

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

INTERACTOME MAPPING OF BMAL1 AND PER2
BY PROXIMITY-DEPENDENT LABELING

Hüseyin GÜL
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
Prof. Dr. NURİ ÖZTÜRK

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

BMAL1 VE PER2 PROTEİNLERİNİN
YAKINLIK BAĞIMLI ETİKETLEME YÖNTEMİ İLE
ETKİLEŞİM PARTNERLERİNİN HARİTALANMASI

HÜSEYİN GÜL
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
Prof. Dr. NURİ ÖZTÜRK

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SUMMARY

Circadian clock is a time length of approximately 24 hours that is driven endogenously without affecting environmental changes. Transcriptome analysis studies revealed that ~10% of genes are regulated by circadian rhythm and considering second-order genes and posttranslational modifications, it could be accepted that 20-40% of signaling mechanisms are under the control of the circadian clock. The mammalian molecular clock is regulated by the transcription–translation feedback loop (TTFL) mechanism which consists of a positive arm and a negative arm. Heterodimeric transcription factor CLOCK/BMAL1 is in the positive arm of the TTFL mechanism and activates up to 10% of expressed genes by binding to E-box-containing promoters. PER and CRY proteins are among clock-controlled genes (CCGs) and play role in the negative arm of the TTFL mechanism. Building detailed protein interactome maps is important for identifying molecular mechanisms and understanding cell function. Conventional interaction techniques have been used to analyze protein interactions for years, but they fall short of detecting dynamic and weak interactions. Proximity-dependent labeling technique allows the detection of weak and transient interactions and thus the detection of new interaction candidates that could not be detected by conventional techniques. We have adapted the novel technique to detect weak and transient interactions of PER2 and BMAL1 in two separate phases (to mimic day and night) in strongly resettable osteosarcoma (U2OS) cells. In addition to known interactions, we have identified several new interaction candidates of PER2 and BMAL1 and verified some PER2 interactions by the conventional co-immunoprecipitation technique. Our results showed that the proximity-dependent labeling technique enables the discovery of new protein interactions in the circadian clock mechanism.

Key Words: Circadian Clock; Proximity-Dependent Labeling, BioID2, Mini Turbo, PPI, PER2, BMAL1.

ÖZET

Sirkadiyen saat, çevresel değişikliklerden etkilemeden endojen olarak sürdürülen yaklaşık 24 saatlik bir süreçtir. Transkriptom analizi çalışmaları, genlerin yaklaşık % 10'unun sirkadiyen ritim tarafından düzenlendiğini ve ikinci dereceden genler ve translasyon sonrası modifikasyonlar dikkate alındığında, sinyal mekanizmalarının %20-40'ının sirkadiyen saatin kontrolü altında olduğu kabul edilmektedir. Memeli moleküler saati, pozitif kol ve negatif koldan oluşan transkripsiyon – translasyon geribildirim döngüsü (TTFL) mekanizması ile düzenlenir. Heterodimerik transkripsiyon faktörü CLOCK/BMAL1, TTFL mekanizmasının pozitif kolundadır ve E-box içeren promoterlere bağlanarak eksprese edilen genlerin % 10'una kadarını aktif hale getirir. PER ve CRY proteinleri saat kontrollü genler (ccgs) arasındadır ve TTFL mekanizmasının negatif kolunda rol alır. Ayrıntılı protein interaktom haritaları oluşturmak, moleküler mekanizmaları tanımlamak ve hücre fonksiyonunu anlamak için önemlidir. Yıllardır protein etkileşimlerini analiz etmek için geleneksel etkileşim teknikleri kullanılmıştır, ancak bunlar dinamik ve zayıf etkileşimleri saptamada yetersiz kalmaktadır. Yakınlığa bağlı etiketleme tekniği, zayıf ve geçici etkileşimlerin saptanmasına ve böylece geleneksel tekniklerle saptanamayan yeni etkileşim adaylarının tespit edilmesine olanak tanır. PER2 ve BMAL1 proteinlerinin zayıf ve geçici etkileşimlerini tespit etmek için iyi resetlendiği bilinen osteosarkom (U2OS) hücrelerinde iki ayrı fazda (gündüz ve geceyi taklit etmek için) yakınlığa bağlı etiketleme tekniğini kullandık. Bilinen etkileşimlere ek olarak, PER2 ve BMAL1 için birkaç yeni etkileşim adayı belirledik ve bazı PER2 etkileşimlerini geleneksel koimünopresipitasyon tekniği ile doğruladık. Sonuçlarımız, yakınlığa bağlı etiketleme tekniğinin sirkadiyen saat mekanizmasında yeni protein etkileşimlerinin keşfedilmesi için uygun gösterdi.

Key Words: Sirkadian Saat; Yakınlığa Bağlı Etiketleme, BioID2, Mini Turbo, PPI, PER2, BMAL1.

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
μg	: Microliter
μl	: Microgram
APS	: Ammonium persulfate
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle Arnt-like 1
BSA	: Bovine serum albumin
ccgs	: Clock controlled genes
CLOCK	: Circadian locomoter output cycles protein kaput
Co-IP	: Co-Immunoprecipitation
CRY	: Cryptochrome
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
EDTA	: Ethylenediaminetetraaceticacid
EGF	: Epithelial Growth Factor
EMT	: Epithelial Mesenchymal Transition
FBS	: Fetal Bovine Serum
HEK293T	: Human embriyonic kidney 293T cells
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid)
HPLC	: High Performance Liquid Chromatography
HRP	: Horseradish peroxidase
LB	: Luria Bertani
MEBM	: Mammary Epithelial Basal Medium
MEGM	: Mammary Epithelial Growth Medium

MMP	:	Matrix metalloproteinases
MS	:	Mass spectrophotometry
MT	:	Mini Turbo
NaCl	:	Sodium chloride
PAS	:	Period-Arnt-Single-minded
PBS	:	Phosphate Buffered Saline
PBS-T	:	Phosphate Buffered Saline with Tween-20
PCR	:	Polimerase chain reaction
PEI	:	Polyethylenimine
PER	:	Period gene
PPI	:	Protein-protein interactions
REV-ERBA	:	Retinoic acid-related orphan nuclear receptors
RHT	:	Retinohypothalamic tract
RIPA	:	Radio Immuno Precipitation Assay
ROR α	:	Retinoid-related orphan receptor α
RT-qPCR	:	Real-time quantitative polymerase chain reaction
SCN	:	Suprachiasmatic nucleus
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	:	Tris/Borate/EDTA
TBS	:	Tris Buffered Saline
TBS-T	:	Tris buffered Saline with Tween 20
TEMED	:	Tetramethylethylenediamine
TRIS	:	Hydroxymethylaminomethane
TTFL	:	Transcription translation feedback loop

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1. INTRODUCTION

Circadian rhythm is a time length of approximately 24 hours that is driven endogenously without affecting environmental changes. Nearly every aspect of human physiology is affected by circadian rhythms including the sleep-wake cycle, daily rhythms in body temperature, hormone production, cell regeneration, and other biological activities. Transcriptome analysis studies revealed that ~10% of genes are regulated by circadian rhythm and considering the second-order genes and posttranslational modifications, it could be accepted that 20-40% of signaling mechanisms are under the control of the circadian clock [Partch et al, 2014]. In mammals, most cells in the brain and peripheral tissues contain a molecular clock consisting of at least 12 specific clock genes and the circadian clock is generated by a cell-autonomous transcriptional autoregulatory feedback loop (TTFL). While BMAL1/CLOCK heterodimer act in the positive arm of the loop, PER/CRY heterodimer negatively regulates the TTFL mechanism. Among the 12 specific circadian clock genes, Per2 and Bmal1 play a central role in the maintaining of circadian phenotypes. [Fu and Lee, 2003]

To understand the role of the circadian clock on human physiology, behavior, and illness, we should elucidate the molecular background of the circadian mechanisms both on the genomic and proteomic levels. Genetic technologies have offered a lot of opportunities to understand the nature of circadian rhythm by doing genome-wide analysis [Reddy, 2013]. In contrast, until the last few years, proteomics approaches in this field have dropped behind due to the limitations in proteomic technologies and techniques. Last developments in technology and new techniques in protein studies have enabled posttranslational analysis (i.e.; protein-protein interaction, PPI) of the circadian clock in a high-throughput manner. Large-scale protein analysis techniques are mainly MS-based. Especially with further improvements, MS-based proteomics techniques allow rapid and high-accuracy analysis of proteomics data [Matthiesen and Bunkenborg, 2020]. Considering the proteomics techniques in circadian clock studies, MS technology

is the main platform of the process. Over the last few years, MS-based techniques in protein interaction studies have displayed increasing progress. Various methods have been established for the purpose of identifying and characterizing protein-protein interactions. This diverse toolbox provides researchers with options to overcome challenges specific to the nature of the proteins under investigation. Among these techniques is a category based on proximity-dependent labeling of proteins in living cells. Discovered in 2012, the BioID technique is an example of a protein interaction technique based on proximity-dependent labeling [Roux et al, 2012]. Compared with the previous PPI techniques like co-IP, BioID allows the detection of transient and weak interactions in living cells. The BioID technique utilizes a mutant form of biotin ligase enzyme (*BirA) derived from *E.coli* to promiscuously biotinylate proximal proteins in the same complex [Roux et al., 2012]. An updated version of the BioID technique, BioID2 was discovered in 2016 by Kim et al. In this method, a different form of biotin ligase enzyme, derived from *Aquifex aeolicus*, is used for proximal biotinylation. Because this enzyme is smaller than *E.coli* *BirA, hybridization of target proteins with *A. aeolicus* BirA has a less adverse effect on protein function and there is a fewer need for extrinsic biotin for the enzyme activity [Kim et al, 2016]. In addition to the BioID2 technique, a mutant version of BirA protein called miniTurbo was generated by Branon et al in 2018. The main advantage of miniTurbo is that it is more rapid than BioID and BioID2 with a 10 min biotinylation speed.

In this study, we aimed to discover new protein interaction partners of both PER2 and BMAL1 by using a proximity-dependent labeling strategy. Unlike previous PPI techniques like co-IP, proximity labeling provide an opportunity to detect transient and weak interactions of the target protein. Therefore we have identified large-scale PPI candidates for both proteins by expressing PER2-BioID2 and miniTurbo-Bmal1 fusion proteins (separately) in the U2OS osteosarcoma cell line. At the final stage of the study, we also validated some of the PER2 interactions using the classical co-IP technique. Our study has clearly indicated that our PPI list for PER2 and BMAL1 serves as high throughput data in circadian proteomics studies.

2. LITERATURE REVIEW

2.1. Mammalian Circadian Clock

The circadian clock is a temporal program that regulates the organisms' cycle of daily rhythms at all levels from gene expression to behavior. It is a time length of approximately 24 hours that is driven endogenously without affecting environmental changes. The day and night cycle causes changes in light-dark and temperature conditions and this affects life in all aspects. Human physiology and behavior like sleep-wake cycles, food intake, digestion, etc. are directly affected by this cycle. At the molecular level, transcriptome analysis studies revealed that ~10% of genes are regulated by circadian rhythm, and considering second-order genes and posttranslational modifications, it could be accepted that 20-40% of signaling mechanisms are under the control of the circadian clock [Hughes et al, 2009].

The mammalian circadian clock consists of two basic components; the Master clock and the peripheral clock. The master clock is located in the SCN and is involved in resetting the clock in peripheral tissues by the day-night cycle. The peripheral clock is the sum of molecular clocks in many organs synchronized by the master clock. The mammalian molecular clock is regulated by a transcription–translation feedback loop (TTFL) (Figure 2.1) [Reppert and Weaver, 2002]. TTFL mechanism consists of a positive arm and the negative arm. Heterodimeric transcription factor CLOCK/BMAL1 is in the positive arm of the TTFL mechanism and activates up to 10% of expressed genes by binding to E-box-containing promoters [Rey et al., 2011]. PER and CRY proteins are among clock-controlled genes (CCGs) and act in the negative arm of the TTFL mechanism. The accumulation of PER and CRY protein in the cytoplasm is controlled by E3-ubiquitin ligases β TRCP1 and FBXL21 [Hirano et al., 2013; Reischl et al., 2007]. When a sufficient amount of protein accumulates in the cytoplasm, protein kinase CK1 δ/ϵ induces the translocation of PER/CRY heterodimers into the nucleus. PER/CRY dimers inhibit the

transcription themselves and other CCGs through CLOCK/BMAL1 in the nucleus [Ko and Takahashi, 2006]. This causes a decrease in PER/CRY level in the cytoplasm and once the CRY proteins are ubiquitinated by FBXL3 E3-ligases, a new cycle is started [Siepka et al., 2007]. An additional feedback mechanism is conducted by nuclear receptor proteins RORs and REV-ERBs through the transcriptional regulation of BMAL1 [Guillaumond et al., 2005]. RORs and REV-ERBs regulate BMAL1 transcription antagonistically by binding to -ERBs/ ROR-binding element (RRE) in the promoter region of BMAL1. One TTFL cycle takes approximately 24h and is the main regulatory mechanism of the mammalian molecular clock in a wide variety of cell types. In addition to TTFL regulation, post-translational modifications and protein-protein interactions are involved in the control of the molecular clock mechanism.

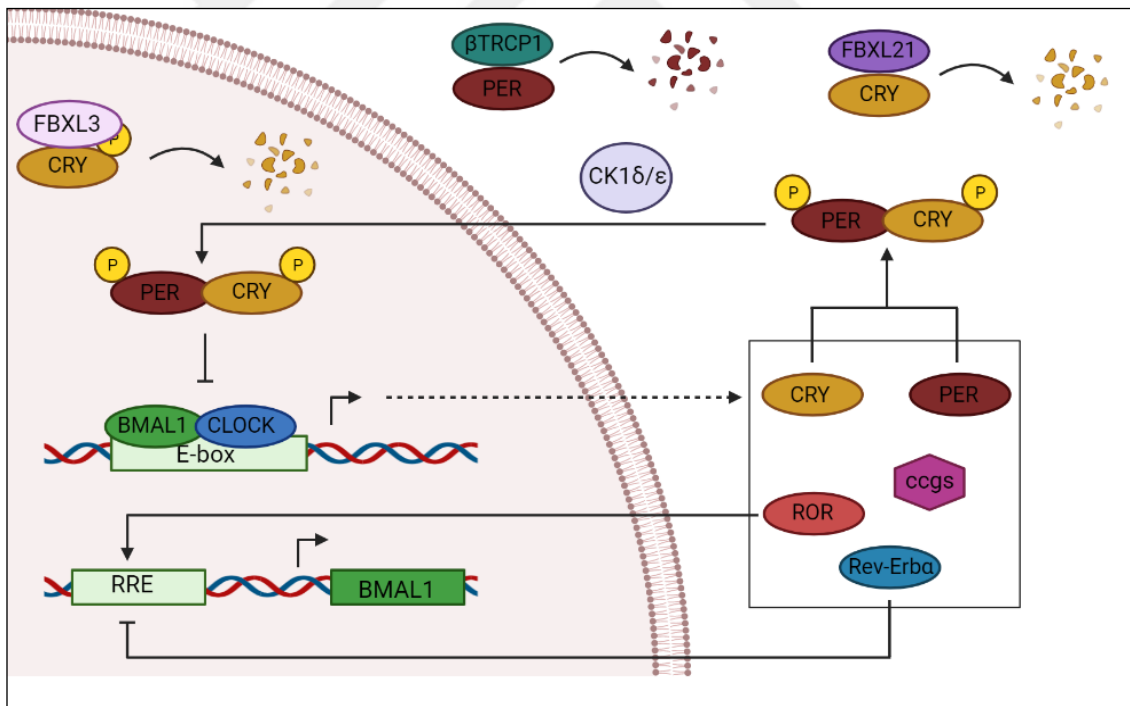


Figure 2.1: TTFL mechanism of the mammalian circadian clock.

Although the TTFL mechanism has been well studied, further studies are needed to understand the post-translational modifications and protein-protein interactions in molecular clock regulation and to elucidate the relationship between the circadian clock and other molecular mechanisms.

2.2. Clock-Controlled Genes

Clock-controlled genes (CCGs) are genes that exhibit 24-hour rhythmic expression and are regulated by circadian clock proteins. CCGs are controlled by the utilization of the three different types of regulatory elements present in their promoters; E-box motifs, RORE motifs, and D-elements [Ueda et al., 2005]. The expression of E-box controlled genes, including *Per* and *Cry* genes, are regulated by CLOCK- NPAS2:BMAL1 heterodimer in a circadian manner. RORE-containing promoters such as the *Bmal1* promoter, are the binding site for ROR and REV-ERB family members. *Bmal1* transcription is activated by ROR α , ROR β , or ROR γ [Akashi and Takumi, 2005; Guillaumond et al., 2005; Sato et al., 2004] and repressed by REV-ERB α or REV-ERB β [Guillaumond et al., 2005; Preitner et al., 2002; Triqueneaux et al., 2004]. D-elements are the binding sites for PAR bZip family members. For example, the D site albumin promoter binding protein (DBP) is a member of the PAR bZip family and activates *Per1* gene expression by directly binding the *Per1* promoter together with the CLOCK-BMAL1 heterodimer [Yamaguchi et al., 2000].

Circadian gene expression is also affected at the epigenetic level. The dynamic modification of chromatin architecture is necessary to facilitate the accession of transcription factors to activate gene expression. The modification of chromatin structure is commonly mediated by the acetylation of histones by histone acetyltransferases (HATs) and the opposing histone deacetylases (HDACs). Histone acetylation results in relaxation of chromatin condensation thereby facilitating gene transcription. Rhythmic gene expression of CCGs is regulated through the rhythmic

histone acetylation at promotor regions of CCGs [Etchegaray et al., 2003, Partch et al., 2014]. Indeed, H3K4me1, H3K4me3, H3K9ac, H3K27ac, and H3K36me3 acetylation show diurnal variation [Vollmers et al., 2012]. Considering the role of CLOCK-BMAL1 in the transcription activation of CCGs, it is not surprising that CLOCK-BMAL1 is associated with HATs or HDACs. For example, CREB-binding protein (CBP), a histone acetyltransferase, is recruited to BMAL1 to control the induction of *Per1* gene transcription [Kalkhoven, 2014]. Another HAT member p300 is associated with CLOCK-BMAL1 heterodimer and activates rhythmic histone acetylation of *Per* and *Cry* promoters [Etchegaray et al., 2003]. Additionally, SIRT1 has rhythmic gene expression and enzyme activity [Asher et al. 2008; Nakahata et al. 2008] and is recruited to the CLOCK/BMAL1 complex in the clock gene promoter via interacting with CLOCK [Nakahata et al. 2008].

As stated above, some CCGs are transcription factors such as DPB, ROR α , and REV-ERB α and they also regulate some other genes' transcription in a circadian manner including *Per1* and *Bmal1*. Additionally, chromatin modification is necessary to facilitate the transcription of CCGs. HAT and HDAC proteins are responsible for chromatin reorganization by histone acetylation and deacetylation respectively. Therefore, transcription factors responsible for CCGs expression, like BMAL1 interact with HAT and HDAC proteins.

2.3. The Significance of Period 2 (PER2) in Circadian Cycles

The *Per* gene was first discovered by Jeffery C. Hall and Michael Rosbash in 1984 through genetic studies on *Drosophila melanogaster* [Reddy et al., 1984; Zehring et al., 1984]. The mammalian *Per2* gene which is a member of the *Period* gene family was discovered in 1997 by researching cDNA homologous to the *Per1* gene [Shearman et al., 1997]. As described above, PER2 is the main component of the negative arm in the TTFL mechanism, so it is expressed in a variety of tissues, including the SCN, where the master clock is located. Mutation analysis of PER2 revealed the role of PER2 in resetting the

circadian clock and regulating the length of the circadian period [Albrecht et al., 2001; Vanselow et al., 2006]. Transfection and mutation studies have shown that PER2 has both nuclear import and export signals (CLD, NLS, and NES1-3), therefore it can be localized in the nucleus and cytoplasm [Yagita et al. 2000, 2002]. PER2 localization is regulated by interaction with cytoplasmic PML proteins [Miki et al. 2016] and PER2 interaction with CRY proteins prevents its ubiquitylation to prolong their presence in the cell [Yagita et al. 2002]. PER2 function is also regulated by phosphorylations. It has been identified that PER2 has more than 20 potential phosphorylation sites [Schlosser et al., 2005; Vanselow et al. 2006]. In fact, regulation of circadian clock by post-translational modification and protein-protein interactions are a common theme in chronobiology [Albrecht et al., 2007]. PER and CRY proteins are translated in the cytoplasm and phosphorylated by various kinases for their nuclear localizations to repress CLOCK-BMAL1 activity [Takahashi, 2015].

PER proteins have an extensive interactome with interactor functions ranging from the immune response to metabolic regulation. A proteome analysis study in human endothelia reveals that PER2 has a role in various metabolic pathways including cholesterol biosynthesis, phagosome maturation, TriCarboxylic Acid (TCA)-cycle, RNA signaling, and tRNA charging [Oyama et al., 2019]. Another interactome study showed that, mouse PER2 protein is in complexes with both polypyrimidine tract binding protein-associated splicing factor (PSF) and SIN3A which are responsible for the gathering of HDACs to the *Per2* promoter region [Duong et al., 2011]. Additionally, PER2 interacts with methyl transferases such as Suv39h1, Suv39h2, HP1 γ , and Kap1 involved in the methylation of H3K9 [Duong and Weitz, 2014]. It is also shown that PER2 has a role in transcription regulation by interacting with various RNA helicases DDX5, DHX9, and SETX, as well as RNA Polymerase II [Padmanabhan et al., 2012]. In addition to the negative feedback role of PER2, it has been reported that PER2 interacts with nuclear receptors including, PPAR α and REV-ERB α , which indicates that PER2 plays a coregulatory role in nuclear receptor-mediated transcriptional regulation via its binding to the *Bmal1* promoter and other nuclear-receptor target genes. [Schmutz et al., 2010].

In general, PER2 appears to be involved in the transcription regulation of genes in the positive arm and other CCGs. Furthermore, the extent of proteins involved in negative arm macromolecular complexes suggests that PER2 has many yet unexplored functions that lead to the proper timing of circadian rhythms. The results obtained with this project contributed to the understanding of the function of PER2 as it revealed new PER2 interactions and offered new approaches to the undiscovered functions of PER2.

2.4. The Significance of Brain and Muscle ARNT-Like 1 (BMAL1) in Circadian Cycles

Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL) or Brain and Muscle ARNT-Like 1 (BMAL1) protein was first discovered in 1997 by John B. Hogenesch under the name Mop3 [Hogenesch et al., 1997]. BMAL1 protein, which is located in the positive arm of the TTFL mechanism, belongs to the transcription factor family that contains the structural domain bHLH-PAS, and forms a heterodimer with the CLOCK protein to control the expression of genes involved in circadian clock such as *Per*, *Cry* and *Rev-Erba* [Ko and Takahashi 2006]. In mammalian cells, BMAL1 is one of the most critical proteins in maintaining the normal circadian rhythm. In fact, it is the only single circadian clock protein that is essential for rhythmic gene expression in the mammalian circadian timing system. BMAL1 damaged mice studies showed that among the clock genes, only the BMAL1 protein affected all rhythmic behavior. [Bunger et al. 2000]. In addition to disturbances in circadian rhythm, some additional problems have also been reported in *Bmal1* null mice such as reproductive problems [Boden and Kennaway 2006], shortening in height, rapid aging [Kondratov 2007] and persistent joint disorders (arthopathy) [Bunger et al. 2005].

In addition to its role in circadian rhythm, BMAL1 plays a role in many cancerous pathways, such as cell division, apoptotic resistance mechanism, invasion and metastasis activation, and rearrangement of energy metabolism [Tang et al. 2017]. It is also described that BMAL1 may play a role in hypertension, diabetes, and obesity [Pappa et

al., 2013]. Using a yeast-two-hybrid approach to detect the direct BMAL1 interactions, BMAL1 has been found to have 150 possible targets, including proteins involved in chromatin modification, regulation of proliferation, DNA repair, and many other pathways [Wallach et al., 2013]. It has also been discovered that 6% of more than 3000 proteins detected by the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) study in mouse liver are rhythmic. Interestingly, many of the rhythmic proteins are encoded by non-rhythmic mRNAs [Robles et al., 2014].

BMAL1 has been reported to present in macromolecular complexes containing many pieces of translational machinery in cytoplasm [R. P. Aryal et al., 2017]. Some of the interactors in these macromolecular complexes are shown that they are the phosphorylation targets of mTOR-effector kinase ribosomal S6 protein kinase 1 (S6K1) [Lipton et al., 2015]. Furthermore, circadian oscillations mediated by BMAL1 were detected in translation which indicates the direct role of BMAL1 in the protein translation process [Lipton et al., 2015]. In addition to the role of BMAL1 in macromolecular complexes in the cytoplasm, it also interacts with various proteins in the nucleus. NOP58, a member of the nucleolar ribonucleoprotein complex that aids in the processing of pre-rRNA has been found to interact with BMAL1 in the nucleus to regulate the U8 small nucleolar RNA level required for the biogenesis of ribosomal RNAs [Cervantes et al., 2020; Boisvert et al., 2007].

Generally, BMAL1 is an important component of the positive arm of the TTFL mechanism with its nuclear and cytoplasmic roles. Besides its circadian clock function, BMAL1 has also roles in various molecular processes from cancerous pathways to rRNA processing. Since it is a transcription activator, BMAL1 regulates the expression of a wide range of genes in a circadian manner. Moreover, its presence in macromolecular complexes in the cytoplasm suggests that BMAL1 has different roles beyond its transcription activator function. Since this study provides high-throughput interactome data, it offers new approaches to the circadian and non-circadian role of BMAL1.

2.5. Proximity Dependent Labeling

Building detailed protein interactome maps is important for understanding cell function and identifying new drug targets. Interactome mapping studies especially in the circadian clock, need to elucidate dynamic and weak interactions in molecular processes. However, previous methods like co-immunoprecipitation (co-IP) and pull-down can only detect strong interactions in living cells. Because these conventional methods require the isolation of intact protein complexes, it is hard to detect weak and transient protein interactions by these methods. Additionally, these conventional approaches use stringent extraction conditions to remove contaminants and this disrupts protein interactions and results in data loss. These limitations have been overcome by the discovery of proximity-dependent labeling methods.

Proximity-dependent labeling offers a new approach to PPI studies. The protein 'fishing' method in the identification of protein interactions has recently been reported [Roux et al., 2012]. This approach, called BioID, utilizes a mutant biotin ligase (BirA*) of *Escherichia coli*, capable of promiscuous biotinylation [Cronan, 2005; Roux et al., 2012]. Biotin ligase enzymes are highly conserved between species and they act as an important role in the biotinylation processes of target proteins [Chapman-Smith and Cronan, 1999]. When expressed in cells, the BirA* fusion protein facilitates the attachment of biotin to its targets, and this attached label permits selective isolation of target proteins with streptavidin binding (Figure 2.2). These proteins are then isolated and subjected to mass spectrometer analysis. MS analysis produces a high-throughput result that needs to be analyzed and validated by statistical and experimental studies.

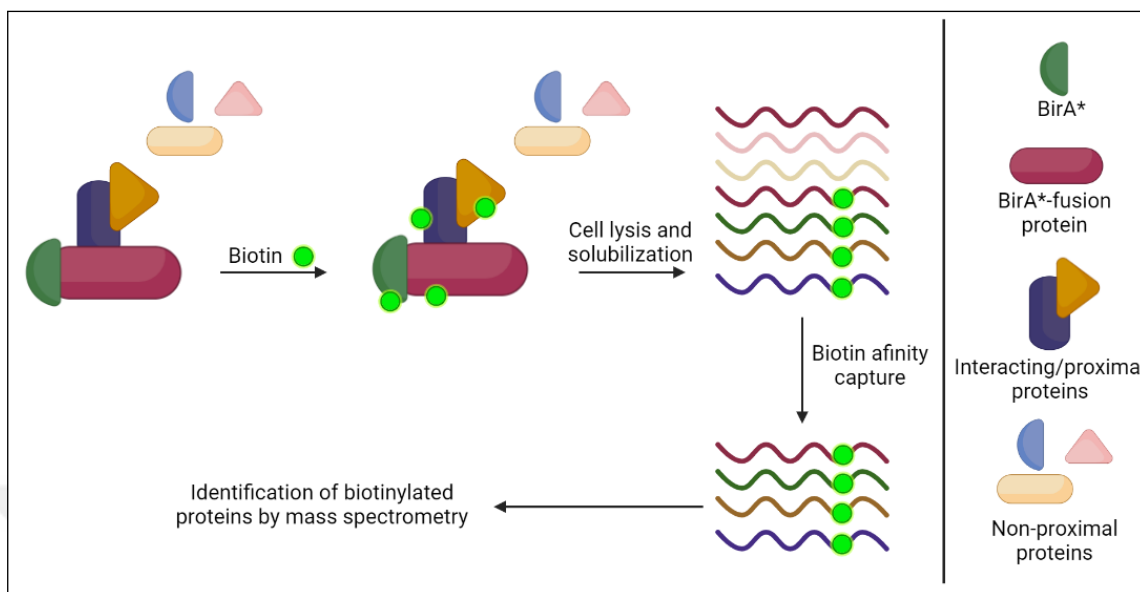


Figure 2.2: Illustration of proximity-dependent labeling technique.

BioID has several key advantages compared with conventional protein interaction methods [Roux et al., 2012]. First, not only interacted proteins but also non-interacting but proximal proteins can be biotinylated by biotin ligase enzyme and this provides more detailed information about the proteomic structure around the fusion (Figure 2.2). Second, dynamic interactions or temporarily proximal proteins can be biotinylated, and the biotin label will not be removed even if the interaction has been interrupted (Figure 2.2). Therefore, BioID is useful for the identification of undetected interactions by previous methods and for performing kinetic studies. Third, the purification of biotinylated proteins from cell lysate by using streptavidin binding does not have a significant effect on experimental efficiency. Hence, BioID proves to be particularly useful in the study of insoluble proteins such as membrane proteins or nuclear lamina. Finally, BioID only requires the expression of an appropriately fused BirA* and target protein. All these advantages have made the BioID technique the most utilized technique in protein interaction studies since its discovery.

Nevertheless, BioID is not without its drawbacks and limitations [Kim et al., 2016; Branon et al., 2018]. The expression level of the fusion protein must be above the threshold level where there is a sufficient amount of labeled protein. Additionally, since the BirA* enzyme expands the size of the fusion protein, it might inhibit the activation or targeting of the fusion protein. As a result of the biotinylation process, irreversible covalent modifications may disrupt the function of labeled proteins by preventing required modifications like phosphorylation or ubiquitination. Another limitation related to the evaluation of results come from MS analysis. Results will include false negatives because there could be naturally biotinylated proteins in the cell. Positive candidates also are not proof of direct interaction with the fusion protein, they could be in the same complex but not in physical interaction as the result of promiscuous biotinylation activity of BioID enzyme.

All limitations that are mentioned above are fundamental problems faced in proximity-dependent labeling methods and they need time to solve. In addition to these fundamental problems, the BioID technique itself has secondary limitations such as the excessive labeling time (24h) of the BioID enzyme and the relatively high biotin requirement (50uM). However, these limitations could have been overcome by the various BioID forms discovered in the last few years. The discovery of mutant BirA* (BioID2) protein in 2016 was the first step toward improvements in the BioID system [Kim et al., 2016]. Different from BioID, the BioID2 technique utilizes the mutant form of the Aquifex aeolicus biotin ligase. It appears that the biotinylation efficiency of BioID2 is enhanced and it requires significantly less biotin (0.1uM) than BioID. This enables the detection of proteins that are difficult to biotinylated with biotin ligases [Kim et al., 2016]. Additionally, with its smaller size (Figure 2.3), BioID2 has a smaller effect on the function and targeting of the fusion protein.

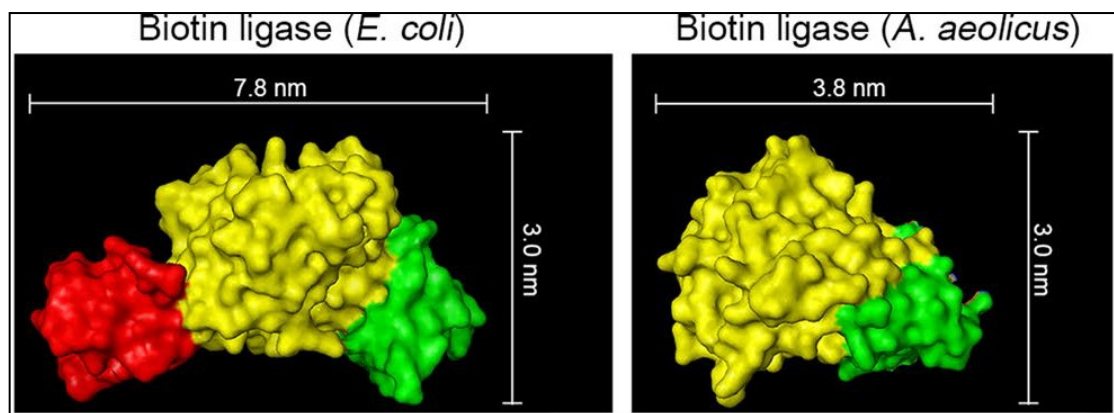


Figure 2.3: The dimensions of *E. coli* (left; PDB ID 1BIA) and *A. aeolicus* (right; PDB ID 2EAY) biotin ligases based on prior structural analyses.

Although BioID2 has many advantages over BioID, the biotinylation rate of mutant BirA* is just under 24 hours (16h). In order to reduce the biotinylation time of the BioID enzyme, new mutations in wt BirA was created and two mutant biotin ligase, MiniTurbo, and TurboID were produced by Branon et. al in 2018 [Branon et al., 2018]. Both enzymes have a significantly less biotinylation time (10-minute) compared to BioID and BioID2 enzymes [Branon et al., 2018].

PPI studies are important to draw interactome mapping of molecular pathways and to highlight the working mechanisms of new drug candidates. Conventional PPI methods include an isolation step that utilizes stringent extraction conditions which only enable the purification of strong interactions. This limits the use of conventional methods in kinetic studies. Proximity-dependent protein labeling methods emerged with the discovery of BioID technique and eliminated various limitations faced in conventional methods. Considering the current developments in proximity-dependent labeling techniques, detecting dynamic and weak interactions, and obtaining high-throughput results have facilitated protein interaction studies. In this context, we used two different proximity-dependent labeling technique (BioID2 and Mini-Turbo) for two circadian clock proteins (PER2 and BMAL1) to identify weak and transient interaction candidates. Although we planned to use MiniTurbo technique for both proteins to analyze the interactions in sub-circadian times (because MiniTurbo has 10-min biotinylation time) ,

we faced some limitations in cloning step of PER2 protein because of its big size (~190kd). Therefore we designed PER2-BioID2 and MiniTurbo-BMAL1 fusion proteins for our study. Although BioID2 and MiniTurbo have various advantages over conventional PPI techniques, the results obtained with these techniques still need to be validated. We analyzed our PER2-BioID2 and MiniTurboBMAL1 interaction candidates by using statistical tools and validated several PER2-BioID2 interaction candidates by co-IP assay.



3. MATERIALS

3.1. Kits

All parental and subclone lines used in the thesis were tested for mycoplasma Contamination using the e-Myco plus Mycoplasma PCR Detection Kit (BocaScientific Inc., USA). Plasmid isolation and gel extraction were performed using Nucleospin Plasmid Kit and PCR clean-up Gel extraction Kit (Macherey Nagel, Germany) respectively. PCR products were column purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Total DNA was isolated by using Nucleospin tissue kit (Macherey Nagel, Germany). All transfections were done using PEI Transfection Reagent (Sigma-Aldrich, USA). Lentivirus infections were done using Polybrene Infection Reagent (Sigma-Aldrich, USA). Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, USA) were utilized to inhibit general degradation of proteins in cell extracts by endogenous proteases. In order to determine protein concentration, Bradford Protein Assay Dye (Bio-Rad, USA) and Pierce™ BCA Protein Assay Kit (Thermo Scientific, US) were used. Protein Molecular Weight Marker (New England Biolabs, USA) was used to monitor the progress of electrophoretic run and analysis conditions of proteins. For DNA analysis, DNA Ladder and DNA Loading Dye (New England Biolabs, USA) were used. NucleoSpin RNA Purification Kit (Macherey Nagel, Germany, cat no: REF 740962) was used for total mRNA isolation. Isolated mRNAs were converted to cDNA using ProtoScript First Strand cDNA Synthesis kit (NEB, Ipswich, MA, cat no: E6300S). RT-qPCR assay was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific, Cat no: K0222). Imaging and detection of proteins by Western Blotting was succeed by using Chemiluminescent HRP Substrate (Advansta, USA) and SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Scientific, US).

3.2. Enzymes

Restriction enzymes (New England Biolabs, USA), Taq DNA Polymerase (M0320S, NEB), T4 DNA ligase (1U/μl) Invitrogen (15224017) and Q5 DNA polymerase (NEB, Ipswich, MA).

3.3. Antibodies

Primary antibodies; Anti-HA tag Ab (1:10000, Abcam, ab13834), Anti-Flag tag Ab (1:2000, Sigma, F1804), Anti-Myc tag (Abcam, Cat: Ab9106), Anti Actin Ab (1:5000, CST, 4967), Anti Bmal1 Ab (1:2000, Bethyl labs, A302-616A), Anti Per2 Ab (1:2000, Bethyl labs, A303-109A), Anti Calnexin Ab (1:10000, Santa Cruz, sc11397) and secondary antibodies; Anti-mouse HRP (1:3000, CST, 7076), Anti-Rabbit HRP (1:3000, CST, 7074), Alexa Fluor 555 conjugated-anti-Rabbit IgG (H + L), F(ab')₂ Fragment (Cell Signaling Technology, cat no: 4413S), anti-GAPDH Ab (Cell Signaling Technology, cat no: 2118S) and anti-Histone-H3 Ab (Abcam, UK, cat no: ab1791) were commercially obtained and used in this study.

3.4. Chemicals

Methanol (947.046, Sigma), ethanol (920.026, Sigma), Agarose (351010, Peqlab), HEPES (P05-01100, PAN), LB Broth Medium (LBP03, Caisson Labs), LB Agar (LBP04, Caisson Labs), 40% Ac-BisAcrilamide (161-0146, Biorad), 2-Propanol (1.00995, Merck), Skim milk (non fat powder, SKI400, Biochop), TRIS (AC228030051, Fisher Scientific), Dimethyl Sulfoxide (BP231, Fisher Scientific), Glycine (G45, Fisher Scientific), NaCl (S271, Fisher Scientific), Sodium Dodecyl Sulfate (BP166, Fisher Scientific), Tween 20 (BP337, Fisher Scientific), TEMED (Tetramethylethylenediamine, A1148, Applichem), 2-Mercaptoethanol (A1108, Applichem), Sodium Hydroxide (106498, Merck), Hydrochloric

acid (A144, Fisher Scientific), IGEPAL CA-630 (I8896, Sigma), Puromycin dihydrochloride (P8833, Sigma), Amphotericin (59349, Sigma), Polybrene (134220, Santa Cruz Biotechnology), Bromophenol Blue (BP115, Fisher Scientific), Boric Acid (B7901, Sigma), Bovine Serum Albumin (A2153, Sigma), Ethylene diamine tetra acetic acid (EDTA, BP118, Fisher Scientific), NP 40 Cell Lysis Buffer (FNN0021, Thermo Fisher), 1× NEB buffer 2 solution (New England Biolabs, U.K.), Biotin (BB0078, Bio Basic Inc.), Dexamethasone (Sigma), HRP-conjugated streptavidin (Abcam, Cat: ab7403), NP40 lysis buffer (Thermo Fisher Scientific, Cat: FNN0021), protease and phosphatase inhibitor cocktail (Sigma, Cat: S8830), MagnaBind Goat Anti-Mouse IgG magnetic beads (Thermo Fisher Scientific, Cat: 21354), streptavidin-coated magnetic beads (New England Biolabs, Cat: S1420S), G418 (Corning, USA, Cat: 61-234-RF), BSA (Sigma, cat no: A2153), DAPI (Thermo Fisher Scientific, Hoechst 33342 cat no: 62249) and Mowiol mounting solution (Sigma-Aldrich, cat no: 81381) were used in this study.

3.5. Cell Culture Supplements and Solutions

Dulbecco's Modified Eagle Medium (DMEM, 41965-039, Gibco, Invitrogen), 100X Penicillin/Streptomycin (15140-122, Gibco, Invitrogen), Fetal Bovine Serum (FBS, 10270-076, Gibco, Invitrogen), 0.05% Trypsin-EDTA (25300-054, Gibco Invitrogen), 10X Phosphate Buffered Saline Liquid Concentrate (6505-OP, Calbiochem), 100 X MEM Non-Essential Amino Acids Solution (11140050, Gibco).

3.6. Buffers and Solutions

Buffers and solutions used in this study are 10X TBE (108g Tris base, 55g Boric acid, 40 ml 0.5M EDTA (pH 8.0), 10X SDS Running Buffer (250mM Tris-Base (pH: 8.3), 1.90 M Glycine, 1% SDS), 10X TBS (20 mM Tris-Cl (pH: 7.6), 1.5 M NaCl), 1X Transfer Buffer (10% 10X SDS Running Buffer, 20% Methanol), 1X TBST (1X TBS, 0.1% Tween-20), 5% Blocking

Solution (5% non-fat dry milk in 1X TBS-T), 1X PBS (137mM NaCl 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄), 1X PBST (1X TBS, 0.1% Tween-20), 5% bovine serum albumin in 1x PBST, 4X Protein Loading Dye (250mM Tris-Cl (pH: 6.8), 8% SDS, 40% Glycerol, 20% β-mercaptoethanol, 0.008% Bromophenol Blue), Cell Lysis Buffer RIPA (750mM NaCl, 250mM Tris (pH: 8.0), 0.1% SDS, 1.0% NP-40), cell freezing solution (10% FBS, 10%DMSO, 80% growth media).

SDS-PAGE Gels were prepared by following the instructions at Table 3.1.

Table 3.1: Stacking and resolving gel recipe in different concentrations.

10% Resolving Gel	8 ml	8% Resolving Gel	8 ml	6% Resolving Gel	8 ml	5% Stacking Gel	2 ml
ddH ₂ O	3.8 ml	ddH ₂ O	4.2 ml	ddH ₂ O	4.6 ml	ddH ₂ O	1.46 ml
40% Acryl:Bis	2 ml	40% Acryl:Bis	1.6 ml	40% Acryl:Bis	1.2 ml	40% Acryl:Bis	250 µl
1.5 M Tris pH8.8	2 ml	1.5 M Tris pH8.8	2 ml	1.5 M Tris pH8.8	2 ml	1 M Tris pH 6.8	250 µl
10% SDS	80 µl	10% SDS	80 µl	10% SDS	80 µl	10% SDS	20 µl
10% APS	80 µl	10% APS	80 µl	10% APS	80 µl	10% APS	20 µl
TEMED	8 µl	TEMED	8 µl	TEMED	8 µl	TEMED	2 µl

3.7. Equipments

Agarose Gel Electrophoresis (1704406, Bio-Rad, USA), Centrifuges (521-1647 refrigerated, Micro Star 17R, VWR; 521-1646 Micro Star 17, VWR; 521-2844 Mini Star, VWR; NF400-Nuve, 02-1827), Chemiluminescence Imaging System (ChemiDoc XRS+ System, Bio-Rad), Incubator (CO₂) (Thermo-Scientific), Electrophoresis System (1658004, Mini-PROTEAN® Bio-Rad), Laminar Flow Cabinet(Hera-Safe Class II Cabinet, Thermo Scientific), Microplate Reader (Varioskan Flash, Thermo Scientific), Power Supply (1645050, Bio-Rad), PCR Thermal Cycler (41798,Bio-Rad), Trans-Blot Transfer System

(1704155, Bio-Rad), NanoDrop (206-26300-8, Shimadzu Biotech) Standard Shaker (VWR) Vortex (Scientific Industries), Water Bath (Wisd), Synergy H1 microplate reader (BioTek Instruments, Inc. USA,) DynaMagTM-2 magnetic rack (Thermo Fisher Scientific, Cat: 12321D), confocal microscopy (Carl Zeiss LSM 880 Airyscan) and ZOE fluorescence cell imager (Bio-Rad) were used in this thesis.

3.8. Cell Lines and Growth Media

U2OS and HEK293T cell lines were from American Type Culture Collection (ATCC; Rockville, MD), and maintained in a humidified incubator at 37°C under 5% CO₂. For growth and experimental procedures of U2OS and HEK293T, DMEM supplemented with 10% FBS, 100U/mL penicillin, 100 µg/mL streptomycin, and 1% non-essential amino acids were used (all from Thermo Fisher Scientific).

3.9. Bacteria

E. coli DH5α cells for transformation step needed for obtaining HEK293T KO cell lines and *E. coli Stb13* cells for transformation step needed for obtaining U2OS recombinant cell lines were used in this study.

3.10. Vectors

To obtain MCS-hPER2-13xLinker-BioID-HA plasmid (Figure 3.1), PER2 coding sequence was cloned into MCS-13XLinker-BioID2-HA (Addgene Plasmid #80899) from pCS-hPER2 plasmid [Partch et al., 2006] which were kindly provided by Carrie Partch (University of California Santa Cruz, CA, USA). MycBioID2-13X Linker-MCS, which expresses BioID2 with a Myc tag at the N-terminus and 13x Linker at the C-terminus (Addgene Plasmid #92308) was used as a mock control in our experiments because MCS-

13X Linker-BioID2-HA required a start codon from the interest of protein (PER2 in our case). pENTER-FLAG-PER2-FLAG-His was generated by inserting FLAG-PER2 coding sequence into NheI-XhoI sites of pENTER plasmid (Vigene Biosciences, Rockville, MD). The interactome candidates (CRY1, CRY2, HK1, MCM3, and IMPDH2) were amplified from U2OS cDNA and cloned into NheI-XhoI site of 3xHA-MiniTurbo-NLS-pcDNA3 plasmid by removing MiniTurbo-NLS cassette to express the candidates with a 3xHA tag at the N-terminus.

To obtain pLentIII-p(Bmal1)-3xHA-MiniTurbo-13xLinker-mBmal1 and pLentIII-CMV-3xHA-MiniTurbo-13xLinker plasmids, we used pLentIII-HA (Applied Biological Materials Inc., USA) vector. MiniTurbo sequence was amplified from 3xHA-miniTurbo-NLS_pCDNA3 (107172, Addgene) vector. pLentIII-p(Bmal1)-3xHA-MiniTurbo-13xLinker and pLentIII-CMV-3xHA-MiniTurbo-13xLinker plasmids were used as mock control in BMAL1 experiments.

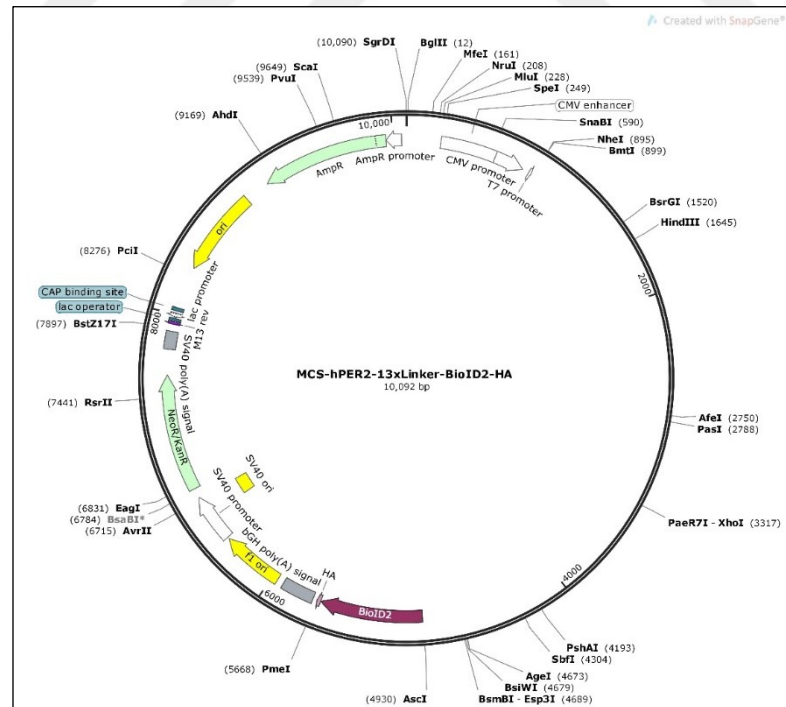


Figure 3.1: MCS-hPER2-13xLinker-BioID2-HA vector map.

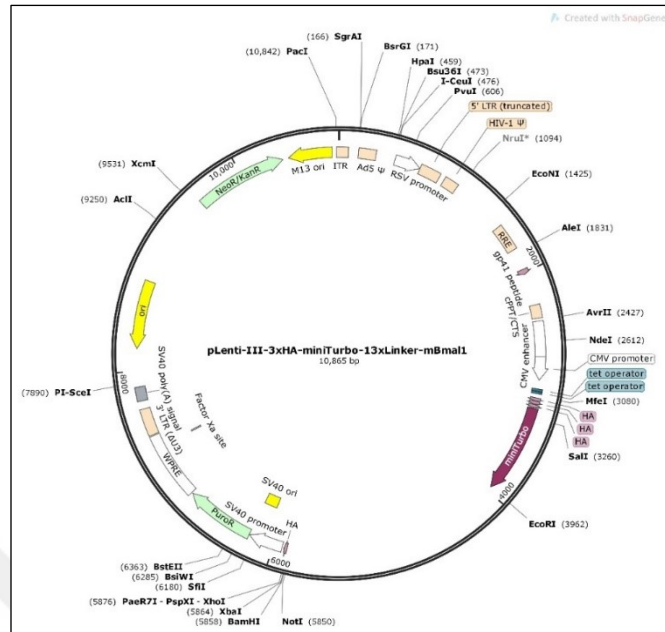


Figure 3.2: pLentiIII- 3xHA-MiniTurbo-13xLinker-mBmal1 vector map.

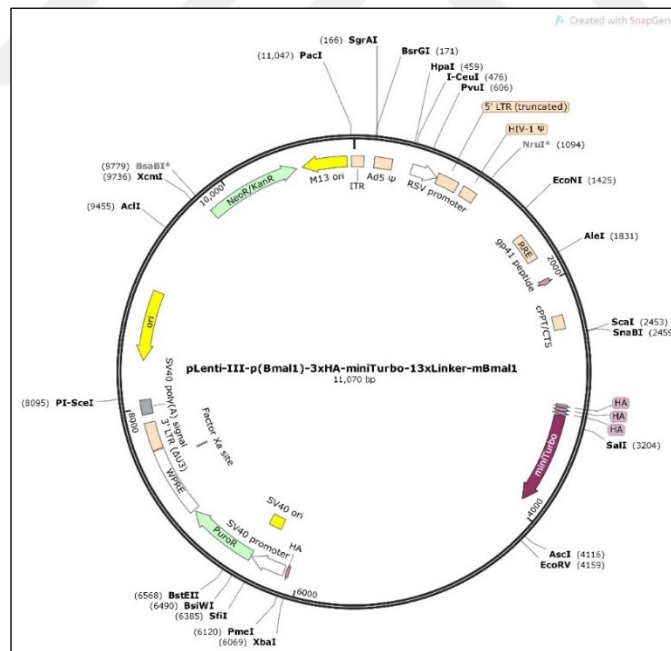


Figure 3.3: pLentiIII-p(Bmal1)-3xHA-MiniTurbo-13xLinker-mBmal1 vector map.

3.11. Oligonucleotides

Table 3.2: Primers used in qPCR analysis and for the design of expression vectors.

Primer Name	Sequence (5'→3')	Purpose	Relevant Sequence
HK1_F	GCAGCTAGCATGATCGCCGCGCAGCTCCT	To amplify HK1 coding sequence	NM_001079525.2
HK1_R	ATGCTCGAGTTAGCTGCTTGCTCTGTGC		
MCM3_F	GCAGCTAGCATGGCGGGTACCGTGGTGCTG	To amplify MCM3 coding sequence	NM_002388.6
MCM3_R	ATGCTCGAGTCAGATGAGGAAGATGATGC		
IMPDH2_F	GCAGCTAGCATGGCCGACTACCTGATTAGT	To amplify IMPDH2 coding sequence	NM_000884.3
IMPDH2_R	ATGCTCGAGTCAGAAAAGCCGCTTCTCAT		
Per2cl_1_F	GCAGGTACCGCTAGCGCCACCATGGATTATA AGGATGACGACGATAAAATGGATACGCGG AATTTC	To amplify FLAG-PER2 for pENTER cloning	NM_02817.3
Per2cl_5_R	CGTACCGGTGTGACCGTCTGCTCTTCGATCC TGTGA		
NheI-Per2_F	CAAGCTAGCGCCACCATGAATGGATACGCGG AATTTC	To amplify PER2 for cloning into MCS-13X Linker-BioID2-HA	NM_022817.3
AgeI-Per2_R	TTTACCGGTTCTGCTCTTCGATCCTGTGA		
hPER2_F	ATGACGTACCAGCTGCCTTC	q-PCR analysis of PER2 expression	NM_022817.3
hPER2_R	CCTGCTTCTGACTCTCCGTG		
hDBP_F	GAGGAACTTAAGCCCAGCC	q-PCR analysis of DBP expression	NM_001352.5
hDBP_R	CCTCGTTGTTCTTGTACC		
hGAPDH_F	TGCACCACCAACTGCTTAGC	q-PCR analysis of GAPDH expression	NM_001357943.2
hGAPDH_R	ACAGTCTTCTGGGTGGCAGTG		

4. METHODS

4.1. Cell Culture

U2OS and HEK293T cells were cultured at 37°C under 5% CO₂ in a Dulbecco's Modified Eagle Medium (DMEM)-based medium supplemented with 1% nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (all from Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged when they reached 80% confluence and their medium was changed every 3-4 days.

The frozen cells were thawed rapidly in a 37°C water bath. Using a pre-warmed growth medium, the thawed cells were diluted in a laminar flow hood. By aspiration of medium following centrifugation at 2500 rpm for 5 minutes, DMSO was removed. Cells were suspended in the appropriate fresh medium required for each cell type and plated in an appropriate cell culture dish at high density to optimize recovery.

For cell passage, the medium was removed in a laminar flow hood, the cells were rinsed once by gently adding 1X PBS to the side of the cell culture dish carefully not to dislodge the cells. Using 0.05% trypsin-EDTA solution, and incubating for 5 min at 37 °C, the adherent cells detached from the dish. By adding warm DMEM, trypsin activity was inactivated, and the cells were transferred to a 15-ml Falcon tube. After centrifuging at 2500 rpm for 5 min., cells were dissociated by pipetting up and down gently in a fresh medium, the cell suspension was obtained and then cells were reseeded into a new dish in an appropriate ratio.

For cell freezing, cell suspension at a high concentration and at as low a passage number as possible, 800µl of cell suspension was dispensed into storage vials. 100 µl of FBS and 100 µl of DMSO were added respectively to storage vials. After a few hours of storage at –20°C, storage vials were transferred to –80°C or to liquid nitrogen.

4.2. Establishment of Stable Cell Lines

4.2.1. Stable Transfection of U2OS Cells

U2OS cells were seeded in 60-mm tissue culture dishes at 40-50% confluence and transfected with the plasmids after overnight incubation. For the transfection procedure of the PER2 study, 3ug of MCS-PER2-13XLinker-BioID2-HA or Myc-BioID2-13xLinker plasmids were dissolved in the 75 µl of transfection buffer (100mM NaCl, 10mM Tris-HCl pH 7.5, and 1mM EDTA) and mixed with pipetting several times. Then, 9 µl of 1 mg/ml PEI (polyethyleneimine from Sigma, USA) were added to this solution, mixed well by pipetting, and incubated for 15 minutes at room temperature. The transfection mixture was mixed with the 3 ml of complete medium and transferred onto the cells. After 16 hours following transfection, the medium was replaced. After 2 days following transfection, cells were seeded into 96 well-plates by serial dilution and, once cells were attached, transfected cells were selected with G418 (Corning, USA, Cat: 61-234-RF) at 400ug/mL concentration for 15 days by changing the medium containing G418 at every 3 days. Single cell-derived clones were picked up and finally screened by immunoblotting using anti-HA tag (Abcam cat: ab137838) or anti-Myc tag (Abcam cat: ab9106) antibodies.

To generate a HEK293T cell line overexpressing FLAG-tagged human PER2 protein, HEK293T cells in a 12-well plate were transfected with 0.5µg of pENTER-FLAG-PER2-FLAG-His using the PEI transfection reagent as described above. The cells were trypsinized/ re-suspended and seeded into 96-well plates by serial dilutions and candidates were selected with puromycin (Sigma cat no: P8833) at 0.5µg/ml concentration for 2 weeks. Single cell-derived colonies were picked up and screened with Western blotting using an anti-FLAG tag antibody (Sigma cat no: F1804). A HEK293T stable cell line was used for co-IP experiments after it was transiently transfected with HA-tagged interactors

4.2.2. Transient Transfection of Hek-293T Cells

Hek-293T cells were seeded in 60-mm tissue culture dishes at 60-70% confluence and transfected with the plasmids after overnight incubation. 3µg of plasmids harboring the target gene were dissolved in the 75 µl of transfection buffer (100mM NaCl, 10mM Tris-HCl pH 7.5, and 1mM EDTA) and mixed with pipetting several times. Then, 9 µl of 1 mg/ml PEI (polyethyleneimine from Sigma, USA) were added to this solution, mixed well by pipetting, and incubated for 15 minutes at room temperature. The transfection mixture was mixed with the 3 ml of complete medium and transferred onto the cells. After 16 hours following transfection, the medium was replaced with a fresh one. 24h later cells were harvested and cell pellets were kept at -20°C for further analysis.

4.2.3. Lentiviral Infection of U2OS Cells

For the production of lentivirus packages, Hek-293T cells were seeded in 60-mm tissue culture dishes at 60-70% confluence in high-glucose complete DMEM and incubated overnight. Cells should be at 80% confluence at the time of transfection. Packaging plasmids at a total of 4µg (P2A/P2B 2:1), 4µg of plasmid harboring the target gene (pLentIII-p(Bmal1)-3xHA-MiniTurbo-13xLinker-mBmal1 or pLentIII-CMV-3xHA-MiniTurbo-13xLinker) and 24µl PEI (3:1 PEI: Total DNA ratio) were mixed into 300µl transfection buffer and incubated at room temperature for 15 min. Mix was transferred into 3ml fresh DMEM and the medium of cells was changed with DMEM containing transfection mix. Cells were then incubated at 37°C for 6 hours and the medium was changed with complete DMEM. After 72-hour incubation following the transfection time point, the culture medium was collected and spun down for 3-min at 3000 rpm. The medium was filtered by using a 0.2 syringe filter and aliquots of virus-containing medium were kept at -80°C until infection.

U2OS cells were seeded in 60-mm tissue culture dishes at 40-50% confluence and incubated at 37°C overnight. Cells were incubated in 3ml of complete medium containing 1.5µl polybrene (100mg/ml) and virus solution (in the determined amount by virus titration) for 24 hours. Cells were then incubated in fresh medium for 48 hours and selected with puromycin at 1µg /ml for 15 days by changing medium containing puromycin (Sigma Cat:P8833) at every 3 days. Single cell-derived clones were picked up and finally screened by immunoblotting using an anti-HA tag (Abcam cat: ab137838).

4.3. Preparation of Cells for Large Scale Mass Analysis

Stable U2OS-PER2-13xLinker-BioID2-HA, U2OS-pLentIII-p(Bmal1)-3xHA-MiniTurbo-Bmal1, U2OS-Myc-BioID2-13xLinker or U2OS-pLentIII-CMV-3xHA-MiniTurbo-13xLinker cells were seeded in fifteen of 100-mm plates at 40% confluence and the circadian rhythms of the cells were synchronized after 24 hours of seeding by treating cells with 100nM of Dexamethasone (Sigma Cat No: D4902) for 1 hour. Biotin (Bio Basic Inc. Cat: BB0078) was added to cells and incubated for a total of 12 hours for BioID2 and 8 hours for MiniTurbo. Time points 36-48h for BioID2 and 32-44h for MiniTurbo represent the middle time points of biotin application. At the end of the labeling process, cells were collected by scraping from plates, pelleted at low speed, washed with ice-cold PBS and frozen at -80°C until the preparation of the samples for MS analysis.

4.4. Streptavidin Bead Enrichment

Cells were harvested from 15 100mm plates. Cell pellets were lysed in 750µl RIPA lysis buffer (50mM Tris pH:8.0, 150mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100, 1X PI, 1mM DTT) by gentle pipetting and incubated for 10 min at 4°C. Cell lysates were transferred to 15 mL conical tube and then sonicated for 6 sessions with 10

pulses using a Branson Sonifier 250 at 30% duty cycle and an output level of 3. The tube was sat on ice for 20 sec between each session to prevent overheating. Lysates were clarified by centrifugation at 13,300 rpm for 15 min at 4°C. 10µl of lysates were removed for BCA assay. For enrichment of biotinylated material, 250 µL streptavidin-coated magnetic beads (NEB S1420S) were washed twice with 1ml RIPA lysis buffer on DynaMag™-2 magnetic rack (Invitrogen). Beads were incubated with clarified lysates (750µl) with rotation overnight at 4°C. The beads were subsequently washed; once with 1mL of RIPA lysis buffer, once with 1mL of 1M KCl, once with 1 mL of 0.1 M Na₂CO₃, and once with 1 mL of 2 M urea in 10mM Tris-HCl (pH 8.0), twice with 1 mL RIPA lysis buffer, once with 1mL of 10mM Tris-HCl (pH 7.4). The beads were then shipped to Koç University on ice for Mass-Spec analysis.

4.5. Mass Spectrometry Analysis of BioID Samples

To identify the biotinylated proteins by mass spectrometry, on-bead tryptic digestion was performed for each sample at the Koç University Proteomics Facility, Istanbul, Turkey. Briefly, protein-beads complexes were washed with 50 mM NH₄HCO₃ once, and then reduced with 100 mM DTT in 50 mM NH₄HCO₃ at 56°C for 45 minutes, and alkylated with 100 mM iodoacetamide at room temperature in darkness for 30 minutes. Mass spectrometry grade Trypsin Protease (Pierce) was added to the beads for overnight digestion at 37°C (1:100 enzyme/protein ratio). Peptides were analyzed by online C18 nanoflow reversed-phase HPLC (2D nanoLC; Eksigent) linked to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The data sets were searched against the human SWISS-PROT database version 2014_08. Proteome Discoverer (version 1.4; Thermo Fisher Scientific) was used to identify proteins.

4.6. Co-Immunoprecipitation

HEK293T cells expressing mPer2-Flag or hPER2-13xLinker-BioID2-HA were seeded in two 60-mm plates and transfected with plasmids that express candidate interactome with either HA, FLAG tags. Appropriately either Per2 or interactome was precipitated with Anti-FLAG antibodies and the interactions were analyzed with anti-HA tag immunoblotting of precipitated complexes. After 2 days of transfections, cells were rinsed in 1x PBS and subsequently lysed in NP40 lysis buffer (Thermo Fisher Scientific, Cat: FNN0021) supplemented with protease and phosphatase inhibitor cocktail (Sigma, Cat: S8830) for 15 minutes on ice with several pipetting at every 5 minutes. Lysates were centrifuged at 16,000g for 10 minutes and supernatants were transferred to new tubes. Meanwhile 25 μ l of slurry MagnaBind Goat Anti-Mouse IgG magnetic beads (Thermo Fisher Scientific, Cat: 21354) per sample were equilibrated with 200 μ l of NP40 lysis buffer containing 1x protease inhibitor, and 1 μ g of Anti-Flag antibody was added into this for a 1-hr incubation by rotation at 4°C. After taking 25 μ l of input sample, 750 μ l of cleared lysates were incubated with Anti-FLAG/MagnaBind complexes at 4°C by a rotation overnight. For the analysis of interactions, beads were collected on a magnetic stand and were washed 4 times with NP40 lysis buffer supplemented with phosphatase inhibitor cocktail. After removing excess liquid, beads were dissolved in 50 μ l of 2x Laemmli-SDS loading buffer. Input samples were also prepared for SDS-PAGE by adding 25 μ l of 2x Laemmli-SDS loading buffer. For each analysis, 10 μ l of input and 20 μ l of complexes were separated with the appropriate SDS-PAGE according to sizes of proteins and analyzed with immunoblotting.

4.7. Western-Blot Analysis

Cells were harvested with 0.05% trypsin-EDTA solution, transferred into 1.5ml tube and centrifuged at 3000rpm 5min to obtain cell pellet. 4 volume 1x lysis buffer was

added on cell pellet and incubated on ice during 20 min. After incubation cell lysate was centrifuged at 13000rpm 15 min and supernatant was transferred to new 1,5ml tube. Then 1/3 volume 4x Laemmli buffer which contain 10% β -mercaptoethanol, was added on supernatant and stored at -20 °C.

Samples were incubated at 95°C for 10 minutes, and then they were shortly spun down. Samples were loaded each well of the gel respectively. 1X SDS Running Buffer was used for electrophoresis. The gel run at 80 V until proteins reach separating gel, then the voltage might be increased up to 120-150V.

Trans-blot Turbo Transfer System (Bio-Rad Trans-blot Turbo Transfer System, Serial No: 690BR011760) was used for semi-dry transfer. 4 blotting papers and 1 nitrocellulose membrane with the same dimensions of SDS PAGE gel were cut. The blotting paper and the nitrocellulose membrane were incubated with 1X transfer buffer and then a transfer sandwich was created.

Assembling the Blotting Sandwich: The components were assembled on the cassette in the order as follow: Top (-) Cassette electrode (cathode)-Two Blotting papers -Gel-Blotting Membrane-Two blotting papers-Bottom (+) cassette electrode (Anode). A roller was used to remove any air trapped between the layers. Transfer was occurred at 12V constant voltage for 60 min at Trans-blot transfer system.

Membrane was taken into a case filled with blocking solution (5% dry-milk in TBS-T) for 1 hour at room temperature after transfer was completed. The primary antibody was diluted in 5% blocking solution as recommended in its datasheet and the membrane was incubated with it over-night at 4°C. The membrane was washed for four times (5-15-5-5 min. respectively) with 1X TBS-T and was then incubated in horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBST as recommended in it datasheet) for 1 hour at room temperature.

By the end of the secondary antibody incubation, the membrane was washed for four times with TBS-T. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation. (In our laboratory, Western Blotting Detection Kit Advansta Cat: K-12043-D10 and Signal Fire™ ECL Reagent, Cell Signaling

Cat: 6883S substrates are used. Western Blotting Detection Kit Advansta includes Western Bright Peroxide (Component 1) and Western Bright Sirius (Component 2). Working solution mix is prepared with equal aliquot of component 1 and 2. The membrane is exposed to the mix for 2 minutes. Chemiluminescent signals were captured by using an imager (BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545).

4.7.1. Analysis of Biotinylation Activity by Western-Blot

SDS-PAGE running of BioID samples were performed as mentioned above. Membrane was taken into BSA blocking solution (5% BSA in PBS-T) for overnight at 4°C after transfer was completed. HRP-conjugated streptavidin was diluted in 5% BSA blocking solution as recommended in its datasheet and the membrane was incubated for 1 hour at room temperature. The membrane was washed for four times (5-15-5-5 min. respectively) with 1X PBS-T and was then analyzed as mentioned above.

4.8. Immunofluorescence Staining

U2OS-BioID2 or U2OS-PER2-BioID2 cells were seeded onto coverslips in 24-well plates. Next day, cells were fixed with -20°C-cold methanol in a freezer for 10 min, and were blocked with the blocking solution that contained 2% BSA in 1X PBS with 0.1% Triton X-100 (PBS-T) for 1 h. After blocking, the cells were incubated with anti-HA antibody (Cell Signaling Technology, Danvers, MA, cat no: C29F4, 1:800 dilution) in PBS-T for 1 h. Following washing three times with PBS-T, the cells were incubated with Alexa Fluor 555 conjugated-anti-Rabbit IgG (H + L), F(ab')₂ Fragment (Cell Signaling Technology, cat no: 4413S) in PBS-T for 45 min. Then the coverslips were washed three times with PBS-T and stained with 0.1% DAPI (Thermo Fisher Scientific, Hoechst 33342 cat no: 62249) for 15 min in the dark at room temperature. Finally, the cells were washed

with PBS-T twice and with distilled water once. Coverslips were closed with 5µl of Mowiol mounting solution (Sigma-Aldrich, cat no: 81381) and observed under confocal microscopy (Carl Zeiss LSM 880 Airyscan) at X63 magnification. The cellular and nuclear localizations were manually counted under ZOE fluorescence cell imager (Bio-Rad).

4.9. Cytosol-Nuclear Fractionation

U2OS-PER2-BioID cells were used to analyze cytosolic and nuclear fractions of PER2-BioID2-HA recombinant protein using a protocol published previously [Doruk et al., 2020]. Fractionation efficiency was checked with Western blotting using anti-GAPDH antibody (Cell Signaling Technology, cat no: 2118S) as cytosolic marker, anti-Histone-H3 antibody (Abcam, UK, cat no: ab1791) as nuclear marker, and anti-HA antibody for detecting PER2-BioID2-HA protein.

4.10. mRNA Analysis With qPCR

U2OS-BioID2 or U2OS-PER2-BioID2 cells were seeded on 60 mm dishes as described previously [Emisoglu-Kulahli et al., 2021]. Briefly, after harvesting the cells, mRNAs were isolated using NucleoSpin RNA Purification Kit (Macherey Nagel, Germany, cat no: REF 740962) and converted to cDNA using ProtoScript First Strand cDNA Synthesis kit (NEB, Ipswich, MA, cat no: E6300S) with oligo dT option. Finally, cDNAs were subjected to SYBR Green-based RT-qPCR assay using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fischer Scientific, Cat no: K0222). GAPDH was used as an internal control. The primers used in RT-qPCR are listed in Table 3.2.

4.11. Analysis of Mass Spectrometry Results

SAINTexpress version 3.6.3 [Teo et al., 2014] was used as a statistical tool to calculate the probability of each potential PPI using default parameters and background contaminants. Proteins with Saint Score >0.5 were mapped into an interactome network using Cytoscape [Shannon et al., 2003] (in PER2 interactome analysis) and web-based visual analytics platform NetworkAnalyst 3.0 (in BMAL1 interactome analysis) [Zhou et al., 2019]. STRING database was used to retrieve PPIs by using interaction data only obtained from experiments with a confidence score of 0.4 or higher.

The cellular location and functional analysis of PER2 CT36 and CT48 interactome was designed using proteins which have SAINT express score >0.5 by finding a relative subnetwork in high confidence using Omics Integrator, with the optimized parameters of $\eta=0.07$, $\beta=150$, $\omega=3$, $D=1$. K-means clustering was applied on the predicted subnetwork, using clusterMaker in Cytoscape program (<https://cytoscape.org/>) [Shannon et al., 2003]. Additionally, cellular components of the proteins in each cluster were determined using the Uniprot website (www.uniprot.org/). Lastly, the highest statistically significant biological process (BP) terms were indicated for each cluster.

Functional enrichment analysis was performed using DAVID Bioinformatics Resources version 2021 [Thompson et al., 2005; Huang et al., 2009]. Proteins in BioID2 only and MiniTurbo only results were excluded from PER2 and BMAL1 lists respectively and proteins with Saint Score >0.5 were analyzed for characteristics of biological process, cellular component and molecular function and the graphs were drawn using the Python Matplotlib library.

KEGG pathway analysis were performed using DAVID Bioinformatics Resources version 2021 and visualized by the algorithm designed by us. The font size of each pathway was determined by their $-\log_{10}$ (p-value) scores using the algorithm that gives the path with the highest $-\log_{10}$ (p-value) the highest font size.

4.12. Statistical Analysis

All data were expressed as means $\pm$ standard error of the means (SEM) for each studied variable unless stated otherwise. Statistical analyses were performed using GraphPad Prism for Windows (GraphPad Software, San Diego, CA). The statistical significance of differences between groups was validated with paired t-test for immunoblotting and qPCR results. P values were indicated in the 33hosph legends.



5. RESULTS

The main purpose of the thesis was to discover new interaction partners of PER2 and BMAL1 proteins. To this end, we adapted proximity-dependent techniques suitable for detecting weak and transient interactions, to our study. As described above, proximity-dependent labeling relies on the biotinylation of proteins at a distance by a biotin ligase. Efficiency and the timing of the biotinylation process depend on the source of the biotin ligase. In our study, we used two different biotin ligases; *A. Aeolicus* BirA* (BioID2) and MiniTurbo, a mutant form of wt *E.coli* BirA, to discover PER2 and BMAL1 interactions respectively.

We firstly expressed the *Per2* gene with the BioID2 enzyme on the C-terminal domain under the CMV promoter in U2OS cells. We collected high-throughput MS data at subjective day and night and validated several PER2 interactions using classical co-IP technique. Having obtained a promising list of interaction candidates for PER2, we then analyzed BMAL1 interaction partners. Since MiniTurbo has 10 min biotinylation time (BioID is 16h), it would be more useful to detect the difference of interaction partners at subjective day and night. Therefore we expressed the BMAL1 gene with the MiniTurbo enzyme in U2OS cells. We cloned the MiniTurbo gene into the N-terminal region of BMAL1 because the transactivation domain (TAD) is located in C-terminal. TAD is a regulatory hub for transcriptional coactivators and repressors and has a role in the period determination of the mammalian circadian clock [Gustafson et al., 2017], so we cloned MiniTurbo to the N-terminal region of the Bmal1 to avoid disruption of the binding of TAD interactors. We firstly expressed MiniTurbo-Bmal1 under the CMV promoter in U2OS cells to test and optimize the MiniTurbo technique for the BMAL1 interactome. Once we obtained a solid interactome map from MT-BMAL1 data, we then cloned MiniTurbo-Bmal1 under the native Bmal1 promoter to compare the results with CMV-MiniTurbo-BMAL1 and to check if there is a difference between opposite circadian

phases. Per2 and Bmal1 results were given under separate headings in this section to avoid confusion and to facilitate interpretation.

5.1. PER2 Interactome Analysis

5.1.1. Stable Expression of PER2-BioID2 and Myc-BioID2 in U2OS Cell Line

As described above, we aimed to identify PER2 interactome partners using a proximity-dependent labeling technique. For this purpose, BioID2-HA was tagged to the C-terminal part of PER2 and stably expressed in U2OS cells which have a high amplitude of the circadian rhythm. We also established a stable U2OS cell line expressing BioID2 only (Myc-BioID2) to use it as a control cell to subtract nonspecific proteins that were randomly tagged by BioID2. We expressed Myc-BioID2 in the control cell line instead of BioID2-HA because it did not contain a start codon. Stable expressions of PER2-BioID2-HA and Myc-BioID2 proteins were analyzed by Western Blot using appropriate antibodies (Figure 5.1A left and middle). In figures and legends, U2OS-PER2-BioID2 and U2OS-BioID2 cell lines were shortly named PER2-BioID2 and BioID2, respectively. Here Myc-BioID2 and PER2-BioID2-HA protein expressions were confirmed and demonstrated with mock control (un-transfected U2OS cells). PER2-BioID2 and endogenous PER2 mRNA levels were relatively compared using quantitative polymerase chain reaction (qPCR). Since total PER2 mRNAs collected from U2OS-PER2-BioID2 contain both endogenous and PER2-BioID2 mRNAs, but mRNAs from U2OS-BioID2 cells only contain endogenous PER2, we compared the amounts of these total mRNAs and we detected approximately 8-fold greater PER2 mRNA expression in U2OS-PER2-BioID2 cells than in U2OS-BioID2 cells (Figure 5.1A right). Because we aimed to identify PER2 interaction partners at opposite time periods, we selected a clone with relatively low expression of PER2-BioID2 since the overexpression of PER2 effect circadian oscillation. To reset the circadian rhythm of U2OS-PER2-BioID2 and U2OS-BioID2 expressing cell lines, we used dexamethasone as a clock-resetting agent and collected protein lysates at CT (circadian time) of 30- and 42-h (CT30 and CT42) after reset (Figure 5.1B). Because the first 16–24

h time slot is not preferred 36hospho analysis of circadian oscillation [Balsalobre et al., 1998] we choose CT30 and CT42. These time points correspond to the middle of the 12-h biotinylation incubations, which will be covered in more depth later in the text. Briefly, CT30 and CT42 samples are representative samples to investigate the oscillation of PER2 stability for CT36 and CT48 biotinylation samples, respectively. We analyzed PER2-BioID2 expression at CT30 and CT42 by Western Blotting using an anti-HA antibody to check if there is an oscillation in recombinant PER2-BioID2 level (Figure 5.1B). Results showed a significant difference in PER2-BioID2 protein level at two different time points (Figure 5.1B, right side—the quantitation graph). Total mRNA analysis of PER2-BioID2 and endogenous PER2 in U2OS-PER2-BioID2 cell line and endogenous PER2 in U2OS-BioID2 cell line at CT30 and CT42 was also consistent with Western Blotting results (Figure 5.1C). Total PER2-BioID2 and endogenous PER2 mRNAs in the U2OS-PER2-BioID2 cell line showed a 3-fold difference between CT30 and CT42. Considering a 3-fold difference in protein level between time points in the U2OS-PER2-BioID2 cell line (Figure 5.1B, right) and 8-fold higher Per2 mRNA level in U2OS-PER2-BioID2 compared with U2OS-BioID2 cell line (Figure 5.1A, right), endogenous PER2 mRNA level was negligible relative to PER2-BioID2 mRNA level. To check the circadian rhythm in the U2OS-PER2-BioID2 cell line with a clock-controlled gene, we analyzed circadian clock-regulated 36hospho D site-binding protein (DBP) mRNA level at CT30 and CT42 time points (Figure 1C, right side). DPB results also proved that CT30 and CT42 were suitable time points to analyze PER2 interactions present at opposite time points. However, since BioID2 has a 12h biotinylation rate (in our study), we started the biotin treatment 6 hours before at CT24 and CT36, covering 30 and 42 hours respectively, and collected samples 6 hours later at CT36 and CT48. In theory, we expected to obtain different PER2 interaction partners at opposite time points even if there were several common proteins, however, most of the interactors were shared by CT36 and CT48 data because the 12-h labeling process was not appropriate to distinguish between the individual circadian phases. When we noticed this situation we continued with MiniTurbo which has a 6h biotinylation rate, to analyze the BMAL1 interactome.

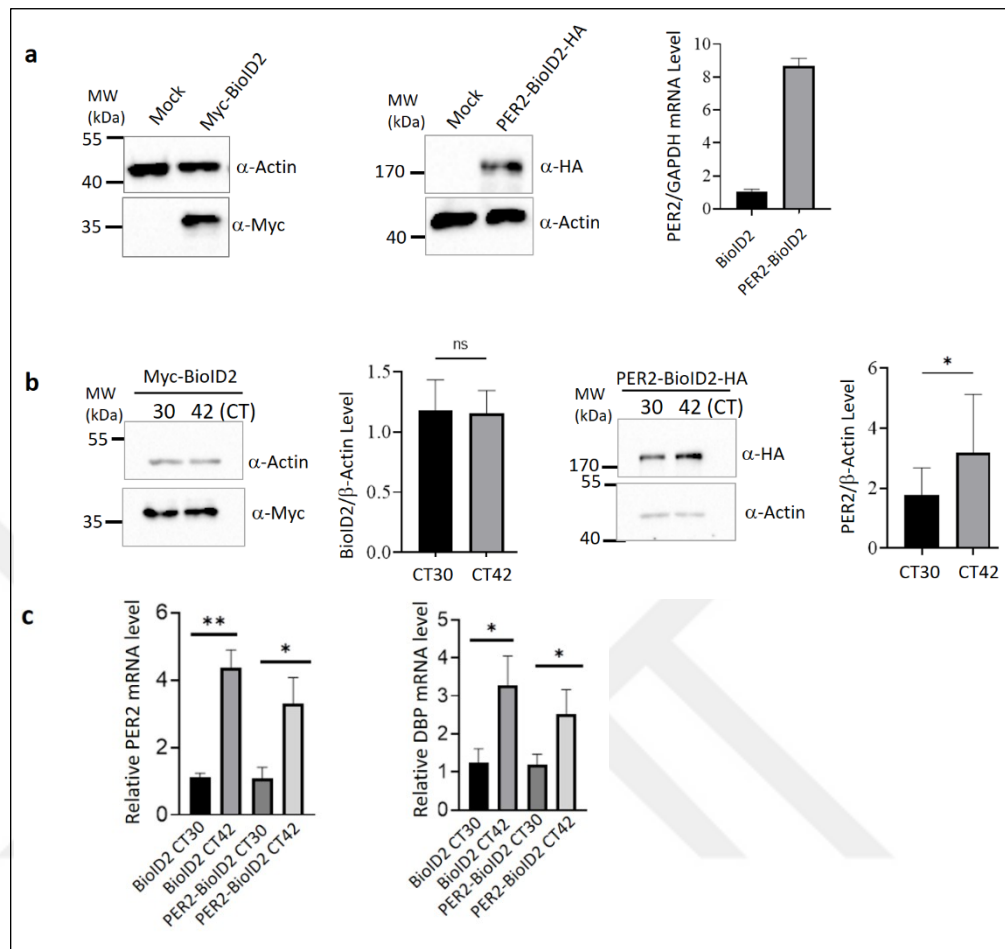


Figure 5.1: Characterization of the stable U2OS cell line expressing U2OS-PER2-BioID2. (A) Western blot analysis of recombinant protein expressions in stable U2OS cell lines. Actin protein was used as loading control. Mock control is un-transfected U2OS cell line. Mean difference at PER2 transcript levels between U2OS-PER2-BioID2 and U2OS-BioID2 was 7.658 (p-value = 0.0019). (B) Differential expression of PER2-BioID2 protein after dexamethasone treatment at CT30 and CT42. Recombinant PER2 level difference between CT30 and CT42 was significant (p-value = 0.0307) in PER2-BioID2 cells while Myc-BioID2 level different between CT30 and CT42 was not significant (p-value = .8940) as anti-Myc or anti-HA signals were normalized to actin levels. MW (molecular weight) are given in kDa. CT, circadian time (in hours). Data were expressed as mean $\pm$ SEM. (C) qPCR analysis of circadian clock-regulated genes after dexamethasone treatment at CT30 and CT42. PER2 expression differences between CT30 and CT42 samples were significantly different between U2OS-BioID2 (p-value = 0.0088) and U2OS-PER2-BioID2 (p-value = 0.0456) cells. DBP expression differences between CT30 and CT42 samples were significantly different in both U2OS-BioID2 (p-value = 0.0235) and U2OS-PER2-BioID2 (p-value = 0.0236) cells. Similar fold inductions (CT42 over CT30) for both DBP and PER2 transcripts were observed in both cell lines.

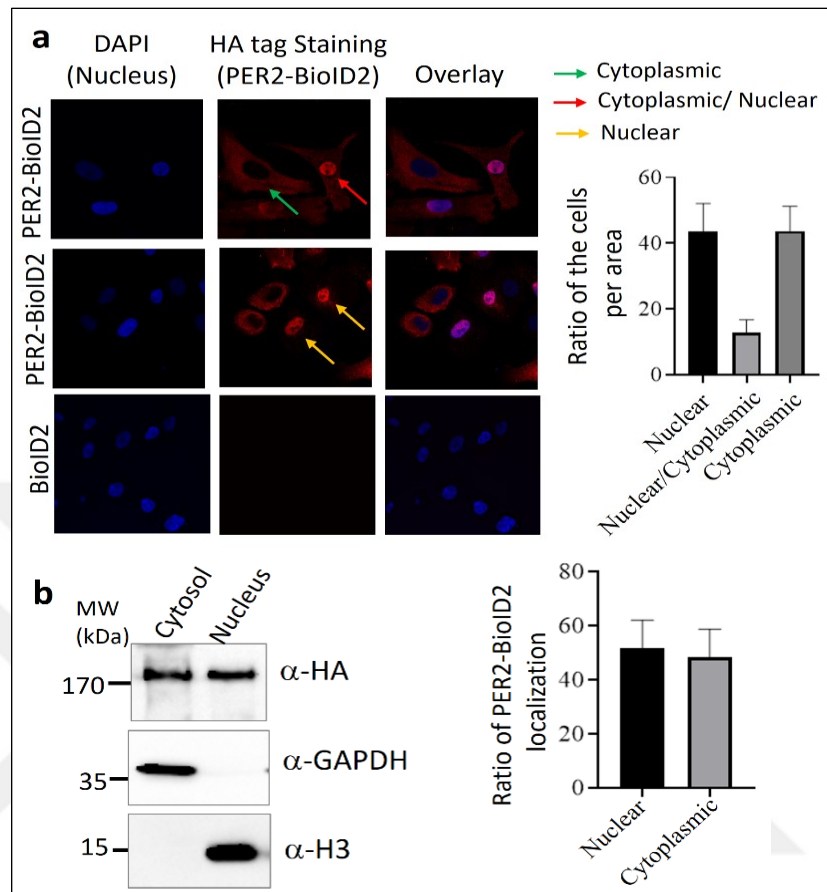


Figure 5.2: Analysis of cellular localization of PER2-BioID2. (a) Representative images of PER2-BioID2 probed with anti-HA antibody in immunofluorescence assay show that PER2-BioID2 can be found in cytoplasm or nucleus. Quantitation from three assays as shown on the right panel indicates that PER2-BioID2 can be found in both cytoplasmic and nuclear subcellular locations. Data were expressed as mean $\pm$ SEM. (b) Determination of cytoplasmic and nuclear fractions of U2OS-PER2-BioID2 using subcellular fractionation. Quantitation of two independent experiments reveals that the recombinant PER2-BioID2 can be found in both fractions (Right panel).

We also analyzed the cellular localization of PER2-BioID2 by immunofluorescence staining (Figure 5.2A) and subcellular fraction assay (Figure 5.2B). PER2-BioID2 stained by anti-HA antibody in immunofluorescence assay showed that PER2-BioID2 can be localized in cytoplasm or nucleus. Cytoplasmic and nuclear fractions of U2OS-PER2-BioID2 cells probed with anti-HA antibody (for PER2-BioID2), anti-H3 antibody (as a nuclear marker), and anti-GAPDH antibody (as a cytoplasmic marker) also indicated that PER2-BioID2 can be found in both.

5.1.2. Optimization of Biotinylation Time and Sample Preparation for MS Analysis

As stated in the introduction section, BioID2 has a 16 hours biotinylation rate, but since we aimed to analyze PER2 interaction partners in individual circadian periods, we tested different biotinylation times and observed a clear pattern of biotinylation with a 12-hour incubation with 50 μ M biotin (Figure 5.3A). After optimizing the biotinylation time to 12 hours, which is suitable for analyzing lower circadian phases, we tested the specificity of the biotin labeling using un-transfected U2OS cells as mock control and found a specific biotinylation pattern in PER2-BioID2 samples compared with mock control and BioID2 control (Figure 5.3B). To analyze the biotinylation pattern at individual circadian times (CT36 and CT48) we incubated U2OS-Per2-BioID2 cells with 50 μ M biotin for 36h and 48h after dexamethasone treatment. Although some protein bands showed distinct band patterns in opposite CT phases (Figure 5.3C, blue arrows), the overall biotinylation profile at the two phases was evidence of a strong biotinylation pattern rather than an oscillation. We then prepared large-scale biotinylation set for MS analysis and isolated biotinylated proteins by pull-down using streptavidin-magnetic beads. We then analyzed the purified samples by Western Blotting to check the yield (if the beads had some biotinylated proteins) before sending them for MS analysis (Figure 5.3D). To identify biotinylated proteins by mass spectrometry, an on-bead tryptic digestion protocol was performed with two technical replicates for each sample as described in the Methods section. The statistical significance of PER2-BioID2 interactions after subtraction of interactions of BioID2 (indicating non-specific interactions) was analyzed using a statistical analysis algorithm as described as follows.

5.1.3. Identification of High Confidence Protein Interactors Using SAINT Express Analysis

We used a label-free quantitation method, the SAINT express algorithm which determines the likelihood of each protein interacting with the bait protein [Teo et al.,

2014]. We identified 46 and 72 proteins for CT36 and CT48, respectively, with a SAINT express score = 1. These candidates were considered to be high-confidence PER2-protein interactors. Additionally, the number of interactome candidates with Saint express score >0.5 and BFDR value <0.05 were clearly higher at CT48 (215) than at CT36 (112) (Figure 5.4A). Interactome candidates of CT36 and CT48 with Saint express score >0.5 and BFDR value <0.05 were listed in Table D1.1 and Table D1.2 respectively.

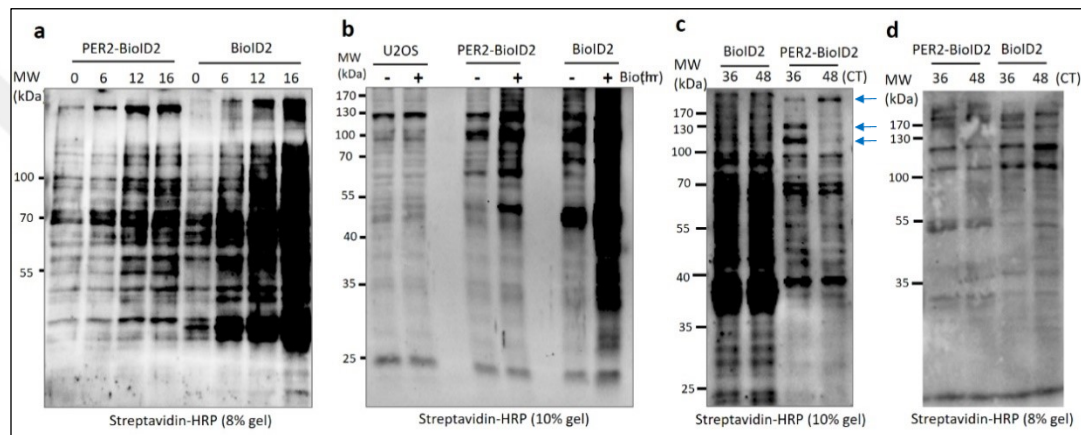


Figure 5.3: Determination of biotinylation conditions and preparation of the cell lines for mass spectrometry analysis. (a) Cells were incubated for different time slots to see whether a biotinylation shorter than 16-h would produce a significant biotinylation pattern. (b) Testing the septicity of the biotin labeling in U2OS-Per2-BioID2 cells comparing with un-transfected U2OS cells (c) Analysis of biotinylation pattern at opposite circadian phases, CT36 and CT48. (d) Confirmation of biotinylated samples on beads before mass spectrometry analysis.

5.1.4. Functional Annotation Clustering of Identified Protein Candidates

We clustered interactome candidates with Saint Scores >0.5 for characteristics of biological process, cellular component, and molecular function (Figure 5.4B–D, respectively and Figure 5.5). We grouped the interactive candidates using the DAVID 2021 bioinformatics resources and the clusterMaker program, and visualized them using the Python Matplotlib library (Figure 5.4B–D) and Cytoscape (Figure 5.5), respectively. We also analyzed the KEGG pathways of PER2-BioID2 interaction partners with Saint Scores >0.5 for both CT phases using DAVID 2021 bioinformatics resources (Figure 5.4E,F). The font size of each pathway was determined by their $-\log_{10}$ (p-value) scores using an algorithm that gives the path with the highest $-\log_{10}$ (p-value) the highest font size. Considering overall data, CT48 had more interactions compared to CT36 and both CT phases share many interaction candidates (Figure 5.4A) which means that 12-h labeling does not allow to separate sub-circadian phases of the interactome. However, they can be considered as biological replicates of each other, possibly with different sample sizes.

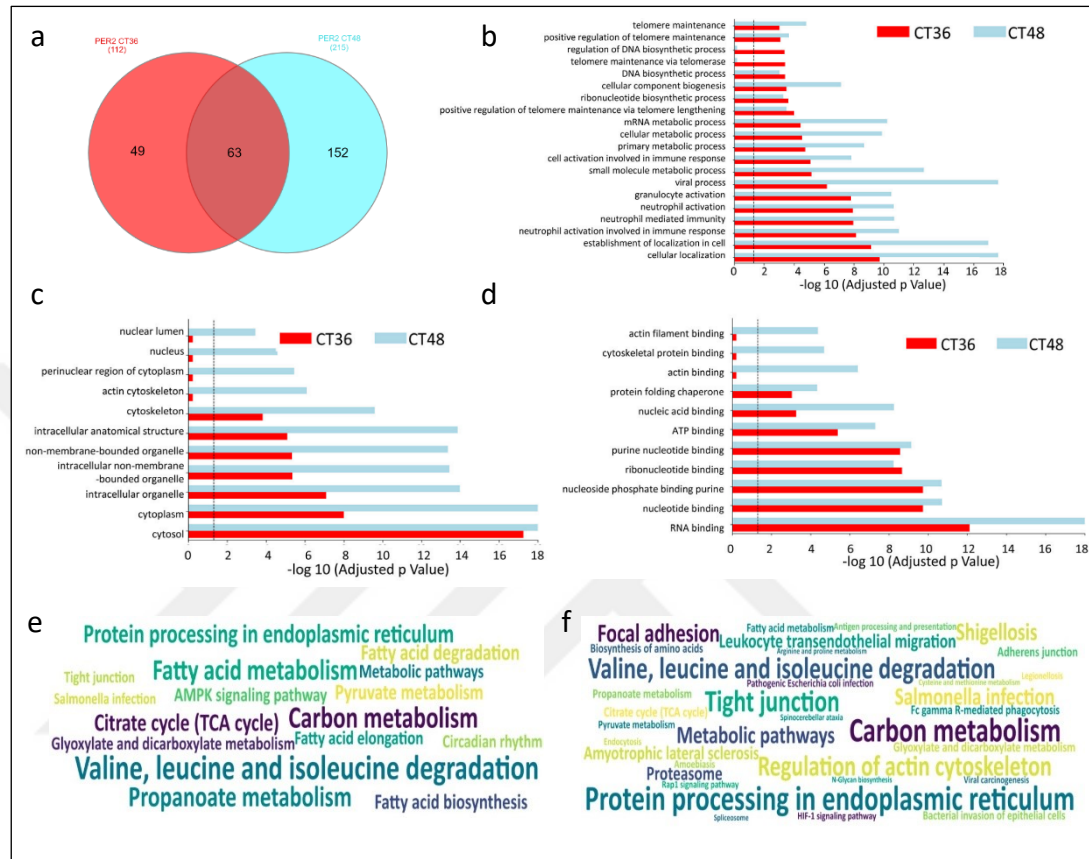


Figure 5.4: Schematic illustration, functional clustering and KEGG pathway analysis of PER2 interaction candidates. Only interaction candidates with Saint express score >0.5 and BFDR value <0.05 in individual CT phases were taken. (A) Common and uncommon interaction numbers at CT36 and CT48 were illustrated using the web-based Interactivenn tool [Heberle et al., 2015]. CT36 and CT48 interactome were analyzed for (B) biological process, (C) cellular component, and (D) molecular function and visualized using Python Matplotlib library. Annotation of the proteins obtained from SAINT express analysis was performed. The representative highest statistically significant terms using a p-value cutoff of 0.1 were demonstrated in the horizontal bar plot for both CT36 and C48 conditions. KEGG pathway analysis of PER2 CT36 I and CT48 (F) interactome was performed using DAVID 2021 bioinformatics resources and visualized using an algorithm created by us. The font size of each pathway was determined by giving the path with the highest $-\log_{10}$ (p-value) the highest font size.

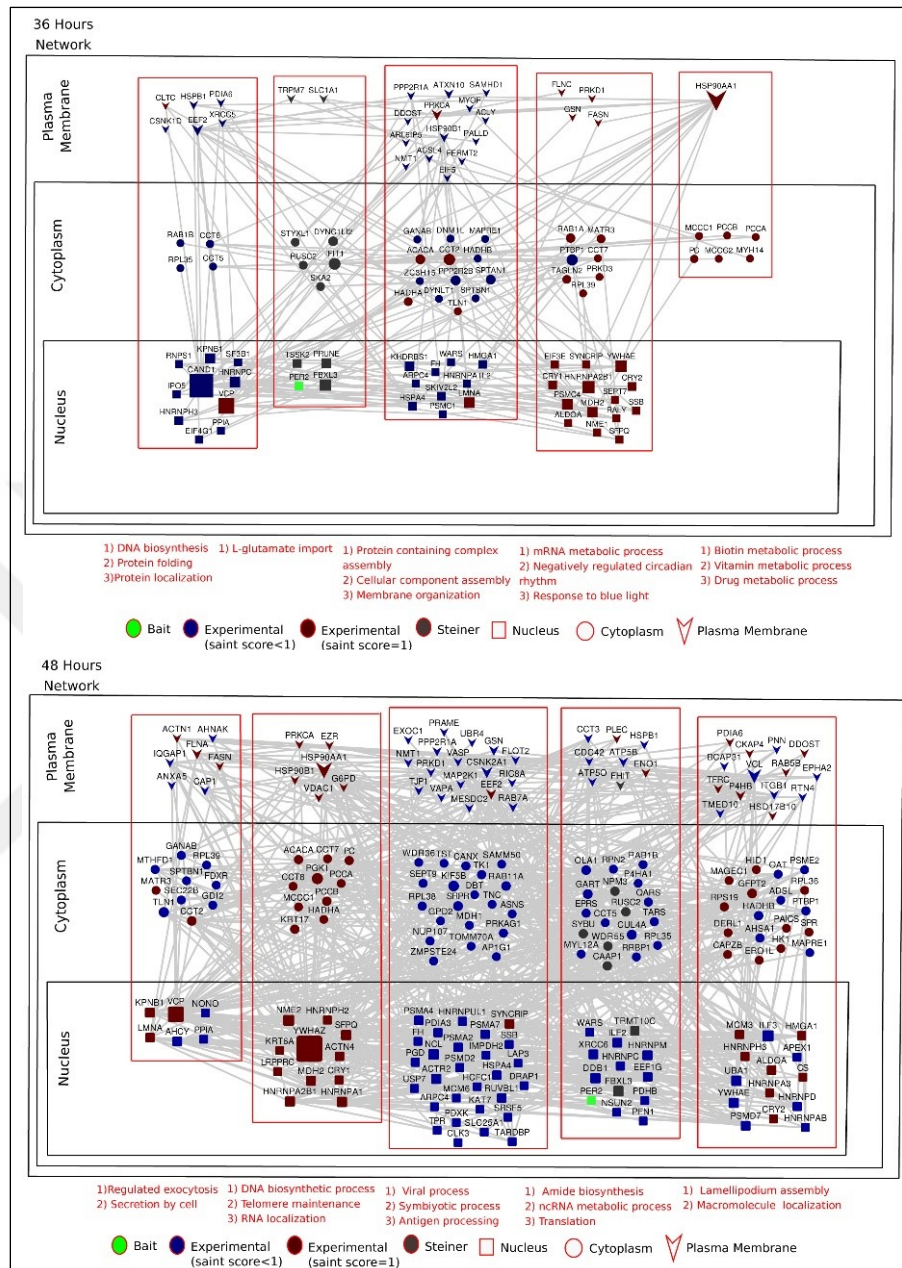


Figure 5.5: The cellular location and functional analysis of PER2 CT36 and CT48 interactome. An interaction network of the proteins which have SAINT express score >0.5 and BFDR value less than 0.05 was predicted by finding a relative subnetwork in high confidence using Omics Integrator, with the optimized parameters of $\eta=0.07$, $\beta=150$, $\omega=3$, $D=1$. K-means clustering was applied on the predicted subnetwork, using clusterMaker in Cytoscape program (<https://cytoscape.org/>). Additionally, cellular components of the proteins in each cluster were determined using the Uniprot website (www.uniprot.org/). Lastly, the highest statistically significant biological process (BP) terms were indicated for each cluster.

5.1.5. Network Analysis of Identified Protein Candidates

We analyzed PER2 interaction partners with SAINT express score >0.5 and BFDR value <0.05 to draw PER2 interactome network (Figure 5.6). STRING database [Szklarczyk et al., 2014] was used to retrieve PPIs by using interaction data only obtained from experiments with a confidence score of 0.4 or higher. Maximum of 50 additional interactors per protein were allowed while constructing the network. Cytoscape was used to visualize and analyze networks. yFiles Organic Layout algorithm was used to cluster genes. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). The height and width of the nodes were calculated according to betweenness centrality and degree, respectively. Proteins identified in our proteomics analysis were shown in blue nodes, however, green nodes are additional proteins added by STRING to construct networks. The highest betweenness values of nodes were used to determine the top bottleneck proteins. Since the interaction of ribosome binding and proteasome binding proteins were highly identified, they occupy most of the PPI network. They were, therefore, excluded from network analysis to show other interacting proteins clearly. Network analysis showed an obvious difference in the node numbers between CT36 and CT48 networks. CT36 network was clearly smaller than CT48; this indicates that the numbers of proteins that are labeled with higher PER2-BioID2 amount in CT48 compared to CT36. However, it was also seen that both CT maps share most of the networks because 12-h labeling did not allow to differentiate circadian phases. Even though HSP90AA1 protein is shared in the top bottleneck proteins of CT36 and CT48 and others are unique to either CT36 or CT48, a few members of heterogeneous nuclear ribonucleoproteins (HNRN) are shared in the top bottleneck proteins of CT36 and CT48. Therefore, it is recommended that PER2 interaction partners in individual circadian phases can be analyzed with another proximity-dependent labeling technique with a shorter biotinylation time, such as MiniTurbo.

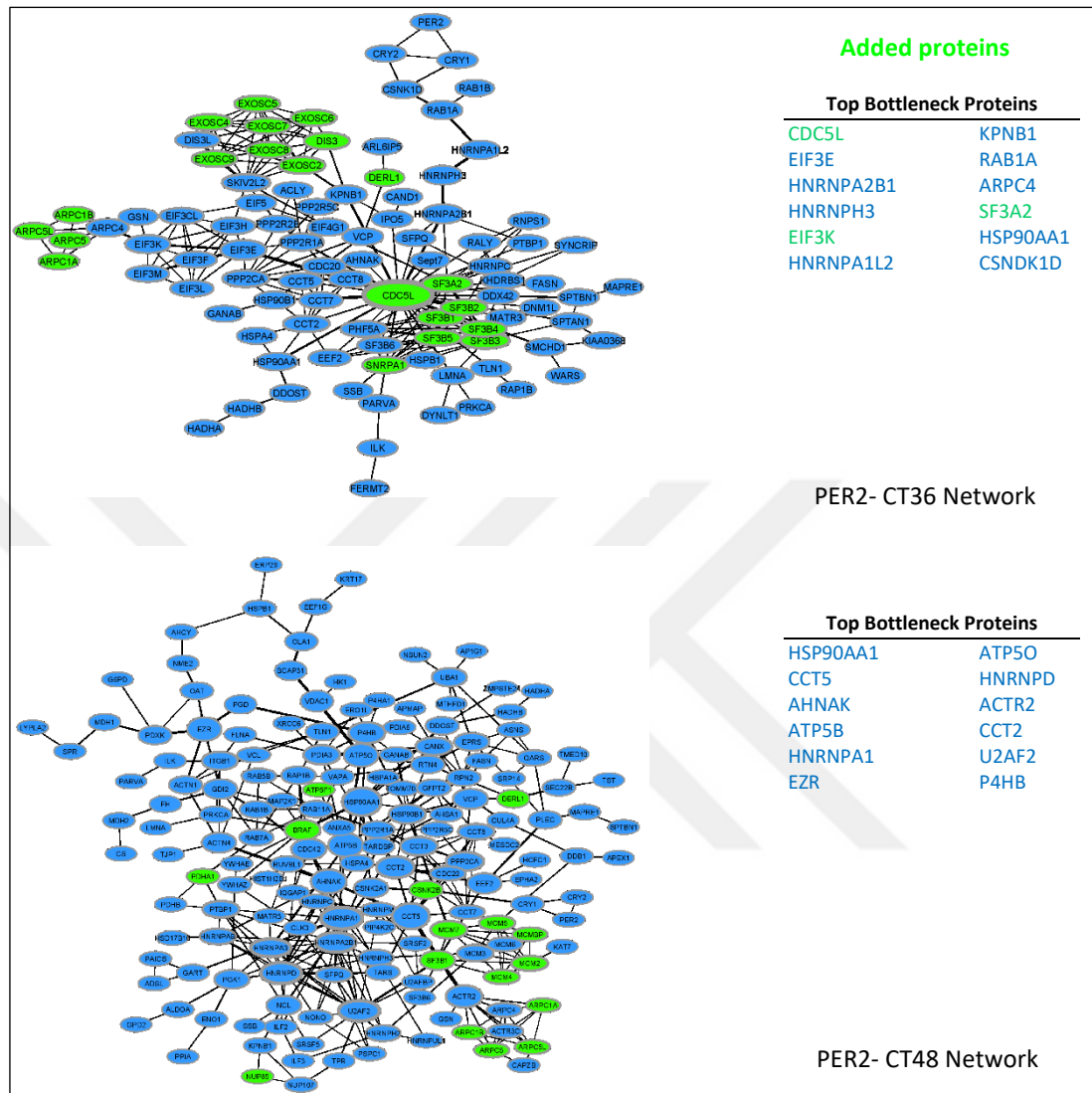


Figure 5.6: Network analysis of PER2 interactome. PER2 interaction partners with Saint express score >0.5 and BFDR value <0.05 were used to draw PER2 interactome network. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). The highest betweenness value of nodes were used to determine the top bottleneck proteins. Ribosome binding and proteasome binding proteins were excluded from the network since they were highly identified and occupy most of the PPI network. Proteins identified in our proteomics analysis were shown in blue nodes, and green nodes are additional proteins added by STRING to construct networks.

5.1.6. Experimental Confirmation of Selected Protein Candidates

We have identified novel PER2 interactors with a SAINT express score = 1 based on SAINT express analysis. We selected five candidates from SAINT express analysis to confirm the interactions using co-IP. Two core circadian clock proteins, CRY1 and CRY2 had a Saint Score = 1. Therefore, we first co-IPed these two proteins to see if we can also confirm these interactions in our experimental conditions. For co-IP studies, we preferred HEK293T cell line over U2OS cell line, because of the higher transfection efficiency and protein yield. Therefore, we immunoprecipitated FLAG-PER2-FLAG from a HEK293T stable cell line using an anti-FLAG antibody and checked the presence of endogenous CRY1 and CRY2 proteins (Figure 5.7A,B). The proteome analysis revealed the core clock components CRY1 and CRY2 as interactors of PER2 but failed to catch BMAL1 as an interactor. Therefore, BMAL1 was probed to find out whether our assay missed some known strong interactors. (Figure 5.7C). This suggested that BioID and classical co-IP coupled to MS analysis could give the best results when they are used as complementary assays. We then transiently expressed the selected novel interactor candidates in the HEK293T-FLAG-PER2-FLAG stable cell line. After immunoprecipitation of PER2 with anti-FLAG antibody, HA-tagged interactors such as HK1 (Hexokinase-1), MCM3 (Minichromosome Maintenance Complex Component 3), and IMPDH2 (Inosine-50 – monophosphate dehydrogenase 2) were probed with anti-HA tag antibody (Figure 5.7D–F). Here, we especially selected candidates with different functions (which is discussed later). These findings were important, especially for the BioID experiment, because co-IP results verified that BioID2 tagging of PER2 at the C-terminus does not significantly interfere with PER2 protein activity. On the other hand, BMAL1 was not in the range of the BioID2 or it was masked by abundant proteins of the interactome that were biotinylated by BioID.

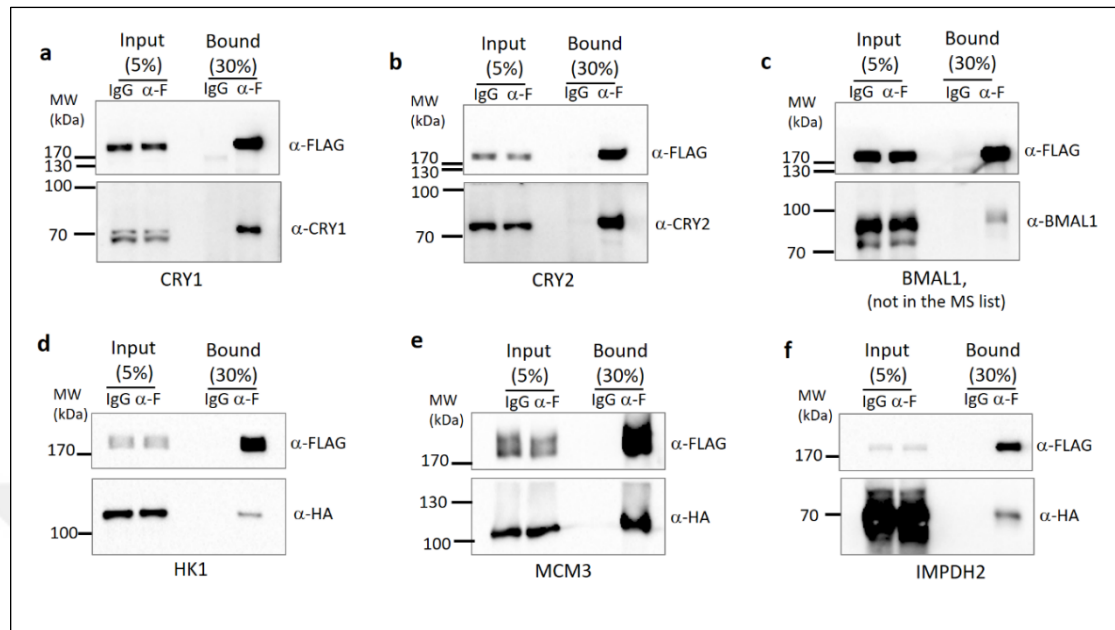


Figure 5.7: Confirmation of selected interactions using the co-immunoprecipitation method. Five proteins from the interactome were selected for validation using the co-immunoprecipitation assay. HEK293T cells expressing FLAG tagged-PER2 (F-PER2-F) was used to 47hosphor4747ipitated PER2 from cell lysates. (A) CRY1 and (B) CRY2 were already known to interact with PER2 strongly and this was confirmed with specific antibodies in the precipitated complex. (C) BMAL1 was not found in BioID2 analysis, but it is known to be found in the PER2 complex. (D–F) Confirmation of selected novel interactors by Western blot analysis. (D) HK1, I MCM3, and (F) IMPDH2 could be detected in FLAG-PER2 precipitated complex as blotted with anti-HA tag antibody. F, Anti-FLAG precipitated complex; IgG, control IgG precipitated complex. Between input and bound samples, one lane was loaded with 1X Laemmli loading dye in order to prevent the masking of signal of bound samples from strong input samples. MW (molecular weight) marker bars represent the protein sizes in kDa.

5.2. (CMV)-MiniTurbo-BMAL1 Interactome Analysis

5.2.1. Stable Expression of (CMV)-MiniTurbo-BMAL1 and HA-MiniTurbo in U2OS Cell Line

After getting promising results from the PER2 interactome obtained by the MS-based proximity-dependent labeling technique, we analyzed the BMAL1 interactome with MiniTurbo labeling, an upgraded version of the BioID technique. For this purpose, HA-MiniTurbo (HA-MT) was tagged to the N-terminal region of BMAL1 and stably expressed in U2OS cells which have a high amplitude of the circadian rhythm. We also established a stable U2OS cell line expressing MiniTurbo only to use it as a control cell to subtract nonspecific proteins that were randomly tagged by MiniTurbo. Stable expressions of HA-MT-BMAL1 and HA-MT proteins were analyzed by Western Blot using appropriate antibodies (Figure 5.8A). In figures and legends, U2OS-HA-MT-BMAL1 and U2OS-HA-MT cell lines were shortly named MT-BMAL1 and HA-MT, respectively. Here HA-MT and MT-BMAL1 protein expressions were confirmed and demonstrated with mock control (un-transfected U2OS cells). To reset the circadian rhythm of U2OS-HA-MT-BMAL1 and U2OS-HA-MT expressing cell lines, we used dexamethasone as a clock-resetting agent and collected protein lysates at CT (circadian time) of 24- and 36-h (CT24 and CT36) after reset (Figure 5.8B). We analyzed MT-BMAL1 expression at CT24 and CT36 by Western Blotting using an anti-BMAL1 antibody to check if there is an oscillation in recombinant MT-BMAL1 level (Figure 5.8B). Results showed there was no difference in MT-BMAL1 protein level at two different time points although there was a relative difference in endogenous BMAL1 expression level in U2OS-HA-MT cell lines (Figure 5.8B, the quantitation graph). In this step, our main purpose was not to analyze BMAL1 interaction partners in their individual circadian phases but to detect new BMAL1 interactome candidates and test MiniTurbo, an upgraded version of the BioID technique, therefore we continued without considering circadian times.

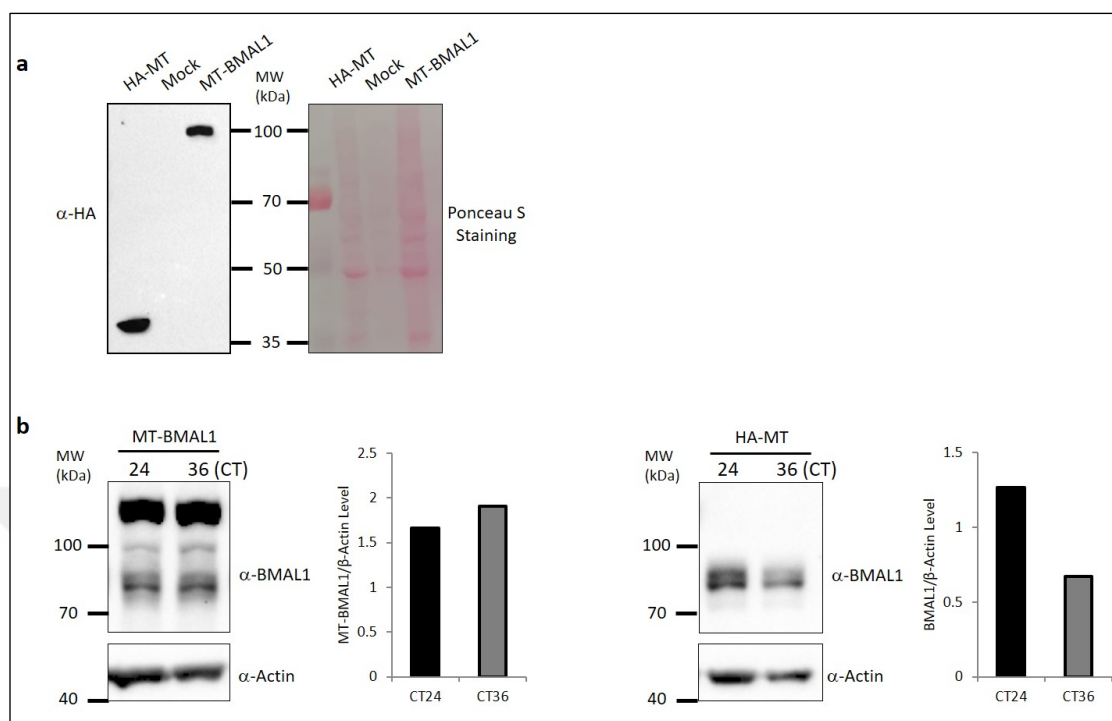


Figure 5.8: Characterization of the stable U2OS cell line expressing U2OS-MT-BMAL1. (A) Western blot analysis of recombinant protein expressions in stable U2OS cell lines. Ponceau S staining was used as a loading control. Mock control is an un-transfected U2OS cell line. (B) Differential expression of MT-BMAL1 protein after dexamethasone treatment at CT24 and CT36. Recombinant BMAL1 level was not different at CT24 and CT36 in U2OS-HA-MT-BMAL1 cells while endogenous BMAL1 level was relatively different between CT24 and CT36 in U2OS-HA-MT cell lines. Anti-BMAL1 signals were normalized to actin levels. MW (molecular weight) is given in kDa. CT, circadian time (in hours).

5.2.2. Optimization of Biotinylation Time and Sample Preparation for MS Analysis

As stated in the introduction section, it is known that MiniTurbo has 10 min biotinylation rate, but we wanted to test its speed in different biotinylation times because it can serve the difference in the stable U2OS lines. We tested 4 different biotinylation times from 0h (10min) to 6 hours with 800 μ M biotin and identify a good biotinylation pattern 6 hours after biotin treatment in U2OS-HA-MT-BMAL1 cell lines (Figure 5.9A). Although MiniTurbo had a 6h biotinylation speed in U2OS-HA-MT-BMAL1

cell lines rather than 10 min [Branon et al., 2018], it was still two times lower than the speed of BioID2. After optimizing the biotinylation time to 6 hours, we tested the specificity of the biotin labeling using un-transfected U2OS cells as mock control and found a specific biotinylation pattern in MT-BMAL1 samples compared with mock control and HA-MT control (Figure 5.9B). We prepared two set of large-scale biotinylation for MS analysis (Figure 5.9C). We isolated biotinylated proteins by pull-down using streptavidin-magnetic beads. To identify biotinylated proteins by mass spectrometry, an on-bead tryptic digestion protocol was performed with two technical replicates for each sample as described in Methods section. The statistical significance of MT-BMAL1 interactions after subtraction of interactions of MT (indicating non-specific interactions) was analyzed using a statistical analysis algorithm as described as follows.

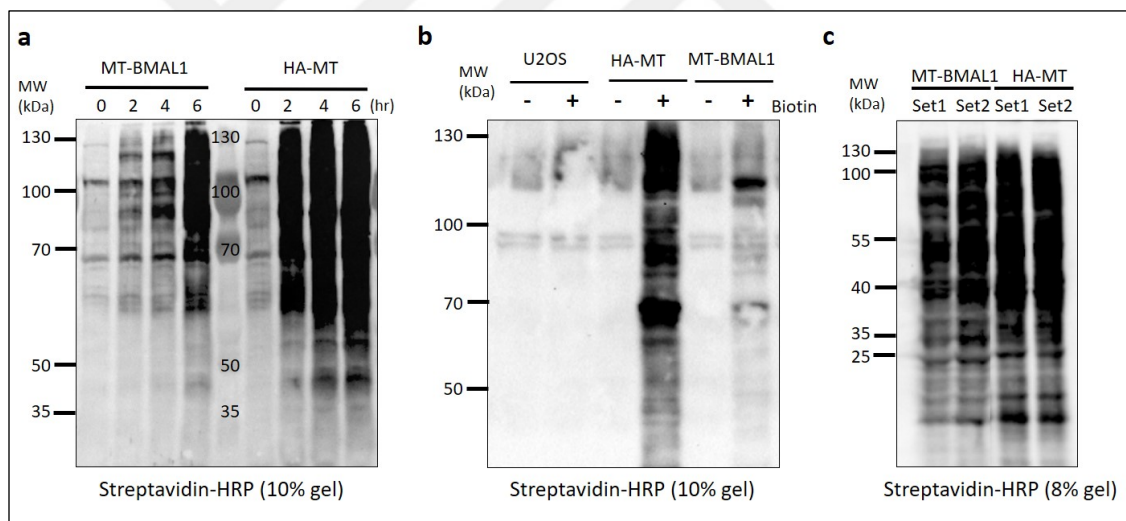


Figure 5.9: Determination of biotinylation conditions and preparation of the cell lines for mass spectrometry analysis. (a) Cells were incubated with 800 μ M biotin for different time slots from 0h (10min) to 6h to test the biotinylation rate of MiniTurbo in U2OS-HA-MT-BMAL1 cell lines. (b) Testing the specificity of the biotin labeling in U2OS-HA-MT-BMAL1 cells compared with un-transfected U2OS cells (c) Screening the biotinylation pattern of two large-scale MT-BMAL1 samples (input samples) before mass spectrometry analysis.

5.2.3. Identification of High Confidence Protein Interactors Using SAINT Express Analysis

We used the SAINT express algorithm as we did for PER2 interactome analysis, to determine the likelihood of each protein interacting with the bait protein. We couldn't identify any interaction partner in Set1 with SAINT express score = 1 and did identify only 5 interactors in Set2. Although most interaction candidates have SAINT express scores lower than 1, we identified BMAL1 itself in Set2 indicating the specificity of the labeling. Interactome candidates of Set1 and Set2 with Saint express score >0.5 were listed in Table D1.3 and Table D1.4 respectively. Here, only top bottleneck proteins identified in network analysis were listed. The rest of the list is planned to be submitted in a later publication.

5.2.4. Functional Annotation Clustering of Identified Protein Candidates

We clustered interactome candidates with Saint Scores >0.5 for characteristics of biological process, cellular component, and molecular function (Figure 5.10A–C, respectively). We merged interactome candidates with Saint Scores >0.5 in two different sets (104 interactions) and grouped them using DAVID 2021 bioinformatics resources and visualized them in excel (Figure 5.10A-C). We also analyzed the KEGG pathways of MT-BMAL1 interaction partners with a Saint score >0.5 using DAVID 2021 bioinformatics resources (Figure 5.10D). The font size of each pathway was determined by their $-\log_{10}$ (p-value) scores using an algorithm that gives the path with the highest $-\log_{10}$ (p-value) the highest font size.

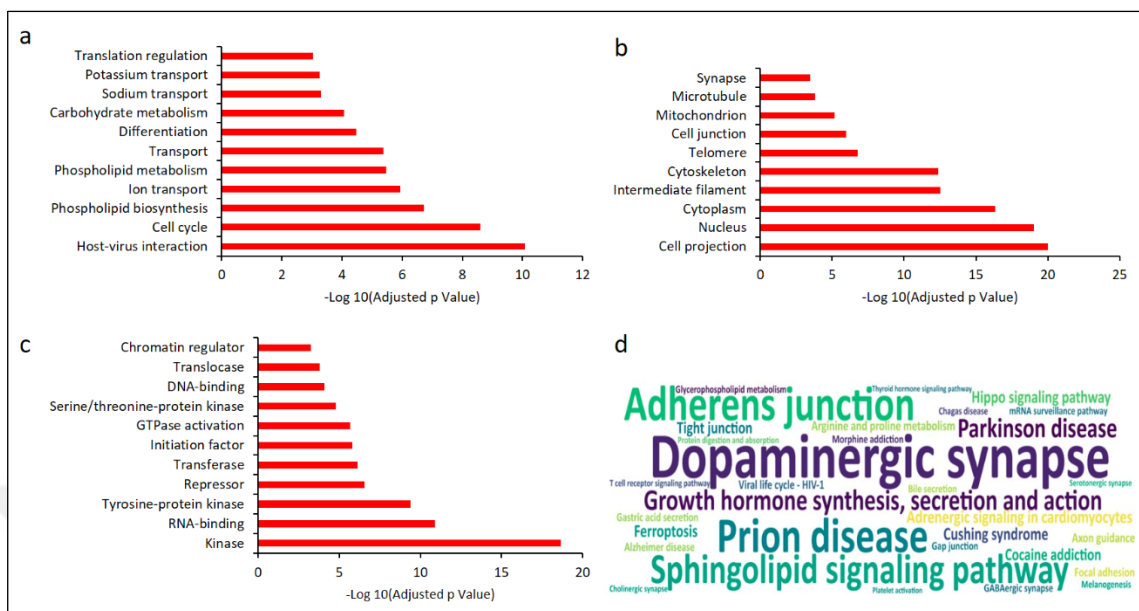


Figure 5.10: Functional clustering and KEGG pathway analysis of BMAL1 interactome candidates. Only interaction candidates with Saint express score >0.5 and BFDR value <0.05 were taken. Merged Set1 and Set2 interactome were analyzed for (a) biological process, (b) cellular component, and (c) molecular function using DAVID 2021 bioinformatics resources and visualized in excel. KEGG pathway analysis of MT-BMAL1 interactome (D). KEGG pathway analysis was performed using DAVID 2021 bioinformatics resources and visualized using an algorithm created by us. The font size of each pathway was determined by giving the path with the highest $-\log_{10}(p\text{-value})$ the highest font size.

5.2.5. Network Analysis of Identified Protein Candidates

We analyzed BMAL1 interaction partners with Saint express score >0.5 to draw a network map by Network Analyst web tool [Zhou et al., 2019] using STRING database [Szklarczyk et al., 2014] to retrieve PPIs by using interaction data only obtained from experiments with a confidence score of 0.4 or higher. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). The size of the nodes and length of the edges were calculated according to degree and betweenness respectively. Proteins identified in our proteomics analysis were shown in blue nodes, however, green nodes are additional proteins added by STRING to construct a solid network. Minimum Network management tool that keeps seed genes as well as non-seed genes that

maintain network connection (suitable for simplifying dense networks) was selected with the degree filter 3.0 to construct the network (Figure 5.10). Top bottleneck proteins were determined by the highest betweenness and degree values of nodes. There were many proteins (green) added by STRING to construct networks because BMAL1 data had only 73 interactors with Saint score >0.5 and BFDR value <0.05 . Although we identified a relatively low number of interaction candidates with a Saint score of >0.5 and a BFDR value of <0.05 in the BMAL1 interactome compared with PER2, we were able to obtain a robust interactome network. Considering the top bottle neck proteins, HSP90AA1 protein is shared in the top bottleneck proteins of BMAL1 and PER2 network. Additionally, a few members of eukaryotic translation initiation factor family are shared in the top bottleneck proteins of BMAL1 (EIF4A) and PER2 (EIF3E, EIF3K).

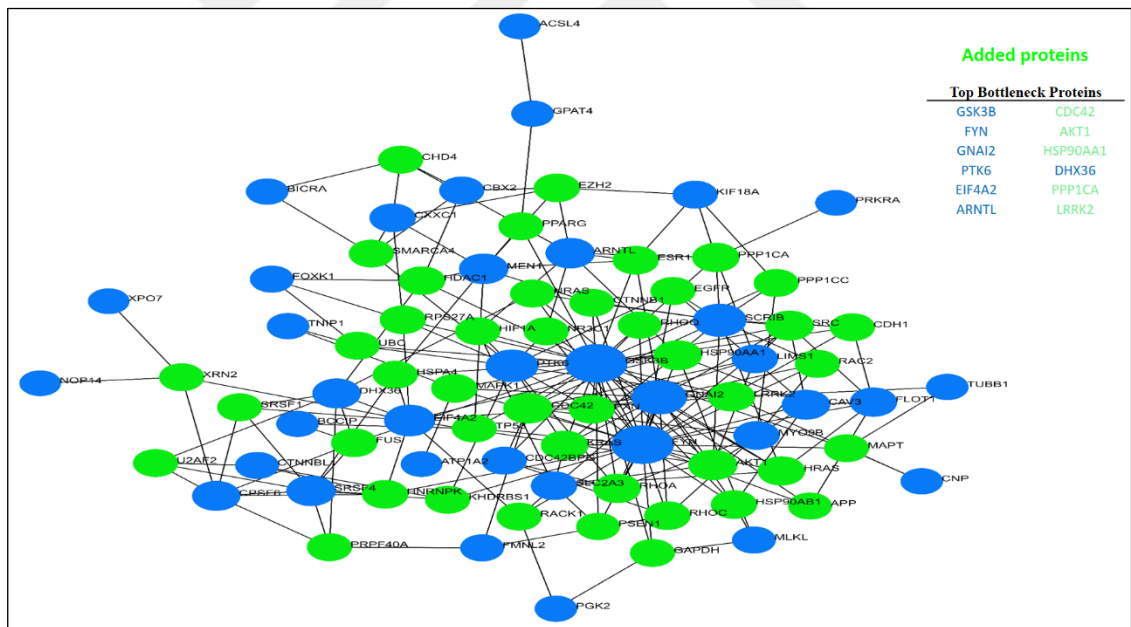


Figure 5.11: Network analysis of MT-BMAL1 interactome. BMAL1 interaction partners with Saint express score >0.5 and BFDR value <0.05 were used to draw BMAL1 interactome network. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). The highest betweenness and degree values of nodes were used to determine the top bottleneck proteins. Proteins identified in our proteomics analysis were shown in blue nodes, and green nodes are additional proteins added by STRING to construct the network.

5.3. (pBMAL1)-MiniTurbo-BMAL1 Interactome Analysis

5.3.1. Stable Expression of (pBMAL1)-MiniTurbo-BMAL1 and HA-MiniTurbo in U2OS Cell Line

After testing MiniTurbo efficacy in BMAL1 interactome analysis, we expressed MT-BMAL1 protein under endogenous Bmal1 promoter to obtain a U2OS cell line expressing oscillating MT-BMAL1 and to check if there is a clear difference between individual circadian interactors of BMAL1. We also established a stable U2OS cell line expressing HA-MiniTurbo under endogenous Bmal1 promoter to use it as control cell line to subtract nonspecific proteins that were randomly tagged by MiniTurbo. Stable expressions of MT-BMAL1 and HA-MT proteins were analyzed by Western Blot using appropriate antibodies (Figure 5.12A). In figures and figure legends, U2OS-p(Bmal1)-MT-BMAL1 and U2OS-p(Bmal1)-HA-MT cell lines were shortly named as p(Bmal1)-MT-BMAL1 and p(Bmal1)-HA-MT, respectively. Here p(Bmal1)-HA-MT and p(Bmal1)-MT-BMAL1 protein expressions were confirmed and demonstrated with mock control (un-transfected U2OS cells). To reset the circadian rhythm of U2OS-p(Bmal1)-MT-BMAL1 and U2OS-p(Bmal1)-HA-MT expressing cell lines, we used dexamethasone as a clock-resetting agent and collected protein lysates at CT28 and CT42 after reset (Figure 5.12B). We analyzed p(Bmal1)-MT-BMAL1 expression at CT28 and CT42 by Western Blotting using anti-BMAL1 antibody to check if there is an oscillation in recombinant MT-BMAL1 level (Figure 5.1B- red arrow). Results showed there was a significant difference in p(Bmal1)-MT-BMAL1 protein level at two different time points normalizing with actin levels (Figure 5.12B, the quantitation graph). In this experimental setup, our priority was to analyze BMAL1 interaction partners in their individual circadian phases, so the oscillation pattern of recombinant p(Bmal1)-MT-BMAL1 protein was clearly enough for our purpose. Additionally, the correlation of oscillation profile of recombinant p(Bmal1)-MT-BMAL1 with endogenous BMAL1 protein (Figure 5.1B- green arrow) was a cross control of the regulation of cloned Bmal1 promoter.

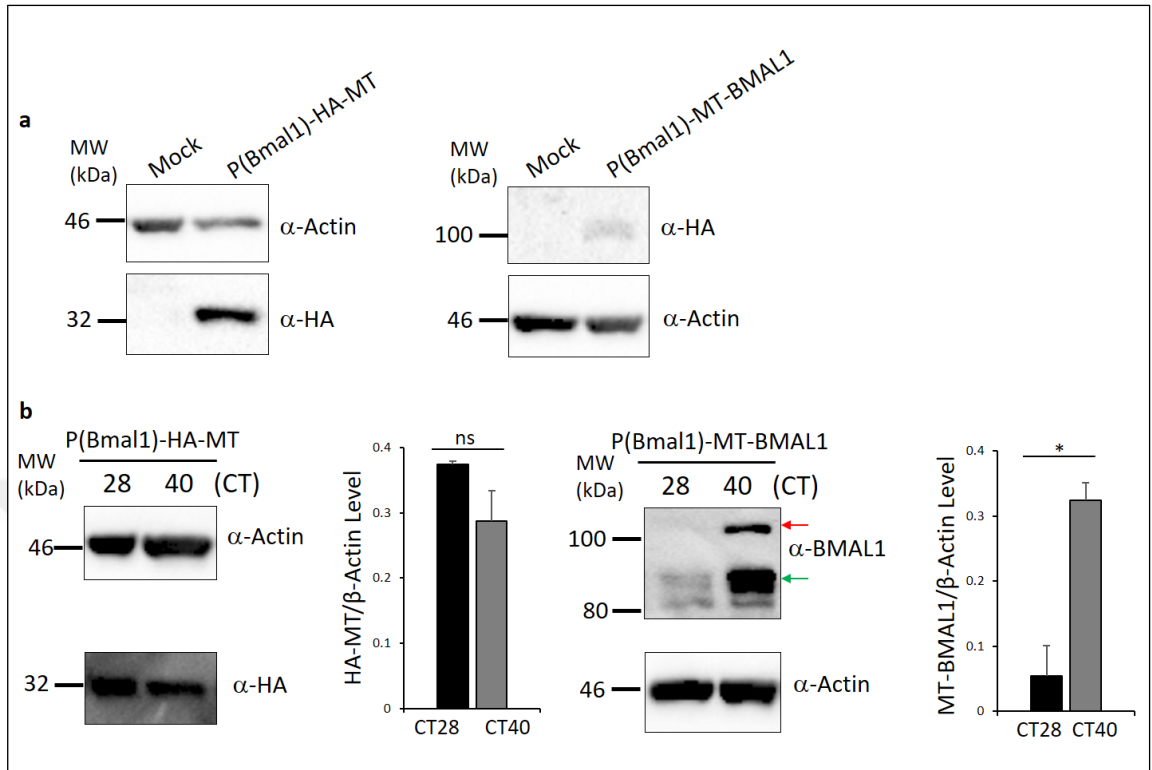


Figure 5.12: Characterization of the stable U2OS cell line expressing U2OS-p(Bmal1)-MT-BMAL1. (A) Western blot analysis of recombinant protein expressions in stable U2OS cell lines. Actin protein was used as a loading control. Mock control is an untransfected U2OS cell line. (B) Differential expression of MT-BMAL1 protein after dexamethasone treatment at CT28 and CT40. Recombinant BMAL1 level difference between CT28 and CT40 was significant (p-value = 0.0191) in U2OS- p(Bmal1)-MT-BMAL1 cells while recombinant HA-MT level difference between CT28 and CT40 was not significant (p-value = 0.1199) as anti-BMAL1 or anti-HA signals were normalized to actin levels. MW (molecular weight) is given in kDa. CT, circadian time (in hours).

5.3.2. Determination of CT Points and Sample Preparation for MS Analysis

As we optimized the biotinylation rate of BMAL1 in stable U2OS cell lines in the previous part, we continued with the same biotinylation time and biotin concentration. Firstly we tested the specificity of the biotin labeling using un-transfected U2OS cells as mock control and found a specific biotinylation pattern in p(Bmal1)-MT-BMAL1 samples compared with mock control and p(Bmal1)-HA-MT control (Figure 5.13A). At the same time, we also analyzed the biotinylation pattern at individual circadian times (CT28 and CT40) with 800 μ M. Although we obtained a clear oscillation profile of recombinant p(Bmal1)-MT-BMAL1, we didn't detect a clear difference between biotinylation patterns at CT28 and CT40 (Figure 5.13A). Additionally, the biotinylation level of p(Bmal1)-MT-BMAL1 was poor compared with PER2 and previous BMAL1 data. This was expected because both recombinant PER2-BioID2 and MT-BMAL1 were expressed under a strong promoter (CMV) but p(Bmal1)-MT-BMAL1 was under the endogenous promoter. Although this relatively poor biotinylation pattern was sufficient to test the specificity of biotinylation and determine the CT phases, we needed a longer biotinylation time (8h) to obtain sufficient protein amount after streptavidin pull-down in large-scale samples preparation for MS analysis. We prepared the large-scale biotinylation set in two different circadian phases, CT32 and CT44 (Figure 5.13B). Since we identified BMAL1 oscillation phases as CT28 and CT40, we began biotin treatment 4 hours before the identified CT phases and collected samples 4 hours later (at CT32 and CT44). We then isolated biotinylated proteins by pull-down using streptavidin-magnetic beads. To identify biotinylated proteins by mass spectrometry, an on-bead tryptic digestion protocol was performed with two technical replicates for each sample as described in Methods section. The statistical significance of p(Bmal1)-MT-BMAL1 interactions after subtraction of interactions of p(Bmal1)-HA-MT (indicating non-specific interactions) was analyzed using a statistical analysis algorithm as described as follows.

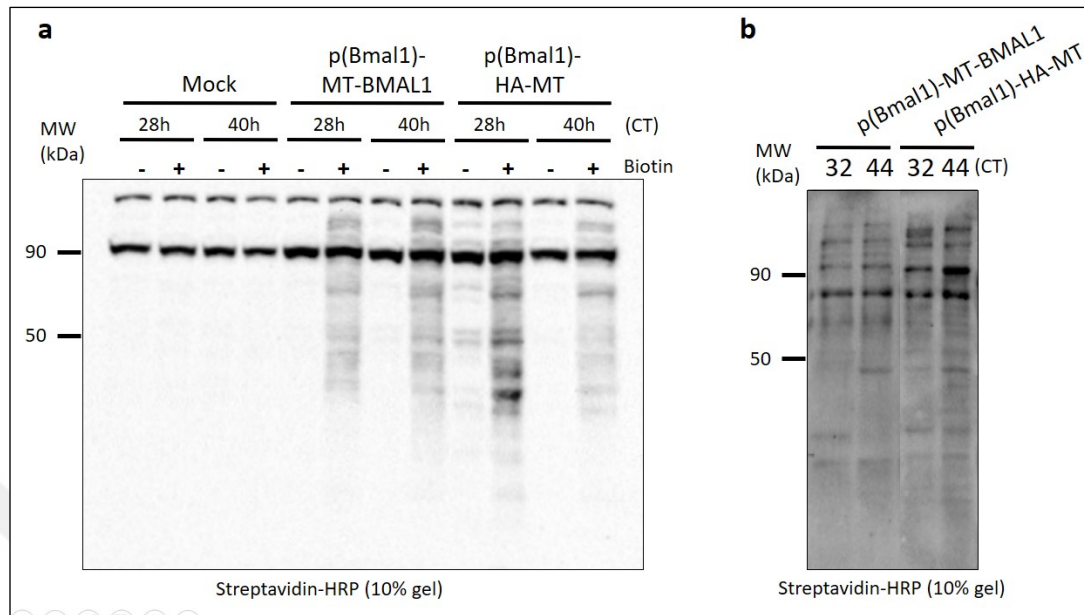


Figure 5.13: Determination of biotinylation conditions and preparation of the cell lines for mass spectrometry analysis. (a) Testing the septicity of the biotin labeling and analysis of biotinylation pattern at opposite circadian phases, CT28 and CT40 in U2OS-p(Bmal1)-MT-BMAL1 cells comparing with un-transfected U2OS cells (b) Confirmation of biotinylated samples on beads before mass spectrometry analysis. Cells were treated with Biotin for 8h at CT28 and CT40 after the reset by dexamethasone to prepare large-scale samples.

6.2.3. Identification of High Confidence Protein Interactors Using SAINT Express Analysis

We used the SAINT express algorithm as we did for PER2 and MT-BMAL1 interactome analysis, to determine the likelihood of each protein interacting with the bait protein. However, we couldn't identify any interactor with Saint express score=1 in CT32 data and only 3 interactors in CT44. Furthermore, there were only 9 interactors with Saint express score >0.5 and only 2 interactors with BFDR <0.05 at CT32. Therefore we didn't perform functional annotation clustering for p(Bmal1)-MT-BMAL1 interactome. Although we were able to identify only several interactome candidates with Saint express score>0.5 in CT32, we constructed the interactome network to compare

individual CT32 and CT44 networks. Interactome candidates of CT32 and CT44 with Saint express score >0.5 were listed in Table D1.5 and Table D1.6 respectively.

5.3.3. Network Analysis of Identified Protein Candidates

We constructed an interactome network for BMAL1 interaction partners with a Saint express score >0.5 obtained from p(Bmal1)-MT-BMAL1 data as described in the MT-BMAL1 section (Figure 5.14). Top bottleneck proteins for CT44 were determined by the highest betweenness and degree values of nodes. We didn't show the top bottleneck proteins for CT32 because it wouldn't be meaningful as it had only several interactions. Network analysis showed an obvious difference in the node numbers between CT32 and CT44 networks. CT32 network was clearly smaller than CT44; this indicates that the numbers of proteins that are labeled with higher MT-BMAL1 amount in CT44 compared to CT32. Compared to the PER2 interactome analysis, the difference in the recombinant p(Bmal1)-MT-BMAL1 level between individual CT phases (Figure 5.12B) was much higher than with recombinant PER2-BioID2 (Figure 5.1B). This was normal because p(Bmal1)-MT-BMAL1 was expressed under the native promoter but PER2-BioID was under CMV. Therefore we saw a remarkable difference between individual CT networks in this study. However, a slight expression of p(Bmal1)-MT-BMAL1 in CT28 (Figure 5.12B) made it difficult to obtain a sufficient amount of labeled protein from large-scale samples, so only several interactions were included in the CT32 network (Figure 5.14-top). Moreover, compared with previous MT-BMAL1 interactome analysis, we were able to identify a relatively low amount of interaction candidates as the endogen Bmal1 promoter was weaker than CMV.

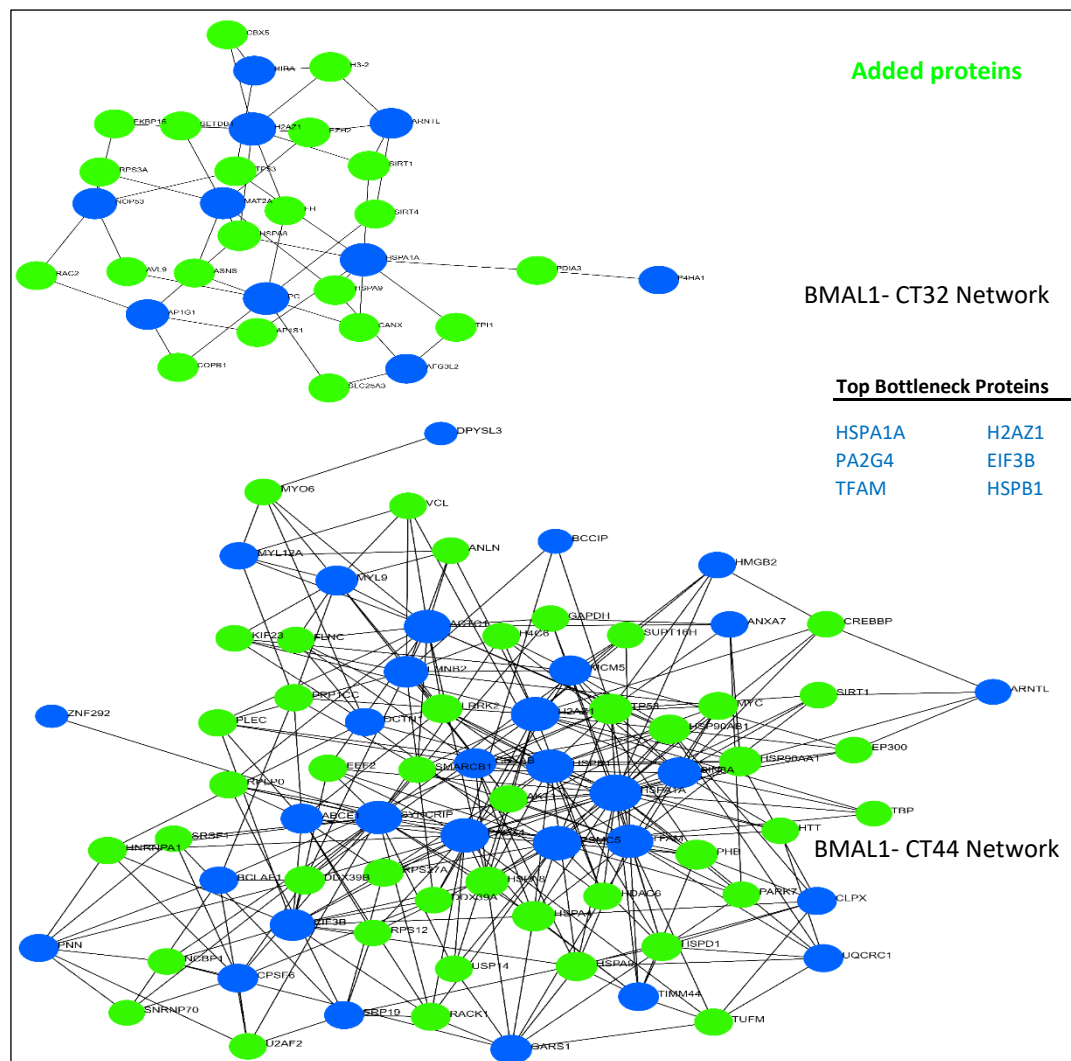


Figure 5.14: Network analysis of p(Bmal1)-MT-BMAL1 interactome. BMAL1 interaction partners with Saint express score >0.5 and BFDR value <0.05 were used to draw BMAL1 interactome network. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). The highest betweenness and degree values of nodes were used to determine the top bottleneck proteins. Proteins identified in our proteomics analysis were shown in blue nodes, and green nodes are additional proteins added by STRING to construct networks.

6. CONCLUSION

Since the first discovery of proteins by Dutch chemist Gerardus Johannes Mulder in the 1830s, great strides have been taken to elucidate the functionality of individual proteins in a biomolecular process. This progress also showed us that proteins do not act alone but work with other proteins in a macromolecular complex to fulfill the needs of a cell (Chautard et al., 2009; Miryala et al., 2018). Learning how these protein complexes work together and how a single protein contributes to this complex is crucial to understanding a molecular mechanism and its connection with other molecular processes. Therefore, protein interaction studies, or in other words, proteome analysis studies, have become a leading research area to elucidate molecular mechanisms and functional changes in a living organism. As described in the introduction section, the circadian clock is a vital biological process since it directly affects 10% of gene regulations and 20-40% of signaling mechanisms through post-translational modifications. To this end, interactome analyses of PER2 and BMAL1 proteins in this study made a significant contribution to the understanding of the circadian clock mechanism.

6.1. Conclusion of PER2-BioID2 Interactome

An excellent experimental setup for identifying the interactome(s) of any core circadian clock protein would be to express a fast biotinylating enzyme in the genome next to the clock protein-coding sequence using CRISPR-mediated gene editing, or to express the biotinylating enzyme and clock protein fusion under that clock gene's native promoter. However, there are some technical issues with the expression of recombinant fusion proteins under their corresponding gene's native promoter. For example, some clock genes require intron sequences for their circadian oscillations when overexpressed. Before moving on to inserting the BioID coding sequence into the genome via CRISPR, we thought that it is important to assess whether BioID enzyme would be functional

when fused to PER2 (or BMAL1 for next part) protein as in the simplest settings. Therefore, we started with the simplest design, at least to determine the interactome of the PER2 protein and whether this is possible in different circadian phases. To this end, we stably overexpressed the circadian core protein PER2 in fusion with BioID2 enzyme from a strong promoter (CMV) in human U2OS osteosarcoma cell line and selected a suitable candidate that expressed PER2-BioID2 at a relatively low level. Here, we observed differential expression in PER2 (both at the protein level as PER2-BioID and at the mRNA level as the sum of recombinant PER2-BioID2 and endogenous PER2) and at the circadian-regulated DBP mRNA level between CT36 and CT48 samples following dexamethasone treatment. Endogenous PER2 and DBP mRNA levels in U2OS-BioID2 cells followed a similar trend (Figure 5.1C). The higher expression of PER2-BioID2 at CT42 compared to CT30 is accompanied by a higher expression of mRNAs for PER2-BioID2 and endogenous PER2 (Figure 5.1B, right side). This differential expression of PER2-BioID2 looks like a specific pattern because Myc-BioID2 levels were not changed at CT36 and CT48 (Figure 5.1B, left side). Even though BioID2 tags were different in PER2 or mock cell lines (HA and Myc tags, respectively; Figure 5.1A), the biotinylation pattern as shown in Figure 5.3 showed that Myc-BioID2 had a higher activity compared to PER2-BioID2 (Figure 5.3A,B). In fact, this increases the reliability of the assay because the proteins that were randomly labeled were subsequently removed from the PER2-BioID2 list.

In agreement with a higher PER2 stability/expression profile (Figure 5.1B, right side), we observed that the size of the interactome at CT48 was larger than that of CT36 (Figure 5.4A). When we analyzed interactome for biological process, cellular component, and molecular function properties (Figure 5.4B-D), we observed a common profile with the exception that CT48 terms were larger than CT36 terms. The common profile between two time points (as if biological replicates) also supported the idea that labeling in the phases of 24–36 h (CT36) and 36–48 h (CT48) were largely merged and 12-h labeling should have been reduced to select the differential interactions at opposite circadian time phases. From the top terms, we focused on the significantly enriched representative ones for two different time points that might help us to understand the

function of the PER2 interactome. For the biological processes (Figure 5.4B), it is not surprising to see the involvement of PER2 in DNA/RNA biosynthetic process and metabolic processes. However, a role of PER2 in telomere maintenance is a novel observation. Although transcriptional regulation of telomerase by CLOCK/BMAL1 has been demonstrated [Chen et al., 2014], PER2 interactions might also directly be involved in telomerase maintenance. On the other hand, the terms related to immune system and circadian clock are also expected. However, the role of PER proteins in the immune system is complex, as PER1 is associated with anti-inflammatory effects, while PER2 is suggested to work as a positive regulator of proinflammatory cytokines [Hergenhan et al., 2020]. This differential function of PER proteins might be explained by the differential interaction networks of PER1 and PER2 even though both function in the negative arm of the TTFL. The last two terms (establishment of localization in cell and cellular localization) can be a reflection of the dynamic subcellular localization of PER2. Once PER2 is synthesized it stays in the cytoplasm but finally translocates into the nucleus in a circadian manner to inhibit BMAL1/CLOCK activity with the help of CRY proteins. During this journey, it is expected that PER2 will interact with the proteins involved in the establishment localization or cellular localization of PER2. For the cellular components terms (Figure 5.4C), the movement of PER2 from the cytoplasm to the nucleus results in the interactions that are shared between two major cellular compartments. In the cytoplasm, it makes complex with CRY proteins and also interacts with the proteins that interact with CRY directly or indirectly in the cytoplasm. In the nucleus, PER2, this time, makes interactions either directly or indirectly through CLOCK/BMAL1 or CRY proteins. It is not surprising that some of the interactors will be enriched in some organelles where PER2 is not located at all because the interactors will continue their movement once they are found around PER2 sometime during a 12-h labeling process. In this respect, an interactor labeled by PER2-BioID2 can be found in an organelle that has nothing with PER2-related signaling network because the biotin label will stay on those interactors even though that interactor was modified by another signaling pathway to direct the interactor to an organelle which has nothing to do with the PER2 function. In this respect,

shorter incubation periods using, for example, MiniTurbo, TurboID or APEX2 enzymes instead of BioID2 would help to define solely PER2-related terms. For molecular function properties of the interactome (Figure 5.4D), actin binding and cytoskeletal binding properties are not surprising. For example, it is known that circadian actin dynamics drive rhythmic fibroblast mobilization during wound healing [Hoyle et al., 2017]. Protein folding chaperone function might reflect the fact that during the folding of overexpressed PER2, fused BioID2 can label proteins around as soon as it folds. Because many of the interactors are involved in DNA/RNA biosynthesis, it is normal to see proteins with binding properties to nucleic acid, ATP, purine nucleotide, ribonucleotide, nucleoside phosphate, nucleotide, and RNA.

Network analysis to find interactions between interactome components or between PER2 and interactome clearly showed that the CT48 interactome was larger (Figure 5.6) and this can be explained by the higher expression of PER2-BioID2 in CT42 (the middle point of CT48 labeling). One of the top bottleneck proteins, HSP90AA1, was shared between CT 36 and CT48 (Figure 5.6). To interpret our STRING data, we have concentrated on top bottleneck proteins in either CT36 or CT48 (Figure 5.6). If we first examine the top bottleneck proteins at CT36, for example, CSNK1D (and CSNK1E) phosphorylates PER2 at two key regions that control a 63hosphor-switch mechanism regulating PER2 stability [Narasimamurthy et al., 2018]. While EIF3A is required for several steps in the initiation of protein synthesis, interestingly knockdown of EIF3A in U2OS cells lead to arrhythmicity according to the Bmal1 promoter-driven luciferase reporter assay [Lee et al., 2011]. On the other hand, heterogeneous nuclear ribonucleoproteins (such as HNRNPA2B1 and HNRNPA1L2) function in a wide range of nuclear processes, including DNA replication and repair, telomere maintenance, transcription, pre-mRNA splicing, and mRNA nucleocytoplasmic export [He and Smith, 2009]. Considering that all these events are also regulated by or associated with the circadian clock, our findings suggest that PER2 interacts with these proteins and HNRNPH3 which is also involved in RNA metabolism. Karyopherin Subunit Beta 1 (KPNB1) functions in nuclear protein import [Chook and Blobel, 2001], and it is possible that PER2-

BioID2 labeled this protein with biotin while it goes into the nucleus (Figure 5.2A, B). Rab1A is a member of the Ras oncogene superfamily and plays an important role in vesicle transport and in mediating dynamic membrane trafficking from the endoplasmic reticulum (ER) to the Golgi [Mukhopadhyay et al., 2011] and recent studies have shown that the Rab1A proteins are also involved in regulating signal transduction and autophagy [Bucci et al., 2014].

Actin-related protein 2/3 complex subunit 4 (ARPC4) gene encodes one of seven subunits of the human Arp2/3 protein complex which controls actin polymerization [Welch et al., 1996]. Cell adhesion also depends on the actin cytoskeleton and that BMAL1/PER/CRY dependent circadian regulation of actin dynamics affects cell adhesion, which is detected directly by the measurement of cellular impedance [Atienza et al., 2005]. Heat shock protein 90 α (Hsp90 α), encoded by the HSP90AA1 gene, is the stress-inducible isoform of the molecular chaperone HSP90 [Blagg and Kerr, 2006] and the main cytoplasmic HSP90 isoform is required for the stabilization of BMAL1 [Schneider et al., 2014]. Here, we can speculate that the stability of cytoplasmic BMAL1 can be regulated through PER2 stability in a circadian manner through PPIs. Super killer viralicidic activity 2-like 2 (SKIV2L2) and RNA helicase, and plays crucial roles in several RNA degradation pathways [Kilchert et al., 2016]. It is difficult to speculate on just one PPI, but here PER2 may involve in the RNA degradation pathway indirectly.

Considering the top bottleneck proteins of CT48, HSP90AA1 was shared with CT36 and discussed already above. Chaperonin containing TCP1 subunit 5 (CCT5) is a molecular chaperone that is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TriC) which is a molecular chaperone complex that assists the folding of proteins upon ATP hydrolysis [Freund et al., 2014]. Neuroblast differentiation-associated protein (AHNAK), also known as desmoyokin is essential for pseudopod protrusion and cell migration [Shankar et al., 2010]. This can be a link from the circadian clock to cell migration through PER2 or the cellular movement can be sensed by the circadian clock through PER2. HNRNPA1 was already mentioned in CT36. EZRIN (EZR) is a protein-tyrosine kinase substrate in microvilli, and is required for the

formation of microvilli and membrane ruffles on the apical pole in epithelial cells [Sauvanet et al., 2015]. Ezrin regulates focal adhesion and invadopodia dynamics by altering calpain activity to promote breast cancer cell invasion [Hoskin et al., 2015]. On the other hand, heterogeneous nuclear ribonucleoprotein D (HNRNPD) may play a role in the regulation of the rhythmic expression of circadian clock core genes and directly binds to the 3' -untranslated region of CRY1 mRNA and induces CRY1 rhythmic translation [Woo et al., 2010]. In this way, PER2 stability indirectly may help CRY1 rhythmic translation. Actin-Related Protein 2 (ACTR2) is not associated with a specific function, but it is a major constituent of the ARP2/3 complex [Welch et al., 1996]. Chaperonin Containing TCP1 Subunit 2 (CCT2) is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (Tric) which is a molecular chaperone complex that assists the folding of proteins [Spiess et al., 2004]. The presence of CCT5 (CT36) and CCT2 (CT48) actually indicates that they are involved in the folding of the overexpressed PER2 or in reverse PER2 may help to regulate folding through these proteins. U2AF2 (Splicing factor U2AF 65 kDa subunit) plays a role in pre-mRNA splicing and 3' -end processing [Glasser et al., 2017]. PER2 can be a part of a complex that is involved in the circadian control of splicing. Prolyl 4-hydroxylase subunit beta (P4HB) is multifunctional and acts as an oxidoreductase for disulfide formation, breakage, and isomerization [Lumb and Bulleid, 2002]. Interaction of circadian clock proteins CRY1 and PER2 is modulated by zinc binding and disulfide bond formation [Schmalen et al., 2014], and it actually could be in close proximity of PER2 due to this post-translational modification. Overall, the examination of top bottleneck proteins for both CT36 and CT48 are in agreement with the grouping of interactors for characteristics of biological process, cellular component, and molecular function as shown in Figure 5.4B-D and Figure 5.5. Additionally, the presence of interactors with very divergent functions in the interactome of PER2 suggests that either PER2 has some non-circadian functions or the circadian clock orchestrates such divergent pathways through the PPIs of PER2.

The confirmation of CRY1 and CRY2 in the PER2 complex by a classical method such as co-IP confirmed the validity of our assay in which CRY1 and CRY2 were identified

as interactors of PER2 (Figure 5.7A,B). Here, we tagged the PER2 with FLAG at both sites because in some interactions one end might be blocked by heavy interactions and, if it were the case, we hoped that the FLAG tag at the other end would be available for anti-FLAG antibody on magnetic beads. The presence of BMAL1 (which could not be identified with BioID method) in FLAG immunoprecipitated PER2 complex suggests that BMAL1 might be masked by other strong interactors in our MS analysis (Figure 5.7C). While the circadian clock regulates many metabolic and physiological events in a very dynamic manner, its core components are also regulated in a circadian manner. Moreover, the clock proteins, as most of the proteins transiently do, interact with many proteins during their journey from translation/post-translational modification to degradation finally. Changes in subcellular localization, such as delayed migration of PER and CRY into the nucleus, contribute to the diversification and enrichment of these dynamic interactions. This further indicates that those interactions can be very transient or unstable on a time scale or even there might be more than one pool of some interactome at the same time. Even though many PPIs have been recognized by a variety of different approaches, novel or more efficient methods are always needed to reveal new interactors which are refractory to be caught by the classical methods.

Although the BioID method is very sensitive to detect weak/transient interactions, it may not cover all interactions due to technical limitations, such as some strong interactors or some abundant proteins nearby may prevent low-abundant ones from detection during identification. For example, CAVIN3 was detected as a cytoplasmic PER2-interacting protein influencing circadian clock properties in NIH3T3 fibroblast cells after pull-down of carboxy-terminally tagged murine PER2 [Schneider et al., 2012] but not in our interactome data. Either because of technical limitations or the use of a different cell line (NIH3T3 vs. U2OS) we could not get CAVIN3 interaction which suggests that multiple approaches should be used in complementation to detect more interactions. As another example of this, we could not get BMAL1 in either CT36 or CT48 interactome with BioID method, however, it coimmunoprecipitated with FLAG-PER2 in HEK293T cells (Figure 5.7C). We cloned HK1, MCM3, and IMPDH2 coding sequences from

the U2OS cell line cDNA to express them in HEK293T-F-PER2-F cell line so we could test their interactions using the co-IP. In three independent experiments, the specific presence of the HA-tagged recombinant interactor proteins on anti-FLAG antibody-coated magnetic beads confirmed that they physically interacted with PER2 (Figure 5.7D–F). HK1 phosphorylates glucose to produce glucose6-phosphate which is the first rate-limiting glycolytic reaction and it is the most abundant and ubiquitously expressed isoform of hexokinases [Wilson, 2003]. MCM3 is involved in the initiation of eukaryotic genome replication and, as a part of CMG (Cdc45-Mcm2-7-GINS) helicase, is also involved in unwinding DNA throughout the entire process of chromosomal DNA replication [Tye and Sawyer, 2000]. On the other hand, IMPDH2 gene encodes the rate-limiting enzyme in the de novo guanine nucleotide biosynthesis and is upregulated in some neoplasms, which suggests a role in malignant transformation [Ruiz-Agudo et al., 2008]. The interactions between PER2 and identified interactome based on the ratio of the input and the bound fractions suggest that the physical interactions indeed varied in strength, even though all these interactions had a SAINT express score = 1 (Figure 5.7D–F). For example, HK1 is expected to interact with only the cytosolic fraction of PER2, moreover, only a certain form of HK1 such as a specific phosphorylated form might be interacting with PER2 or vice versa. Therefore, it is not surprising to see weak interactions between PER2 and novel interactors. On the other hand, PER2 and CRY1/CRY2 co-exist in the molecular core clock in both cytoplasm and nucleus while PER2 is expected to bind BMAL1/CLOCK in nucleus at a certain circadian time point. In this respect, the interaction between PER2 and BMAL1 is expected to be weaker (as seen in Figure 5.7C) compared to that of between PER2 and CRY1/CRY (as seen in Figure 5.7A, B).

Circadian clock might regulate the other pathways through interactions with the critical proteins/enzymes. For example, CRY1 has posttranslational functions out of the context of the core clock mechanism and binds to G-proteins and cysteine beta-synthase and regulates their activity and, in turn, regulates cellular metabolism [Cal-Kayitmazbatir et al., 2021; Zhang et al., 2019]. In a similar way, the circadian clock might regulate HK1 activity by PER2-HK1 interaction to regulate glycolysis, which is needed to be shown

experimentally. Considering just interactors which were here used to confirm the validity of our interactome, it is possible that PER2, in addition to being a main component of the core circadian clock, might be interacting or functioning in very diverse pathways such as metabolism and replication. Moreover, the network analysis suggests a more comprehensive involvement of PER2 in various metabolic processes and different cellular locations.

6.2. Conclusion of MT-BMAL1 Interactome

As discussed in the PER2 interactome analysis section, there are some technical issues with the expression of recombinant fusion proteins under their corresponding gene's native promoter. Therefore we firstly designed a simple strategy for BMAL1 interactome analysis by expressing recombinant MT-BMAL1 under the constitutive CMV promoter in U2OS cells. In this study, our main purpose was to test the MiniTurbo technique for BMAL1 interactome analysis and optimize the biotinylation time of recombinant MT BMAL1 protein. Although HA-MT showed a 10 min biotinylation rate in HEK-293 cells [Branon et al., 2018], we were able to detect a proper biotinylation rate for MT-BMAL1 after 6h of biotinylation in U2OS cells (Figure 5.9A). Considering 12h BioID2 biotinylation time, 6h was half of this time and it would be useful for the analysis of sub-circadian interactions. However, overexpressed recombinant MT-BMAL1 protein level was not different between CT24 and CT36 (Figure 5.8B) unlike the overexpressed PER2-BioID2. Moreover, it can be concluded that recombinant MT-BMAL1 overexpression disrupts the endogenous BMAL1 oscillation (Figure 5.8B) even more repeats are needed to draw a significant graph. Therefore, we only focused on identifying the BMAL1 interactome using the MiniTurbo technique, without considering the circadian phases. Here, we observed that the biotinylation pattern in HA-MT was higher compared to MT-BMAL1 (Figure 5.9A,B). In fact, this increases the reliability of

the assay because the proteins that were randomly labeled were subsequently removed from the MT-BMAL1 list.

We prepared two sets of large-scale samples for MS analysis (Figure 5.9C) and analyzed the interactome, obtained from MS data, for biological process, cellular components, and molecular function properties (Figure 5.10A-C). For the biological processes (Figure 5.10A), it is not surprising to see the involvement of BMAL1 in the translation regulation process because it is known that BMAL1 regulates translation in response to S6K1-mediated phosphorylation and interacts with eukaryotic initiation factors family members, eIF4E, eIF4A, eIF4G and eIF3B [Lipton et al., 2015]. Our interactome list also included some members of the eukaryotic initiation factors family such as eIF2B3 with Saint score=0.6 and eIF4A2 with saint score=1 which was also one of the bottleneck proteins (Figure 5.11). Although the circadian clock is known to be involved in the regulation of the ion transport via rhythmic transcription of the SLC12A2 (Na-K-Cl cotransporter) [Milićević et al., 2019], the role of BMAL1 in potassium transport, sodium transport or ion transport at PPI level is a novel observation. Additionally, the role of BMAL1 protein in the differentiation process is not surprising. BMAL1 deletion in embryonic stem cells (ESCs) causes the deregulation of genes from the three germ layers (i.e., ectoderm, mesoderm, and endoderm) and the absence of BMAL1 affects embryoid body formation and gastrulation [Ameneiro et al., 2020]. Moreover, it has been reported that BMAL1 deletion leads to impairment of cartilage development through the overexpression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factors α (HIF α) genes resulting in Runx2 enhancement [Ma et al., 2019]. Therefore, the identification of BMAL1 interaction partners involved in differentiation processes offers a mechanistic perspective on the role of BMAL1. For the cellular component analysis of BMAL1 interactions (Figure 5.10B), it is expected to see cytosolic and nuclear localization because BMAL1 is firstly localized in the cytoplasm and then moved to the nucleus to activate gene expression by binding E-box response elements in dimer with CLOCK/NPAS2. In fact, this analysis doesn't mention the subcellular localization of the BMAL1 protein but its interaction partners. As mentioned above, biotinylated proteins

continue their movement once they are found around BMAL1 sometime during a 6-h labeling process of MiniTurbo. Although the 6-h biotinylation time of MiniTurbo is 2 times lower than BioID2, still it is possible to see subcellular compartments where BMAL1 interactions are located but BMAL1 itself is not. Therefore shorter incubation periods would help to define solely BMAL1-related terms. This can be achieved using again MiniTurbo but in Hek293 cells by ignoring sub-circadian interactions. Additionally, TurboID or Apex might also serve a shorter biotinylation time even in U2OS cells but they need to be tested to be sure. Going back to the biomolecular enrichment analysis of PER2 interactions, the telomere maintenance role of PER2 was a novel observation. Here we also observed telomere term in the cellular component analysis of BMAL1 interactions. Both individual results indicate the role of circadian proteins in a molecular mechanism involved in telomere (maintenance) and this is worthy of further investigation in future studies. For molecular function properties of the interactome (Figure 5.10C), it is expected to see DNA binding or chromatin regulator properties because of the transcription factor role of BMAL1. Serine/threonine kinase or tyrosine kinase is also not surprising because BMAL1 undergoes extensive posttranslational modification in cells, including phosphorylation. For example, phosphorylation of BMAL1 on Ser17 and Thr21 residues by GSK3 β targets BMAL1 for ubiquitination and subsequent degradation [Sahar et al., 2010] or phosphorylation on Ser90 by Casein kinase 2 α (CK2 α) induce nuclear localization of BMAL1 [Tamaru et al., 2009]. Additionally, tyrosine phosphorylation of BMAL1 such as Thr534 phosphorylation by mitogen-activated protein kinase (MAPK) negatively regulates the transactivation potential of the CLOCK/BMAL1 complex at E-boxes [Sanada et al., 2002]. Detecting RNA-binding or initiation factor properties may also be considered normal because recombinant MT-BMAL1 may have biotinylated proximal RNA-binding proteins or eukaryotic initiation factors immediately after translation.

Although we couldn't detect core clock proteins except BMAL1 itself in our interaction list, we were able to build a solid interactome network including proteins in our data and proteins retrieved from the STRING database to construct the network

(Figure 5.11). Considering the top bottleneck proteins, It is not surprising to see BMAL1 itself on this list. HSP90AA1 which is also a member of the top bottleneck proteins of PER2 interactome encodes the Heat shock protein 90 α (Hsp90 α) required for the stabilization of BMAL1 [Schneider et al., 2014] and it is consistent to see it among BMAL1 upper bottleneck proteins. As stated above, BMAL1 undergoes extensive posttranslational modification in cells for its functional regulation and subcellular localization. Therefore, several serine/threonine kinase or tyrosine kinase proteins are the members of top bottleneck proteins; glycogen synthase kinase-3 beta (GSK3 β), proto-oncogene tyrosine-protein kinase FYN, RAC (Rho family)-alpha serine/threonine-protein kinase AKT1, leucine-rich repeat kinase 2 (LRRK2), and tyrosine-protein kinase 6 (PTK6). As discussed above, GSK3 β induces proteolytic degradation of BMAL1 by phosphorylating its Ser17 and Thr21 residues [Sahar et al., 2010]. In fact, GSK3 β can be considered a hub protein in the regulation of the circadian clock mechanism. It has a role not only in BMAL1 regulation but also in PER2 nuclear localization [Iitaka et al., 2005], CLOCK, and CRY2 degradations [Spengler et al., 2009; Harada et al., 2005]. However, GSK3 β activation is negatively regulated by AKT serine/threonine kinase which is another member of top bottleneck proteins (AKT1). It is reported that BMAL1 stability is enhanced by CA-Akt (a constitutively active form of AKT) treatment, which causes an increase in GSK3 β phosphorylation and consequent inactivation [Sahar et al., 2010]. BMAL1 phosphorylation by mammalian target of rapamycin (mTOR) serine/threonine kinase is also known to regulate BMAL1 degradation. [Dadon-Freiberg et al., 2021]. FYN tyrosine kinase is reported that binds to mTOR and activates the mTOR/S6K pathway regulating dopaminergic neuronal apoptosis in animal and cell models [Tan et al., 2021]. Although we could not identify mTOR in our interactome analysis, the presence of FYN in the top bottleneck list indicates that BMAL1, FYN, and mTOR may be in the same complex. LRRK2 protein shows encased kinase activity in Parkinson's disease and Gly2019Ser mutations in LRRK2 are the most common cause of autosomal-dominant Parkinson's disease [Gilks et al., 2005]. Although the molecular mechanism is not clear, the circadian clock is disrupted in Parkinson's disease and the inactivation of BMAL1 in

1-methyl-4-phenyl-1,2,4,5-tetrahydropyridine (MPTP)-treated mice causes various neurologic defects including loss of dopaminergic neurons [Liu et al., 2020]. Additionally, as described above, BMAL1 deletion leads to overexpression of HIF α genes and causes impairment of cartilage development [Ma et al., 2019]. HIF α stabilization is reported to be increased with phosphorylation at Tyr565 and Ser797 by PTK6 and LRRK2 respectively [Lin et al., 2016]. These reported findings and the presence of both PTK6 and LRRK2 proteins in the BMAL1 top bottleneck interaction list suggest that BMAL1-mediated PTK6 and LRRK2 signaling in cartilage development and/or Parkinson's disease is worthy of further investigation. Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PPP1CA) is one of the three catalytic subunits of protein phosphatase 1 (PP1). PP1 has been co-immunoprecipitated with PER2 in HEK293 cells and shown to have a role in the dephosphorylation and stabilization of PER2 protein. [Gallego et al., 2006]. Moreover, PP1 increases the cytoplasmic localization of PER2 in neuroblastoma cells expressing PP1 inhibitors [Schmutz et al., 2011]. Although we couldn't obtain a PP1 protein subunit in our PER2 interactome data, we detected protein phosphatase 2 (PP2) subunits, PPP2R2B and PPP2R1A with saint scores =0.97 and 0.98 respectively. PP2 has not been shown to directly interact with PER2 but is speculated to regulate the stability of PP1 [Piggins, 2006]. Therefore, it can be assumed that BMAL1, PER2, PP1, and PP2 are present in the same complex (even for a moment) and PP1 regulates BMAL1 stability or subcellular localization besides PER2. Since BMAL1 has been reported to interact with DEAD-box RNA helicase MVH in the chromatoid body, a cytoplasmic organelle plays an important role in RNA post-transcriptional and translation regulation [Peruquetti et al., 2021], it is not surprising to see probable ATP-dependent RNA helicase DHX36 on top bottleneck proteins. Furthermore, considering the PER2 top bottleneck member SKIV2L2 RNA helicase, our data provide consistent output on the regulation of RNA processing by the circadian clock mechanism. Eukaryotic initiation factor 4A2 (EIF4A2) is required for several steps in the initiation of protein synthesis and is already known to be rhythmically associated with BMAL1 as discussed above [Lipton et al., 2015]. Guanine nucleotide-binding protein G(i), alpha-2 subunit (GNAI2) is associated with RGS16 protein in SCN,

and the Rgs16 gene is known to regulate by BMAL1 through its E-Box regulatory element [Ishida et al., 2011]. As GNAI2 is a member of top bottleneck proteins, we speculate that BMAL1 may also regulate GNAI2 function/localization directly or indirectly at the post-translational level. Cell division control protein 42 homolog (CDC42) is a small GTPase of the Rho family. Microarray analysis of clock-regulated gene expressions has shown the oscillation of Cdc42 in rat-1 fibroblasts [Duffield et al., 2002]. Similar to GNAI2, CDC42 can be directly or indirectly regulated by BMAL1 protein at the post-translational level.

Overall, the examination of top bottleneck proteins for BMAL1 interactome is in agreement with the grouping of interactors for characteristics of biological process, cellular component, and molecular function as shown in Figure 5.10A-C. Additionally, the presence of interactors with very divergent functions in the interactome of BMAL1 suggests that either BMAL1 has some non-circadian functions or the circadian clock orchestrates such divergent pathways through the PPIs of BMAL1.

6.3. Conclusion of p(Bmal1)-MT-BMAL1 Interactome

Once we obtained a solid interactome network from MT-BMAL1 data then we intended to identify sub-circadian interactions for BMAL1 expressing recombinant MT-BMAL1 protein under the native Bmal1 promoter. Here, we observed differential expression in p(Bmal1)-MT-BMAL1 protein level between CT28 and CT40 samples following dexamethasone treatment (Figure 5.12B) in contrast to the protein level of CMV-controlled recombinant MT-BMAL1 (Figure 5.8B). Since our main purpose in this step was to detect BMAL1 interaction partners in individual circadian phases we analyzed the individual CT phases to detect the exact oscillation pattern of BMAL1 protein. We observed a more precise difference in BMAL1 protein levels between CT28-CT40 than between CT24-CT36 and we continue with 28- and 40-h circadian times. On the other hand, since the p(Bmal1) promoter is weaker than CMV, we were able to obtain a proper biotinylation profile after 8 hours of biotinylation instead of 6 hours (Figure 13A).

Therefore we collected large-scale samples in CT32 and CT44 after the resetting as the lowest and highest amplitude of the BMAL1 circadian oscillation was obtained at 28- and 40-h respectively. Consistent with recombinant MT-BMAL1 protein levels at opposite circadian phases, the CT40 interaction number was higher than CT28 (Figure 5.14) and this can be explained by the higher expression of MT-BMAL1 in CT40. Although the total number of interactions in MT-BMAL1 and p(Bmal)-MT-BMAL1 are almost the same (about 1300 and 1000 interactions respectively), only a small amount of interaction candidates were obtained with Saint express score=1 in this study. This is because p(Bmal1) is weaker than CMV and therefore the number of biotinylated proteins is not sufficient to obtain a significant readout in MS analysis. However, we wanted to construct interactome networks for both CT phases to show the difference between the sizes of the networks of the individual phases (Figure 5.14). Since the CT32 network was very small, it wouldn't be meaningful to determine the top bottleneck proteins for this network so we only showed that of CT44. Here we determined the member of heat shock proteins Heat shock 70 kDa protein 1 (HSPA1A) and heat shock protein beta-1 (HSPB1) in the top bottleneck proteins of CT48 as in the top bottleneck proteins of MT-BMAL1 (HSP90AA1). We didn't determine any translation initiation factor in the previous top bottleneck list of the BMAL1 network but different members of the EIF3 family were shared with PER2-BioID2 (EIF3E and EIF3K) and p(Bmal1)-MT-BMAL1 (EIF3B) analysis. Different from previous top bottleneck proteins, we determined a histone protein (H2AZ1), an RNA binding protein (PA2G4), and a mitochondrial transcription factor protein (TFAM). Histone H2A.Z protein is expressed by the H2AZ1 gene and works with chromatin remodelers in chromatin remodeling [Jin et al., 2009]. H2A.Z ChIP analysis from mouse liver chromatin has revealed a rhythmic expression of H2A.Z protein and CLOCK-BMAL1 DNA binding promotes rhythmic chromatin remodeling incorporation of the histone variant H2A.Z [Menet et al., 2014]. Proliferation-associated protein 2G4 (PA2G4) is an RNA-binding protein that is involved in growth regulation. We couldn't find information about the direct/indirect regulation of PA2G4 gene expression or protein function in the literature but our molecular function clustering analysis showed RNA

binding in the MT-BMAL1 interactome (Figure 5.10C) which increase the consistency of the network analysis. Furthermore, as discussed above BMAL1 is known to physically interact with ATP-dependent DEAD-box RNA helicase MVH protein and is involved in RNA metabolism in chromatid bodies [Peruquetti et al., 2021]. Mitochondrial transcription factor A (TFAM) is a key activator of mitochondrial transcription and is also involved in mitochondrial DNA replication [Picca and Lezza, 2015] and TFAM expression level is reported to change with the expression level of PGC-1 α regulated by BMAL1 in renal IRI [Ye et al., 2022].

6.4. General Conclusion

Overall, in this study, we obtained high-throughput data for PER2 and BMAL1 interaction candidates using a novel approach, proximity-dependent labeling, which allows the identification of weak and transient interactions. For this purpose, we tested two different types of this technique; BioID2 for PER2 interactome analysis and MiniTurbo for BMAL1 interactome analysis. Firstly we analyzed the PER2 interactome using BioID2 and obtained a robust interactome network. Although the identification of CRY1 and CRY2 proteins in this study improved the reliability of our data, other core clock proteins BMAL1, CLOCK, and PER2 itself couldn't be detected in our interactome list. Similarly, we couldn't detect core circadian clock proteins in BMAL1 interactome analysis except for BMAL1 itself in MT-BMAL1 interactome. This may be because other interacting proteins mask the labeling of these core clock proteins or even themselves.

The classical methods have also already identified many proteins as the interactors of PER2 and BMAL1 (Figure 6.1). For example; β -TrCP (β -transducin repeat-containing protein), a ubiquitin adapter protein, that binds CKI ϵ phosphorylated PER2 and it was identified as PER2 interactors using cell culture-based biochemical assays [Eide et al., 2005]. The ubiquitin-protein ligase UBE3A (as well as casein kinase 1 delta) is recognized as a light-induced protein in SCN by the AutoProteome method which is an

automatic sample processing step followed by LC-MS/MS with two stages of peptide separation [Tian et al., 2011]. NONO (SAINT express score = 0.99 at CT48 and 0.49 at CT36, in this study) was identified as an interactome of PER1 (which makes heterodimers with PER2) using rat cell lines stably expressing exogenous tagged PER1 protein [Brown et al., 2005]. It is possible that NONO was in close proximity to PER2 in this study because of PER1. The presence of ubiquitin-specific protease 7 (USP7) in CT48 interactome (SAINT express score = 0.98, in this study) indicates that BioID2 also labels the proteins that are in the interaction with PER2 through CRY2 (SAINT express score = 1 in both CT36 and CT48) because it was shown that USP7 directly interacts with CRY2 [Hirano et al., 2016]. On the other hand, Poly(ADP-Ribose) Polymerase 1 (PARP-1) interacts with CLOCK-BMAL1 heterodimer and poly(ADP-ribosyl)ates CLOCK in a circadian manner to modulate DNA-binding activity of BMAL1-CLCOK and further interaction with negative arm proteins PER2 and CRY [Asher et al., 2010]. SIRT-1 has been reported to be expressed in a circadian manner and involved in chromatin reorganization. , PER2 acetylation and degradation by interacting with BMAL1-CLCOK heterodimer [Jin et al., 2009]. It is also submitted that it modulates PER2 acetylation and further degradation through BMAL1-CLCOK interaction [Asher et al., 2008]. Mitogen-activated Protein Kinase (MAPK) is another interacting protein with BMAL1 identified by the classical yeast two-hybrid technique and it is shown that BMAL1 is phosphorylated and negatively regulated by MAPK in the nucleus [Sanada et al., 2002]. Additionally, as we discussed above, some proteins have already been identified both in classical PPI techniques and in our BMAL1 interactome analysis such as GSK3 β (Saint express score= 0.94) [Tamaru et al., 2009]. Therefore considering previous interactors of BMAL1 and PER2 identified by both classical techniques and in our findings, we recommend using proximity-dependent labeling and classical techniques together to obtain a more solid network.

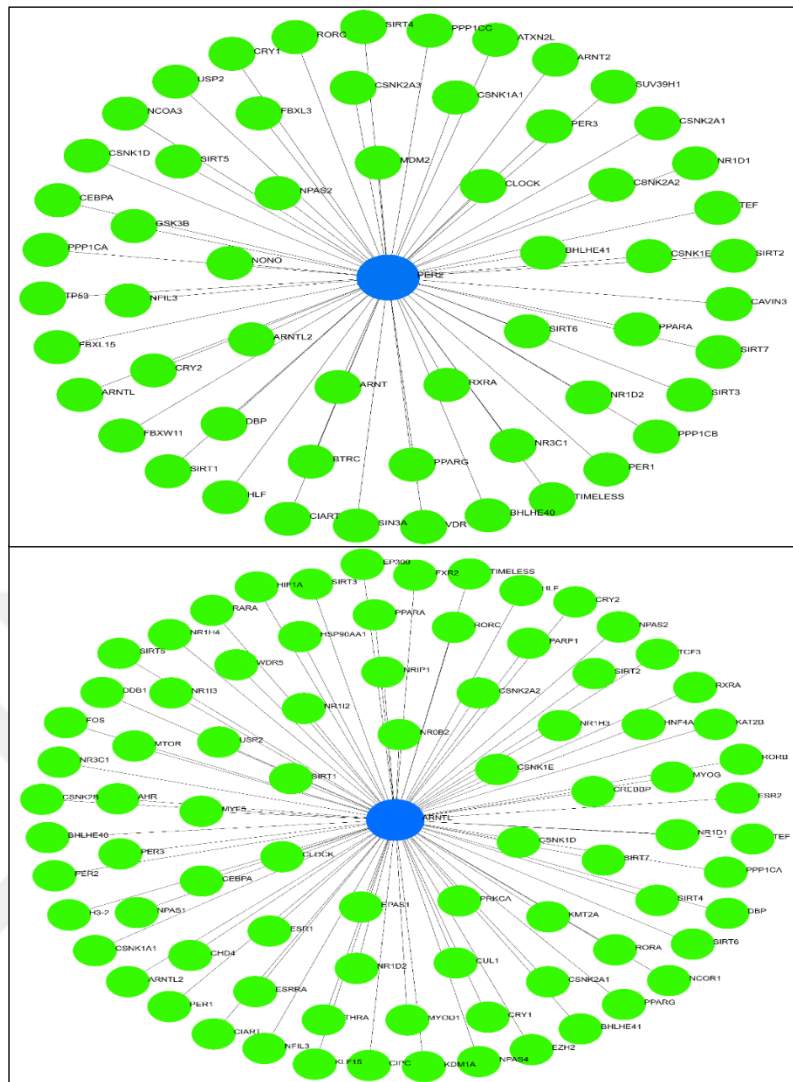


Figure 6.1: Identified PER2 and BMAL1 interaction partners by classical methods. Interactions were visualized by the web-based visual analytics platform NetworkAnalyst 3.0. STRING database was used to retrieve PPIs by using interaction data only obtained from experiments with a confidence score of 0.4 or higher. Proteins were visualized as ellipse nodes, and interactors were connected via lines (edges). PER2 and BMAL1 were shown in the blue nodes, and green nodes are additional proteins added by STRING to construct the networks.

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BIOGRAPHY

Huseyin GUL completed his undergraduate education at Cumhuriyet University, Department of Molecular Biology and Genetics in 2011. In the same year, he started his master's education at Gebze Institute of Technology, Department of Molecular Biology and Genetics. He has been a part of the Ph.D. program of Molecular Biology and Genetics at Gebze Technical University since 2014 under the supervision of Prof Nuri ÖZTÜRK. He also worked as a research assistant at Kilis 7 Aralik University in 2012 and at Gebze Technical University between 2013 and 2020. He won a scholarship from the Turkish Government to conduct Ph.D. studies in the UK and currently, he is a Ph.D. student in the Center for Discovery Brain Sciences at the University of Edinburgh under the supervision of Prof. Jamie Davies.

APPENDICES

APPENDIX A: The Paper Published within the Scope of the Thesis

Gul H., Selvi S., Yilmaz F., Ozcelik G., Olfaz-Aslan S., Yazan S., ... Ozturk, N. (2022), "Proteome analysis of the circadian clock protein PERIOD2", *Proteins: Structure, Function, and Bioinformatics*, 90(6), 1315-1330.

APPENDIX B: Mycoplasma Testing

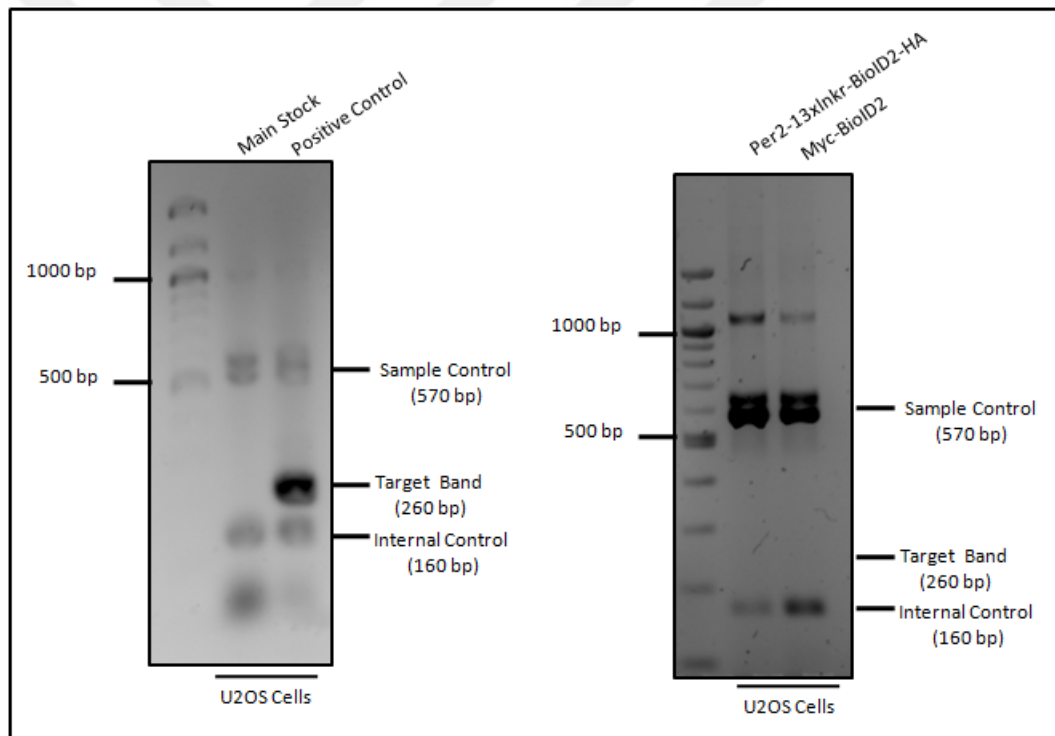


Figure B1.1: Mycoplasma testing of maintenance U2OS cells and stable PER2-BioID2 and Myc-BioID2 clones.

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Figure C1.2: pLenti-III-3xHA-miniTurbo-13xLinker-mBmal1 Plasmid Sequence.

APPENDIX D: Saint Express Analysis Results

Table D1.1: Saint express analysis of PER2-BioID2 CT36 interactome data.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	PER2	P11498	PC	303	151.5	1	1	1	1	1	206.97	1515	0
2	PER2	P37802	TAGLN2	12	6	1	1	1	1	1	6.95	60	0
3	PER2	P05165	PCCA	74	37	1	1	1	1	1	51.96	370	0
4	PER2	P05166	PCCB	50	25	1	1	1	1	1	34.5	250	0
5	PER2	Q96RQ3	MCCC1	87	43.5	1	1	1	1	1	64.93	435	0
6	PER2	P15531	NME1	14	7	1	1	1	1	1	10.26	70	0
7	PER2	P00387	CYB5R3	18	9	1	1	1	1	1	10.26	90	0
8	PER2	P62820	RAB1A	15	7.5	1	1	1	1	1	11.87	75	0
9	PER2	P07900	HSP90AA1	54	27	1	1	1	1	1	40.35	270	0
10	PER2	P62891	RPL39	8	4	1	1	1	1	1	6.95	40	0
11	PER2	Q9HCC0	MCCC2	40	20	1	1	1	1	1	27.12	200	0
12	PER2	P78371	CCT2	18	9	1	1	1	1	1	5.93	18	0
13	PER2	P17252	PRKCA	37	18.5	1	1	1	1	1	5.85	12.33	0
14	PER2	P55072	VCP	36	18	1	1	1	1	1	13.37	9	0
15	PER2	P62258	YWHAE	12	6	1	1	1	1	1	10.26	60	0
16	PER2	P04075	ALDOA	10	5	1	1	1	1	1	8.63	50	0
17	PER2	P05455	SSB	10	5	1	1	1	1	1	6.95	50	0
18	PER2	P22626	HNRNPA2B1	8	4	1	1	1	1	1	6.95	40	0
19	PER2	Q9UKM9	RALY	10	5	1	1	1	1	1	6.95	50	0
20	PER2	P40926	MDH2	10	5	1	1	1	1	1	6.95	50	0
21	PER2	Q16526	CRY1	25	12.5	1	1	1	1	1	16.56	125	0
22	PER2	Q00610	CLTC	40	20	1	1	1	1	1	5.44	3.64	0
23	PER2	P12268	IMPDH2	10	5	1	1	1	1	1	6.95	50	0
24	PER2	P02545	LMNA	24	12	1	1	1	1	1	6.05	12	0
25	PER2	Q99832	CCT7	12	6	1	1	1	1	1	10.26	60	0
26	PER2	P40939	HADHA	24	12	1	1	1	1	1	4.8	12	0
27	PER2	Q09666	AHNAK	36	18	1	1	1	1	1	4.88	4	0
28	PER2	P14625	HSP90B1	21	10.5	1	1	1	1	1	15.01	105	0
29	PER2	Q16181	SEPT7	12	6	1	1	1	1	1	10.26	60	0
30	PER2	P60228	EIF3E	10	5	1	1	1	1	1	6.95	50	0
31	PER2	P43243	MATR3	15	7.5	1	1	1	1	1	10.26	75	0
32	PER2	Q9NWT8	AURKAIP1	10	5	1	1	1	1	1	6.95	50	0
33	PER2	P06396	GSN	12	6	1	1	1	1	1	8.63	60	0
34	PER2	O60506	SYNCRIP	12	6	1	1	1	1	1	10.26	60	0

Table D1.1: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
35	PER2	Q7Z406	MYH14	92	46	1	1	1	1	1	69.24	460	0
36	PER2	Q15139	PRKD1	12	6	1	1	1	1	1	10.26	60	0
37	PER2	Q49AN0	CRY2	8	4	1	1	1	1	1	6.95	40	0
38	PER2	P43686	PSMC4	12	6	1	1	1	1	1	10.26	60	0
39	PER2	Q13085	ACACA	20	10	1	1	1	1	1	8.84	20	0
40	PER2	Q94806	PRKD3	8	4	1	1	1	1	1	6.95	40	0
41	PER2	P23246	SFPQ	8	4	1	1	1	1	1	6.95	40	0
42	PER2	Q8IYJ1	CPNE9	8	4	1	1	1	1	1	6.95	40	0
43	PER2	P49327	FASN	18	9	1	1	1	1	1	13.45	90	0
44	PER2	P06744	GPI	10	5	1	1	1	1	1	6.95	50	0
45	PER2	Q9Y490	TLN1	15	7.5	1	1	1	1	1	7.38	15	0
46	PER2	A6NHR9	SMCHD1	12	6	1	1	1	1	1	10.26	60	0
47	PER2	Q14315	FLNC	20	10	1	1	1	1	1	16.56	100	0
48	PER2	P17096	HMGA1	6	3	0.98	1	0.98	1	0.98	3.36	30	0
49	PER2	Q15287	RNPS1	17	8.5	0.98	1	0.98	1	0.98	3.13	5.67	0
50	PER2	P23381	WARS	6	3	0.98	1	0.98	1	0.98	3.36	30	0
51	PER2	A6NIZ1	RP1BL	6	3	0.98	1	0.98	1	0.98	3.36	30	0
52	PER2	B2RPK0	HMGB1P1	6	3	0.98	1	0.98	1	0.98	3.36	30	0
53	PER2	O00429	DNM1L	6	3	0.98	1	0.98	1	0.98	3.36	30	0
54	PER2	P26599	PTBP1	8	4	0.98	1	0.98	1	0.98	3.36	40	0
55	PER2	P34932	HSPA4	6	3	0.98	1	0.98	1	0.98	3.36	30	0
56	PER2	Q96HE7	ERO1L	6	3	0.98	1	0.98	1	0.98	3.36	30	0
57	PER2	P42285	SKIV2L2	6	3	0.98	1	0.98	1	0.98	3.36	30	0
58	PER2	P55084	HADHB	6	3	0.98	1	0.98	1	0.98	3.36	30	0
59	PER2	P63172	DYNLT1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
60	PER2	P13639	EEF2	28	14	0.97	0.99	0.97	0.99	0.97	2.84	3.5	0
61	PER2	O75915	ARL6IP5	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
62	PER2	Q15691	MAPRE1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
63	PER2	P48643	CCT5	16	8	0.97	0.97	0.97	0.97	0.97	3.53	8	0
64	PER2	P30084	ECHS1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
65	PER2	P59998	ARPC4	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
66	PER2	Q07666	KHDRBS1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
67	PER2	P00846	MT-ATP6	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
68	PER2	P07954	FH	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0

Table D1.1: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
69	PER2	Q9NVD7	PARVA	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
70	PER2	O75718	CRTAP	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
71	PER2	P55010	EIF5	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
72	PER2	F8W031	Uncharacterized protein	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
73	PER2	Q8IV32	CCDC71	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
74	PER2	Q00005	PPP2R2B	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
75	PER2	Q32P51	HNRNPA1L2	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
76	PER2	P30419	NMT1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
77	PER2	P30153	PPP2R1A	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
78	PER2	A6NE09	RPSAP58	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
79	PER2	Q9COH2	TTYH3	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
80	PER2	Q07065	CKAP4	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
81	PER2	O60488	ACSL4	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
82	PER2	P39656	DDOST	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
83	PER2	Q9Y3Z3	SAMHD1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
84	PER2	Q8WU90	ZC3H15	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
85	PER2	Q96AC1	FERMT2	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
86	PER2	P43003	SLC1A3	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
87	PER2	O00116	AGPS	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
88	PER2	Q9UBB4	ATXN10	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
89	PER2	Q14697	GANAB	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
90	PER2	Q8WX93	PALLD	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
91	PER2	Q13813	SPTAN1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
92	PER2	P53396	ACLY	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
93	PER2	Q01082	SPTBN1	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
94	PER2	Q5VYK3	ECM29	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
95	PER2	Q9NZM1	MYOF	4	2	0.97	0.97	0.97	0.97	0.97	3.36	20	0
96	PER2	Q15084	PDIA6	8	4	0.96	0.96	0.96	0.96	0.96	3.06	8	0.02
97	PER2	P27708	CAD	16	8	0.95	0.95	0.95	0.95	0.95	2.88	4	0.02
98	PER2	P50990	CCT8	14	7	0.94	0.97	0.94	0.97	0.94	2.21	7	0.02
99	PER2	P07477	PRSS1	57	28.5	0.94	1	0.94	1	0.94	2	3.17	0.02
100	PER2	Q14974	KPNB1	28	14	0.92	1	0.92	1	0.92	1.64	3.5	0.02
101	PER2	P42766	RPL35	26	13	0.91	0.98	0.91	0.98	0.91	1.64	3.25	0.02
102	PER2	Q04637	EIF4G1	12	6	0.9	0.9	0.9	0.9	0.9	2.21	6	0.02
103	PER2	Q9H0U4	RAB1B	21	10.5	0.89	0.91	0.89	0.91	0.89	1.97	5.25	0.02
104	PER2	P04792	HSPB1	14	7	0.79	0.95	0.79	0.95	0.79	0.52	3.5	0.02

Table D1.1: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
105	PER2	P13010	XRCC5	16	8	0.71	0.88	0.71	0.88	0.71	0.15	4	0.02
106	PER2	P31942	HNRNPH3	8	4	0.71	0.71	0.71	0.71	0.71	0.89	4	0.02
107	PER2	P48730	CSNK1D	12	6	0.63	0.63	0.63	0.63	0.63	0.52	3	0.03
108	PER2	O75533	SF3B1	12	6	0.63	0.63	0.63	0.63	0.63	0.52	3	0.03
109	PER2	O00410	IPO5	12	6	0.63	0.63	0.63	0.63	0.63	0.52	3	0.03
110	PER2	Q86VP6	CAND1	7	3.5	0.61	0.71	0.61	0.71	0.61	0.03	3.5	0.04
111	PER2	P62937	PPIA	17	8.5	0.6	0.75	0.6	0.75	0.6	-0.16	2.83	0.04
112	PER2	P07910	HNRNPC	10	5	0.59	0.76	0.59	0.76	0.59	-0.27	3.33	0.04

Table D1.2: Saint express analysis of PER2-BioID2 CT48 interactome data.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	PER2	P11498	PC	353	176.5	1	1	1	1	1	253.93	1765	0
2	PER2	P05166	PCCB	103	51.5	1	1	1	1	1	72.47	515	0
3	PER2	P06899	HIST1H2BJ	116	58	1	1	1	1	1	83.92	580	0
4	PER2	P05165	PCCA	167	83.5	1	1	1	1	1	116.69	835	0
5	PER2	Q9HCC0	MCCC2	134	67	1	1	1	1	1	93.91	670	0
6	PER2	Q96RQ3	MCCC1	141	70.5	1	1	1	1	1	103.88	705	0
7	PER2	P22392	NME2	25	12.5	1	1	1	1	1	18.46	125	0
8	PER2	P06733	ENO1	48	24	1	1	1	1	1	8.68	3.69	0
9	PER2	Q13085	ACACA	219	109.5	1	1	1	1	1	157.82	1095	0
10	PER2	P63104	YWHAZ	23	11.5	1	1	1	1	1	18.46	115	0
11	PER2	P17096	HMGA1	8	4	1	1	1	1	1	7.31	40	0
12	PER2	P13639	EEF2	60	30	1	1	1	1	1	7.61	15	0
13	PER2	Q15149	PLEC	378	189	1	1	1	1	1	32.9	3.02	0
14	PER2	P00558	PGK1	20	10	1	1	1	1	1	16.92	100	0
15	PER2	P09651	HNRNPA1	27	13.5	1	1	1	1	1	19.98	135	0
16	PER2	P22626	HNRNPA2B1	18	9	1	1	1	1	1	13.81	90	0
17	PER2	P07900	HSP90AA1	51	25.5	1	1	1	1	1	36.33	255	0
18	PER2	P78371	CCT2	26	13	1	1	1	1	1	6.27	5.2	0
19	PER2	P21796	VDAC1	16	8	1	1	1	1	1	13.81	80	0
20	PER2	B2RPK0	HMGB1P1	12	6	1	1	1	1	1	8.99	60	0
21	PER2	Q15084	PDIA6	13	6.5	1	1	1	1	1	10.63	65	0
22	PER2	P00387	CYB5R3	14	7	1	1	1	1	1	10.63	70	0
23	PER2	P17252	PRKCA	33	16.5	1	1	1	1	1	25.99	165	0
24	PER2	P02545	LMNA	30	15	1	1	1	1	1	7.36	6	0

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
25	PER2	P55795	HNRNPH2	16	8	1	1	1	1	1	10.63	80	0
26	PER2	P40926	MDH2	16	8	1	1	1	1	1	10.63	80	0
27	PER2	P39019	RPS19	8	4	1	1	1	1	1	7.31	40	0
28	PER2	Q9Y3U8	RPL36	8	4	1	1	1	1	1	7.31	40	0
29	PER2	P40939	HADHA	30	15	1	1	1	1	1	24.5	150	0
30	PER2	Q99714	HSD17B10	12	6	1	1	1	1	1	10.63	60	0
31	PER2	P23246	SFPQ	16	8	1	1	1	1	1	10.63	80	0
32	PER2	P21333	FLNA	102	51	1	1	1	1	1	30.04	5.67	0
33	PER2	P61020	RAB5B	8	4	1	1	1	1	1	7.31	40	0
34	PER2	P0DMV8	HSPA1A	22	11	1	1	1	1	1	16.92	110	0
35	PER2	Q14974	KPNB1	30	15	1	1	1	1	1	4.95	5	0
36	PER2	P04075	ALDOA	10	5	1	1	1	1	1	8.99	50	0
37	PER2	Q9HDC9	APMAP	8	4	1	1	1	1	1	7.31	40	0
38	PER2	P11413	G6PD	17	8.5	1	1	1	1	1	13.81	85	0
39	PER2	P55072	VCP	45	22.5	1	1	1	1	1	4.88	11.25	0
40	PER2	P51991	HNRNPA3	9	4.5	1	1	1	1	1	7.31	45	0
41	PER2	P43243	MATR3	24	12	1	1	1	1	1	5.41	6	0
42	PER2	P15311	EZR	22	11	1	1	1	1	1	18.46	110	0
43	PER2	P50990	CCT8	16	8	1	1	1	1	1	12.23	80	0
44	PER2	Q99832	CCT7	18	9	1	1	1	1	1	13.81	90	0
45	PER2	P35270	SPR	8	4	1	1	1	1	1	7.31	40	0
46	PER2	P02538	KRT6A	24	12	1	1	1	1	1	16.92	120	0
47	PER2	P47756	CAPZB	7	3.5	1	1	1	1	1	5.57	35	0
48	PER2	P14625	HSP90B1	34	17	1	1	1	1	1	23	170	0
49	PER2	Q04695	KRT17	18	9	1	1	1	1	1	13.81	90	0
50	PER2	Q16526	CRY1	20	10	1	1	1	1	1	16.92	100	0
51	PER2	P12814	ACTN1	24	12	1	1	1	1	1	7.68	6	0
52	PER2	A6NIZ1	RP1BL	8	4	1	1	1	1	1	7.31	40	0
53	PER2	Q9NWT8	AURKAIP1	12	6	1	1	1	1	1	10.63	60	0
54	PER2	P22234	PAICS	14	7	1	1	1	1	1	10.63	70	0
55	PER2	Q75390	CS	12	6	1	1	1	1	1	10.63	60	0
56	PER2	O43707	ACTN4	22	11	1	1	1	1	1	16.92	110	0
57	PER2	O60506	SYNCRIP	14	7	1	1	1	1	1	6.29	14	0
58	PER2	Q86YQ8	CPNE8	12	6	1	1	1	1	1	10.63	60	0
59	PER2	P49327	FASN	35	17.5	1	1	1	1	1	13.73	8.75	0
60	PER2	P31942	HNRNPH3	8	4	1	1	1	1	1	7.31	40	0

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
61	PER2	Q49AN0	CRY2	8	4	1	1	1	1	1	7.31	40	0
62	PER2	P07237	P4HB	10	5	1	1	1	1	1	7.31	50	0
63	PER2	P39656	DDOST	8	4	1	1	1	1	1	7.31	40	0
64	PER2	P42704	LRPPRC	16	8	1	1	1	1	1	10.63	80	0
65	PER2	P19367	HK1	12	6	1	1	1	1	1	10.63	60	0
66	PER2	P02786	TFRC	8	4	1	1	1	1	1	7.31	40	0
67	PER2	O60732	MAGEC1	12	6	1	1	1	1	1	10.63	60	0
68	PER2	Q07065	CKAP4	14	7	1	1	1	1	1	7.31	70	0
69	PER2	P25205	MCM3	8	4	1	1	1	1	1	7.31	40	0
70	PER2	Q96HE7	ERO1L	8	4	1	1	1	1	1	7.31	40	0
71	PER2	O94808	GFPT2	8	4	1	1	1	1	1	7.31	40	0
72	PER2	O94806	PRKD3	8	4	1	1	1	1	1	7.31	40	0
73	PER2	Q8IV36	HID1	12	6	1	1	1	1	1	5.57	60	0
74	PER2	P08708	RPS17	20	10	0.99	1	0.99	1	0.99	3.89	10	0
75	PER2	P51572	BCAP31	7	3.5	0.99	1	0.99	1	0.99	3.72	35	0
76	PER2	Q14103	HNRNPD	12	6	0.99	1	0.99	1	0.99	3.72	60	0
77	PER2	P62258	YWHAE	10	5	0.99	1	0.99	1	0.99	3.72	50	0
78	PER2	P61927	RPL37	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
79	PER2	P50395	GDI2	14	7	0.99	1	0.99	1	0.99	3.86	7	0
80	PER2	Q9UL46	PSME2	6	3	0.99	1	0.99	1	0.99	3.72	30	0
81	PER2	Q15233	NONO	19	9.5	0.99	0.99	0.99	0.99	0.99	4.54	9.5	0
82	PER2	P26599	PTBP1	8	4	0.99	1	0.99	1	0.99	3.72	40	0
83	PER2	P30566	ADSL	6	3	0.99	1	0.99	1	0.99	3.72	30	0
84	PER2	Q14697	GANAB	18	9	0.99	1	0.99	1	0.99	4.63	6	0
85	PER2	Q09666	AHNAK	39	19.5	0.99	1	0.99	1	0.99	4.35	9.75	0
86	PER2	P49755	TMED10	6	3	0.99	1	0.99	1	0.99	3.72	30	0
87	PER2	O95433	AHSA1	6	3	0.99	1	0.99	1	0.99	3.72	30	0
88	PER2	Q15691	MAPRE1	6	3	0.99	1	0.99	1	0.99	3.72	30	0
89	PER2	Q12906	ILF3	11	5.5	0.99	1	0.99	1	0.99	3.72	55	0
90	PER2	Q99729	HNRNPAB	6	3	0.99	1	0.99	1	0.99	3.72	30	0
91	PER2	Q9H307	PNN	7	3.5	0.99	1	0.99	1	0.99	3.72	35	0
92	PER2	P04181	OAT	6	3	0.99	1	0.99	1	0.99	3.72	30	0
93	PER2	P27695	APEX1	6	3	0.99	1	0.99	1	0.99	3.72	30	0
94	PER2	P51665	PSMD7	6	3	0.99	1	0.99	1	0.99	3.72	30	0
95	PER2	P05556	ITGB1	6	3	0.99	1	0.99	1	0.99	3.72	30	0
96	PER2	P22314	UBA1	6	3	0.99	1	0.99	1	0.99	3.72	30	0

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
97	PER2	Q9NQC3	RTN4	6	3	0.99	1	0.99	1	0.99	3.72	30	0
98	PER2	Q3KQU3	MAP7D1	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
99	PER2	P55084	HADHB	11	5.5	0.99	1	0.99	1	0.99	3.72	55	0
100	PER2	P18206	VCL	6	3	0.99	1	0.99	1	0.99	3.72	30	0
101	PER2	P06396	GSN	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
102	PER2	O43747	AP1G1	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
103	PER2	P29317	EPHA2	8	4	0.99	1	0.99	1	0.99	3.72	40	0
104	PER2	P24821	TNC	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
105	PER2	Q5T4S7	UBR4	5	2.5	0.99	1	0.99	1	0.99	3.72	25	0
106	PER2	P62937	PPIA	18	9	0.98	1	0.98	1	0.98	3.24	9	0
107	PER2	P63173	RPL38	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
108	PER2	P12956	XRCC6	32	16	0.98	0.98	0.98	0.98	0.98	3.76	3.2	0
109	PER2	Q9P0L0	VAPA	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
110	PER2	K7EPI3	Unchrctrzd protein	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
111	PER2	Q9P2X0	DPM3	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
112	PER2	Q9Y3B4	SF3B6	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
113	PER2	P25787	PSMA2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
114	PER2	Q16762	TST	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
115	PER2	P04183	TK1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
116	PER2	O14818	PSMA7	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
117	PER2	Q14696	MESDC2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
118	PER2	P51149	RAB7A	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
119	PER2	O00764	PDXK	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
120	PER2	P54619	PRKAG1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
121	PER2	Q02750	MAP2K1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
122	PER2	Q14919	DRAP1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
123	PER2	Q9C0K3	ACTR3C	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
124	PER2	P59998	ARPC4	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
125	PER2	P68400	CSNK2A1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
126	PER2	P52209	PGD	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
127	PER2	Q9BW60	ELOVL1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
128	PER2	P62491	RAB11A	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
129	PER2	P07954	FH	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
130	PER2	O95251	KAT7	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
131	PER2	P40925	MDH1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
132	PER2	P53007	SLC25A1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
133	PER2	P30040	ERP29	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
134	PER2	P25789	PSMA4	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
135	PER2	P61160	ACTR2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
136	PER2	P50552	VASP	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
137	PER2	Q9UHD8	SEPT9	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
138	PER2	Q8TBX8	PIP4K2C	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
139	PER2	P05455	SSB	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
140	PER2	O75844	ZMPSTE24	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
141	PER2	Q13243	SRSF5	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
142	PER2	P08240	SRPR	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
143	PER2	Q14254	FLOT2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
144	PER2	Q8IV32	CCDC71	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
145	PER2	Q9Y512	SAMM50	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
146	PER2	P49761	CLK3	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
147	PER2	P19338	NCL	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
148	PER2	Q9Y265	RUUBL1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
149	PER2	O94826	TOMM70A	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
150	PER2	P11182	DBT	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
151	PER2	Q13148	TARDBP	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
152	PER2	P30419	NMT1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
153	PER2	P78395	PRAME	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
154	PER2	P30153	PPP2R1A	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
155	PER2	P33176	KIF5B	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
156	PER2	Q9BUJ2	HNRNPUL1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
157	PER2	P27824	CANX	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
158	PER2	P30101	PDIA3	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
159	PER2	Q01082	SPTBN1	12	6	0.98	1	0.98	1	0.98	3.42	12	0
160	PER2	Q13200	PSMD2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
161	PER2	P51659	HSD17B4	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
162	PER2	Q15139	PRKD1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
163	PER2	P34932	HSPA4	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
164	PER2	Q9NV70	EXOC1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
165	PER2	Q9NPQ8	RIC8A	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
166	PER2	P08243	ASNS	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
167	PER2	Q8NI36	WDR36	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
168	PER2	P12268	IMPDH2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
169	PER2	P28838	LAP3	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
170	PER2	P57740	NUP107	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
171	PER2	Q14566	MCM6	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
172	PER2	Q6PI48	DARS2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
173	PER2	Q93009	USP7	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
174	PER2	P43304	GPD2	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
175	PER2	Q07157	TJP1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
176	PER2	P51610	HCFC1	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
177	PER2	P12270	TPR	4	2	0.98	0.98	0.98	0.98	0.98	3.72	20	0
178	PER2	P62891	RPL39	8	4	0.97	0.97	0.97	0.97	0.97	3.42	8	0.01
179	PER2	P07910	HNRNPC	17	8.5	0.97	0.99	0.97	0.99	0.97	3.24	4.25	0.01
180	PER2	P52272	HNRNPM	30	15	0.97	0.99	0.97	0.99	0.97	2.89	3.33	0.01
181	PER2	P08758	ANXA5	14	7	0.95	0.98	0.95	0.98	0.95	2.58	7	0.01
182	PER2	P46940	IQGAP1	14	7	0.95	0.98	0.95	0.98	0.95	2.58	7	0.01
183	PER2	P42766	RPL35	29	14.5	0.94	1	0.94	1	0.94	2	3.62	0.01
184	PER2	P11586	MTHFD1	8	4	0.94	0.99	0.94	0.99	0.94	2.02	8	0.01
185	PER2	P60953	CDC42	15	7.5	0.93	0.96	0.93	0.96	0.93	2.09	3.75	0.01
186	PER2	O75396	SEC22B	12	6	0.93	0.93	0.93	0.93	0.93	2.58	6	0.01
187	PER2	P49368	CCT3	20	10	0.93	0.93	0.93	0.93	0.93	2.65	3.33	0.01
188	PER2	Q9Y490	TLN1	24	12	0.93	1	0.93	1	0.93	1.77	4.8	0.01
189	PER2	P23526	AHCY	13	6.5	0.88	0.99	0.88	0.99	0.88	1.25	6.5	0.01
190	PER2	P22570	FDXR	12	6	0.88	0.98	0.88	0.98	0.88	1.25	6	0.01
191	PER2	Q01518	CAP1	10	5	0.87	0.87	0.87	0.87	0.87	1.9	5	0.02
192	PER2	Q9NTK5	OLA1	15	7.5	0.85	0.99	0.85	0.99	0.85	0.89	3.75	0.02
193	PER2	P04792	HSPB1	17	8.5	0.84	0.91	0.84	0.91	0.84	1.15	4.25	0.02
194	PER2	P22102	GART	14	7	0.84	0.96	0.84	0.96	0.84	0.89	3.5	0.02
195	PER2	Q9Y6V0	PCLO	7	3.5	0.83	0.99	0.83	0.99	0.83	0.68	7	0.02
196	PER2	P32322	PYCR1	6	3	0.82	0.97	0.82	0.97	0.82	0.68	6	0.02
197	PER2	P13674	P4HA1	8	4	0.78	0.78	0.78	0.78	0.78	1.25	4	0.02
198	PER2	P04844	RPN2	8	4	0.78	0.78	0.78	0.78	0.78	1.25	4	0.02
199	PER2	P26639	TARS	8	4	0.78	0.78	0.78	0.78	0.78	1.25	4	0.02
200	PER2	P48643	CCT5	20	10	0.77	0.99	0.77	0.99	0.77	0.21	3.33	0.02
201	PER2	P26641	EEF1G	34	17	0.76	0.93	0.76	0.93	0.76	0.33	2.83	0.02
202	PER2	P07737	PFN1	8	4	0.73	0.87	0.73	0.87	0.73	0.4	4	0.02
203	PER2	P07814	EPRS	8	4	0.73	0.87	0.73	0.87	0.73	0.4	4	0.02
204	PER2	Q12905	ILF2	12	6	0.71	0.71	0.71	0.71	0.71	0.89	3	0.03

Table D1.2: Continued.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
205	PER2	Q9H0U4	RAB1B	27	13.5	0.69	0.83	0.69	0.83	0.69	0.19	3.38	0.03
206	PER2	O95372	LYPLA2	4	2	0.66	0.66	0.66	0.66	0.66	0.68	4	0.03
207	PER2	P09211	GSTP1	4	2	0.66	0.66	0.66	0.66	0.66	0.68	4	0.03
208	PER2	O43795	MYO1B	16	8	0.66	0.98	0.66	0.98	0.66	-0.72	3.2	0.04
209	PER2	Q9NVD7	PARVA	4	2	0.66	0.66	0.66	0.66	0.66	0.68	4	0.03
210	PER2	Q08J23	NSUN2	4	2	0.66	0.66	0.66	0.66	0.66	0.68	4	0.03
211	PER2	Q16531	DDB1	4	2	0.66	0.66	0.66	0.66	0.66	0.68	4	0.03
212	PER2	P19105	MYL12A	22	11	0.63	0.88	0.63	0.88	0.63	-0.48	2.75	0.04
213	PER2	Q9P2E9	RRBP1	19	9.5	0.62	0.81	0.62	0.81	0.62	-0.27	3.17	0.04
214	PER2	P37108	SRP14	6	3	0.6	0.6	0.6	0.6	0.6	0.4	3	0.04
215	PER2	P06576	ATP5B	27	13.5	0.58	0.93	0.58	0.93	0.58	-1.17	2.7	0.04

Table D1.3: Saint express analysis of MT-BMAL1 Set1 top bottleneck proteins.

	Bait	Accession	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	BMAL1	Q13882	PTK6	3	3	0.99	0.99	0.99	0.99	0.99	4.45	30	0
2	BMAL1	P06241	FYN	3	3	0.99	0.99	0.99	0.99	0.99	4.45	30	0
3	BMAL1	Q9H2U1	DHX36	2	2	0.93	0.93	0.93	0.93	0.93	2.61	20	0.02
4	BMAL1	P04899	GNAI2	2	2	0.93	0.93	0.93	0.93	0.93	2.61	20	0.02

Table D1.4: Saint express analysis of MT-BMAL1 Set2 top bottleneck proteins.

	Bait	Prey	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	BMAL1	Q14240	EIF4A2	10	10	1	1	1	1	1	15.9	100	0
2	BMAL1	Q9H2U1	DHX36	3	3	0.99	0.99	0.99	0.99	0.99	4.54	30	0
3	BMAL1	P49841	GSK3B	2	2	0.94	0.94	0.94	0.94	0.94	2.7	20	0.01
4	BMAL1	O00327	ARNTL	2	2	0.94	0.94	0.94	0.94	0.94	2.7	20	0.01

Table D1.5: Saint express analysis of p(Bmal1)-MT-BMAL1 CT32 interactome data.

	Bait	Prey	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	BMAL1	P0C055	H2AZ1	56	28	0.92	1	0.92	1	0.92	1.62	3.29	0
2	BMAL1	P0DMV8	HSPA1A	6	3	0.79	0.98	0.79	0.98	0.79	0.39	30	0
3	BMAL1	P11498	PC	15	7.5	0.64	0.99	0.64	0.99	0.64	-0.88	7.5	0.21
4	BMAL1	P31153	MAT2A	4	2	0.6	0.6	0.6	0.6	0.6	0.39	20	0.28
5	BMAL1	P54198	HIRA	4	2	0.6	0.6	0.6	0.6	0.6	0.39	20	0.28
6	BMAL1	Q9NZM5	NOP53	4	2	0.55	0.55	0.55	0.55	0.55	0.22	20	0.08

Table D1.5: Continued.

	Bait	Prey	PreyGene	Spec	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
7	BMAL1	Q9Y4W6	AFG3L2	2 2	2	0.55	0.55	0.55	0.55	0.55	0.22	20	0.08
8	BMAL1	O43747	AP1G1	2 2	2	0.55	0.55	0.55	0.55	0.55	0.22	20	0.08
9	BMAL1	P13674	P4HA1	6 6	6	0.51	0.51	0.51	0.51	0.51	0.02	6	0.35

Table D1.6: Saint express analysis of p(Bmal1)-MT-BMAL1 CT44 interactome data.

	Bait	Prey	PreyGene	Spec	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
1	BMAL1	P08708	RPS17	16 13	14.5	1	1	1	1	1	7.36	29	0
2	BMAL1	O60361	NME2P1	9 9	9	1	1	1	1	1	5.5	9	0
3	BMAL1	P0DMV8	HSPA1A	12 16	14	1	1	1	1	1	18.59	140	0
4	BMAL1	P24844	MYL9	12 11	11.5	0.99	1	0.99	1	0.99	4.48	5.75	0
5	BMAL1	P19105	MYL12A	22 18	20	0.98	1	0.98	1	0.98	3.25	3.64	0.01
6	BMAL1	P20648	ATP4A	3 3	3	0.98	0.98	0.98	0.98	0.98	4.18	30	0
7	BMAL1	P02511	CRYAB	7 7	7	0.97	0.97	0.97	0.97	0.97	3.43	14	0.01
8	BMAL1	P0C055	H2AZ1	29 25	27	0.96	1	0.96	1	0.96	2.53	3.18	0.01
9	BMAL1	P35659	DEK	15 11	13	0.96	1	0.96	1	0.96	2.43	4.33	0.01
10	BMAL1	Q14919	DRAP1	2 4	3	0.95	1	0.95	1	0.95	2.34	30	0.02
11	BMAL1	P20073	ANXA7	2 3	2.5	0.95	0.98	0.95	0.98	0.95	2.34	25	0.02
12	BMAL1	O94806	PRKD3	3 2	2.5	0.95	0.98	0.95	0.98	0.95	2.34	25	0.02
13	BMAL1	P26583	HMGB2	6 6	6	0.94	0.94	0.94	0.94	0.94	2.72	12	0.03
14	BMAL1	Q03252	LMNB2	5 6	5.5	0.94	0.97	0.94	0.97	0.94	2.26	7.33	0.03
15	BMAL1	P04792	HSPB1	18 24	21	0.91	1	0.91	1	0.91	1.5	3.82	0.06
16	BMAL1	O14602	EIF1AY	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
17	BMAL1	P09132	SRP19	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
18	BMAL1	Q9P287	BCCIP	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
19	BMAL1	P61221	ABCE1	5 6	5.5	0.91	0.94	0.91	0.94	0.91	1.98	11	0.06
20	BMAL1	Q16630	CPSF6	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
21	BMAL1	Q92604	LPGAT1	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
22	BMAL1	Q14699	RFTN1	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
23	BMAL1	Q00059	TFAM	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
24	BMAL1	Q14195	DPYSL3	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
25	BMAL1	Q8WVX9	FAR1	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
26	BMAL1	O76031	CLPX	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
27	BMAL1	P41250	GARS1	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
28	BMAL1	Q8N2M8	CLASRP	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
29	BMAL1	Q965T3	SIN3A	2 2	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03

Table D1.6: Continued.

	Bait	Prey	PreyGene	Spec Sum	Avg Spec	AvgP	MaxP	Topo AvgP	Topo MaxP	Saint Score	logOdds Score	Fold Change	BFDR
30	BMAL1	Q9HCE3	ZNF532	4	2	0.91	0.91	0.91	0.91	0.91	2.34	20	0.03
31	BMAL1	Q9H307	PNN	20	10	0.88	0.88	0.88	0.88	0.88	2	3.64	0.06
32	BMAL1	P33992	MCM5	10	5	0.85	0.97	0.85	0.97	0.85	0.96	6.67	0.06
33	BMAL1	O60506	SYNCRIP	24	12	0.71	0.99	0.71	0.99	0.71	-0.28	3.43	0.07
34	BMAL1	Q14203	DCTN1	4	2	0.63	0.63	0.63	0.63	0.63	0.52	8	0.07
35	BMAL1	O60281	ZNF292	4	2	0.63	0.63	0.63	0.63	0.63	0.52	8	0.07
36	BMAL1	P55884	EIF3B	7	3.5	0.62	0.88	0.62	0.88	0.62	-0.54	7	0.09
37	BMAL1	Q9UQ80	PA2G4	10	5	0.6	0.6	0.6	0.6	0.6	0.4	5	0.1
38	BMAL1	P31930	UQCRC1	6	3	0.57	0.77	0.57	0.77	0.57	-0.54	6	0.11
39	BMAL1	Q9NYF8	BCLAF1	28	14	0.55	0.99	0.55	0.99	0.55	-2.21	3.11	0.11
40	BMAL1	P62195	PSMC5	17	8.5	0.52	0.88	0.52	0.88	0.52	-1.64	3.09	0.12
41	BMAL1	O43615	TIMM44	8	4	0.52	0.52	0.52	0.52	0.52	0.06	4	0.13
42	BMAL1	P68032	ACTC1	165	82.5	0.51	1	0.51	1	0.51	-3.73	2.77	0.14