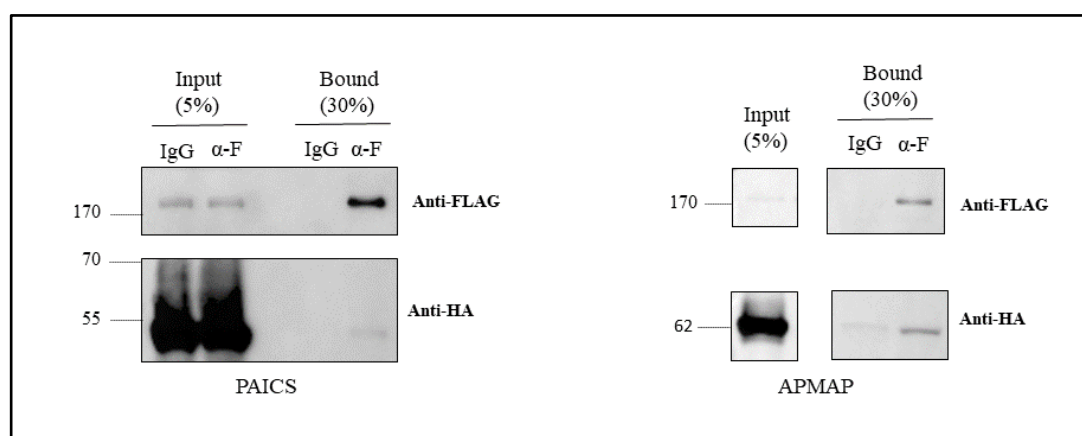


Figure 5.11: Western blot analysis of co-IP samples of PAICS and APMAP proteins. Cell lysates of HEK293T-FLAG-hPER2-FLAG which were transfected with plasmids to express HA-tagged interactors were incubated with anti-FLAG antibody-coated magnetic beads (or IgG as control) to precipitate target protein and Western blotting was performed with anti-FLAG and anti-HA antibodies. Input represents the sample from total protein lysate, bound samples represent immunoprecipitated samples. The control used was IgG, and α-F represents an anti-FLAG antibody. 8% SDS-PAGE gel was used to run the samples. The marker used was the PageRuler Prestained Protein Ladder.

In western blot analysis with anti-FLAG antibody, a band above 170 kDa was observed in input and co-IP samples. In western blot analysis with anti-HA antibody, it was observed that HA-tagged PAICS and APMAP recombinant proteins were precipitated by binding to FLAG-hPER2-FLAG protein, the absence of the same band in the IgG bound lane indicated the specificity of the interaction.

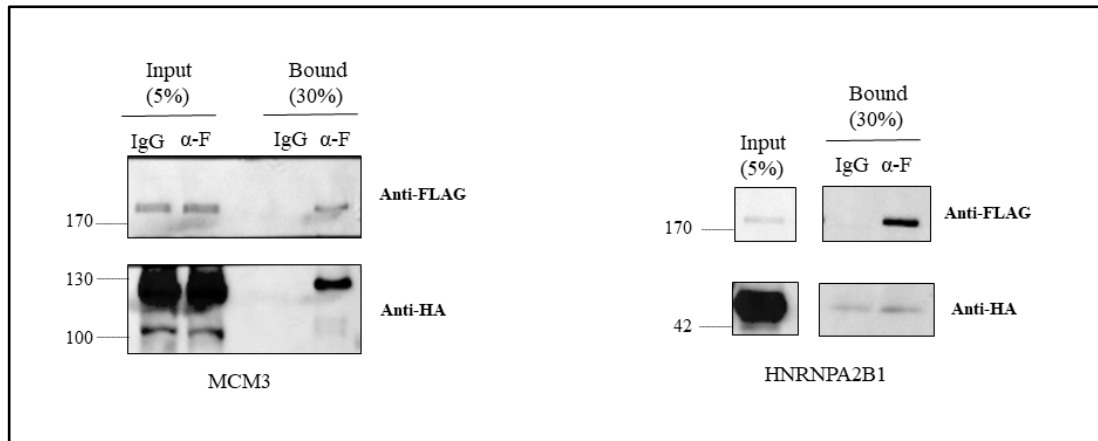


Figure 5.12: Western blot analysis of co-IP samples of MCM3 and HNRNPA2B1 proteins. Cell lysates were incubated with anti-FLAG antibody-coated magnetic beads (or IgG as control) to precipitate the hPER2 complex and Western blotting was performed with anti-FLAG and anti-HA antibody. Input represents the sample from total protein lysate, bound samples represent immunoprecipitated samples. The control used was IgG, and a-F represents an anti-FLAG antibody. 8% SDS-PAGE gel was used to run the samples. The marker used was the PageRuler Prestained Protein Ladder.

In western blot analysis with anti-FLAG antibody, a band above 170 kDa was observed as FLAG-hPER2-FLAG. In western blot analysis with anti-HA antibody, it was observed that HA-tagged MCM3 and HNRNPA2B1 recombinant proteins were precipitated with FLAG-hPER2-FLAG.

6. DISCUSSION

The circadian clock regulates physiological and biochemical activities of organisms, and its disruption is associated with many diseases such as cancer, diabetes, and obesity. At the molecular level, the circadian clock is generated by a TTFL mechanism. The positive arm of TTFL contains transcription factors CLOCK and BMAL1, while the negative arm contains PERIOD and CRYPTOCHROME. The *PER2* clock gene is essential for the robust maintenance of the circadian rhythm. Any disruption in *PER2* expression alters circadian oscillation. The pathways that are regulated by or interact with the circadian clock are controlled through transcriptional regulation as well as PPIs. Thus, it is important to study PPIs of the biological processes in which the circadian clock is involved to find out the interaction between the circadian clock and other biological pathways.

PPIs are critical for cells to survive, grow, divide, differentiate and perform their specific functions. These interactions regulate intracellular and intercellular signaling, play a role in cell cycle control, regulate metabolism, and affect gene expression. Advances in technology have led to the identification of an increasing number of PPIs and the development of a more comprehensive network of interactome. Recently, proximity labeling-based techniques have replaced traditional approaches, such as immunoprecipitation followed by MS analysis and yeast-two hybrid analysis, which were the primary means of characterizing PPIs so far. Proximity labeling-based techniques operate on the principle of using an enzyme fused to a protein of interest to covalently modify nearby proteins. Subsequently, the labeled proteins can be purified and analyzed by mass spectrometry (MS). The kinetics of the labeling process is dependent on the type of enzyme used. Biotin ligases (BioID/BioID2, miniTurbo, TurboID) and peroxidases (APEX/APEX2, HRP) are the most used enzyme classes in proximity labeling techniques. The labeling efficiency of the enzymes varies from 10 minutes to 24 hours. In addition, this technique offers a highly efficient approach as it resists harsh conditions that disrupt protein-protein interactions or integrity of organelles once the labeling is complete. On the other hand, one of the limitations of this approach is the lack of knowledge regarding the nature of the interaction between the proteins of interest. Because it may label proteins in proximity even the proteins that do not interact with the bait. Therefore, the (candidate) interactors identified by

proximity-dependent labeling should be confirmed by classical methods such as co-IP.

In a previous study conducted in our laboratory, the BioID2 enzyme-based BioID technique was used to identify the interactome of the PER2 protein, providing a long labeling window [Gul et al., 2022]. BioID2 randomly biotinylates proteins within a 10 nm radius after biotin is added. The interacting proteins were identified by MS after enrichment with streptavidin magnetic beads of the samples from U2OS osteosarcoma cell line that expressed the PER2 protein fused to the BioID2 enzyme. The SAINT (Significance Analysis of INteractome) statistical calculation tool was used to select important interactors from the MS Data where the Saint score =1 indicates strong interaction. As a result of SAINT analysis, a list of proteins which interacted with PER2 with a high probability was created. In this thesis, our aim was to confirm the physical interactions of some of the proteins with SAINT Express score = 1 from the PER2 interactome using the co-immunoprecipitation (co-IP) method. The Co-IP assay is a simple and inexpensive technique in which interacting partners are immunoprecipitated using an antibody specific for the protein of interest (bait) from cell lysate. The cell line used in this thesis was the human embryonic kidney 293T (HEK293T) cell line, which is relatively easy to culture compared to the U2OS osteosarcoma cell line. We cloned 11 candidate interactor genes selected for this study and tagged them with HA-tag. HA-tagged interactors were then transiently transfected into the HEK293T- FLAG-PER2-FLAG cell line. For the co-IP study, we used anti-FLAG antibody alongside with anti-IgG antibody as control. We analyzed PER2 and interactors by western blot using anti-FLAG antibody and anti-HA antibody, respectively.

Considering the functions of HNRNPA2B1, MCM3, PAICS, APMAP proteins in the interactome list, we hypothesized that these proteins might interact with PER2 while the others were studied by other members of the laboratory. Therefore, we validated interactions by co-IP method by selecting these proteins. The first of these proteins, heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), is a member of the RNA-binding protein family involved in the regulation of gene expression. These proteins are associated with pre-mRNA and are involved in functions such as pre-mRNA processing, mRNA metabolism and transport. The HNRNPA2 and HNRNPB1 proteins are involved in packaging, alternative splicing, and cytoplasmic RNA trafficking, translation, and stabilization of nascent mRNA.

HNRNPA2 and HNRNPB1 also appear to function in telomere maintenance, cell proliferation and differentiation, and glucose transport [Dreyfuss et al., 2002], [Geuens et al., 2016]. One study showed that while the circadian clock protein PER regulates the circadian rhythm, cytoplasmic trafficking is important for the functioning of a regular rhythm, and disruptions of cytoplasmic trafficking of PER cause circadian sleep disorders [Beesley et al., 2020]. Since the circadian clock has a regulatory effect on these processes [McGlinchy et al., 2012], [Torres et al., 2018], we tested their interaction with PER2 with co-IP (Figure 5.12). Here, although the expression level of the interactor was strong, there was a weak interaction in the co-IP.

Another protein on the list is DNA replication licensing factor MCM3 (MCM3), one of the highly conserved mini-chromosome maintenance proteins (MCM) that plays an important role in initiating eukaryotic genome replication. The hexameric protein complex formed by MCM proteins is an essential component of the pre-replication complex (pre-RC) and may be involved in the formation of replication forks and activation of other DNA replication-related proteins. In recent studies, it has been found that MCM3 is associated with the proliferation of cancer cells [Gato et al., 2018], [Nodin et al., 2012], [Løkkegaard et al., 2021]. It is thought that MCM3 and PER2 may meet at a common point in terms of possible interaction, cell proliferation and apoptosis, and cell cycle regulation. A strong interaction of MCM3 was observed in the co-IP study (Figure 5.12).

Phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) is a multifunctional enzyme involved in the biosynthesis of nucleotides. PAICS expression is upregulated in several types of cancer and has been shown to be associated with resistance to chemotherapy [Agarwall et al., 2020]. It is known that the expression levels of Per2 decrease in most cancer types, and it has a tumor suppressor function by suppressing cell proliferation [Fu et al., 2002]. These two proteins appear to have different functions in the pathways that lead the cell to proliferate or metastasize. According to our co-IP results, a weak interaction between PAICS and PER2 was confirmed. (Figure 5.11). This suggests that it might come with another protein that interacts with PER2 or just weakly interacts with PER2.

Adipocyte plasma membrane-associated protein (APMAP) is a protein associated with the plasma membrane of adipocytes, which are fat-storing cells in the body. APMAP has been implicated in a variety of biological processes, including

glucose uptake, insulin sensitivity, and energy metabolism. Studies have shown that APMAP is upregulated in mouse and human adipocytes and in white adipose tissue (WAT) of obese mice and is a target of peroxisome proliferator-activated receptor gamma (PPAR γ) [Bogner-Strauss et al., 2010]. Another study showed that PPAR γ plays a role in proinflammatory activation of Per1/2 disrupted macrophages [Xu et al., 2014]. Our study, which confirmed that PER2 interacts with APMAP (Figure 5.11), suggests that PPAR γ -PER2-APMAP can regulate inflammatory responses in adipocytes.

In this study, some of the proteins in the PER2-interacting protein list created in line with the MS results were validated with co-IP technique. Some of the proteins we studied showed high interaction, while others showed relatively weaker interactions. This may be due to indirect interactions between that interactor and PER2 or just low affinity of the interaction. The proteins we selected for co-IP mostly function in the nucleus. After PER2 protein is expressed, it forms a dimer structure in the cytoplasm with its partner CRY and translocates to the cell nucleus, while its amount increases in the cell depending on the circadian time and decreases to a minimum in the morning hours. The weak interactions here may be due to interactions of these proteins with PER2 at different times within the cell, or it may be an indirect rather than a direct interaction. Nuclear Magnetic Resonance (NMR) and Surface Plasmon Resonance (SPR) experiments can be performed to examine the interactions of these proteins in more detail. These techniques allow us to learn more about the structure of protein complexes, their conformational changes, interaction sites, and the real-time kinetics of proteins.

Confirmation of the physical interaction between PER2 and candidate interactors by co-IP method will enable the discovery of new pathways involving the circadian clock and the expansion of the list of genes controlled by the circadian clock. The discovery of interactions between the circadian clock and the new biological pathways will allow to elucidate the cellular processes by these new biological pathways. In addition, it can be thought that the clock may be related to other pathways in which the mentioned pathways are indirectly or directly involved.

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BIOGRAPHY

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APPENDICES

Appendix A: Protein and DNA Markers

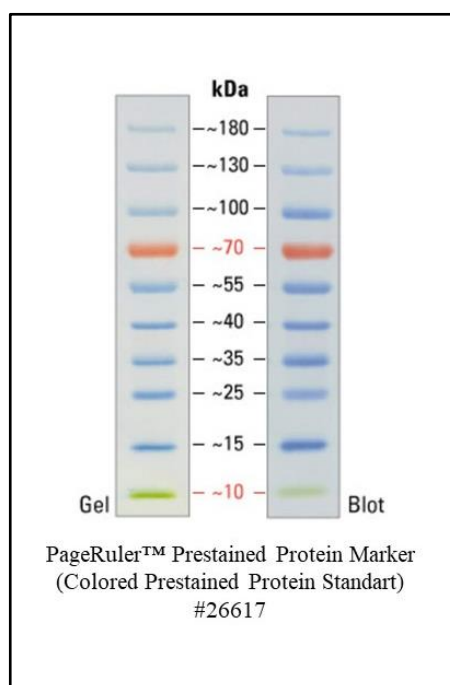


Figure A1.1: Protein Ladder.

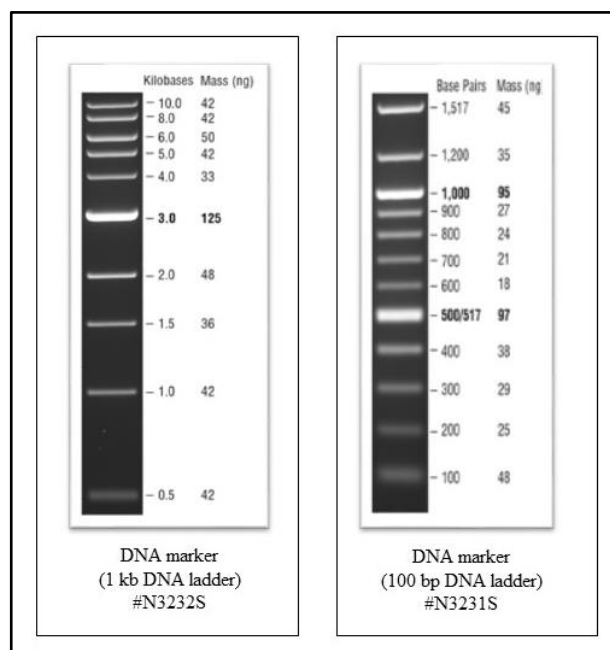


Figure A1.2: DNA Ladder.

Appendix B: List of PER2 interactors generated by Saintexpress Analysis

Table B1.1: A list of PER2 interactors was created using SAINTexpress software (saint score =1).

Gene Name	SaintScor	Gene Name	SaintScore	Gene Name	SaintScore
PC	1	AURKAIP1	1	HSD17B10	1
TAGLN2	1	GSN	1	FLNA	1
PCCA	1	SYNCRIP	1	RAB5B	1
PCCB	1	MYH14	1	HSPA1A	1
MCCC1	1	PRKD1	1	KPNB1	1
NME1	1	CRY2	1	APMAP	1
CYB5R3	1	PSMC4	1	G6PD	1
RAB1A	1	ACACA	1	HNRNPA3	1
HSP90AA1	1	PRKD3	1	EZR	1
RPL39	1	SFPQ	1	CCT8	1
MCCC2	1	CPNE9	1	SPR	1
CCT2	1	FASN	1	KRT6A	1
PRKCA	1	GPI	1	CAPZB	1
VCP	1	TLN1	1	KRT17	1
YWHAE	1	SMCHD1	1	ACTN1	1
ALDOA	1	FLNC	1	RAP1B	1
SSB	1	HIST1H2BJ	1	PAICS	1
HNRNPA2B1	1	NME2	1	CS	1
RALY	1	ENO1	1	ACTN4	1
MDH2	1	YWHAZ	1	CPNE8	1
CRY1	1	HMGA1	1	HNRNPH3	1
CLTC	1	EEF2	1	P4HB	1
IMPDH2	1	PLEC	1	DDOST	1
LMNA	1	PGK1	1	LRPPRC	1
CCT7	1	HNRNPA1	1	HK1	1
HADHA	1	VDAC1	1	TFRC	1
AHNAK	1	HMGB1P1	1	MAGEC1	1
HSP90B1	1	PDIA6	1	CKAP4	1
SEPT7	1	HNRNPH2	1	MCM3	1
EIF3E	1	RPS19	1	ERO1L	1
MATR3	1	RPL36	1	GFPT2	1
HID1	1				