

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**KNOCKOUT AND COMPLEMENTATION OF BMAL1 GENE
FOR CIRCADIAN RESEARCH**

BÜŞRA TİRYAKİ

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

ADVISOR: PROF. DR. NURİ ÖZTÜRK

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

BMAL1 GENİNİN SİRKADİYEN ARAŞTIRMALAR İÇİN
NAKAVT VE KOMPLEMENTASYONU

BÜŞRA TİRYAKİ

YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMAN: PROF. DR. NURİ ÖZTÜRK

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*To those who burn both ends of
the candle for the sake of science*

ABSTRACT

The circadian clock ensures the organism's harmony with environment. The disfunction or disruption of the circadian clock in some way has many consequences on the health of organisms as such, the importance of the circadian clock in the scientific studies, diagnosis and treatment of most diseases that threaten human health is an indisputable fact. At the molecular level, the circadian clock is generated by a transcription translation feedback loop (TTFL). In the positive arm of the TTFL, CLOCK and BMAL1 transcription factors transactivate the expression of the circadian clock-controlled genes (CCGs). The products of two of the CCGs, CRYPTOCHROME and PERIOD, which make the negative arm of the TTFL, translocate into nucleus, and inhibits the CLOCK-BMAL1 activity by protein-protein interaction. This activation and inhibition generate an oscillatory pattern in the CCGs which are further tuned by secondary loops and post-translational modifications. Studying the effects of variations in clock genes is a hot topic because of its relation to human health. However, the effect of variation of small changes such as SNPs might be difficult to observe in the presence of the wild-type genes. Therefore, the generation of knockout cells and then adding back of the variants of the target genes to study their direct effect without any interference from wild-type protein is a promising strategy.

In this study, we developed a system to dissect the BMAL1 function in the circadian clock. For this purpose, we knocked out *BMAL1* gene in U2OS cells using the CRISPR/Cas9 gene editing system and confirmed the disruption of circadian oscillation. Subsequently, *BMAL1* coding sequence was added back into the knockout cell line to see the recovery of the oscillation. The main purpose of the generation of this cell line is to study the effects of *BMAL1* variations such as SNPs on the circadian oscillation. This strategy is now available to study the functional relevance of genetic alterations of *BMAL1* gene and can be applied for other circadian clock genes or even to any genes.

Key Words: Gene Editing, CRISPR/Cas9, Circadian clock, BMAL1, SNPs.

ÖZET

Sirkadiyen saat organizmanın çevre ile uyumunu sağlar. Sirkadiyen saatin bir şekilde işlevsiz kalması veya bozulması organizmaların sağlığı üzerinde birçok sonuç doğurur, bu nedenle insan sağlığını tehdit eden çoğu hastalığın bilimsel çalışmalarında, teşhis ve tedavisinde sirkadiyen saatin önemi tartışılmaz bir gerçektir. Moleküler düzeyde, sirkadiyen saat bir transkripsiyon çeviri geri besleme döngüsü (TTLF) tarafından oluşturulur. Transkripsiyon çeviri geri besleme döngüsünün pozitif kolunda CLOCK ve BMAL1 transkripsiyon faktörleri sirkadiyen saat kontrollü genlerin (CCG'ler) ifadesini transaktive eder. TTFL'nin negatif kolunu oluşturan CCG'lerden ikisinin ürünleri, CRYPTOCHROME ve PERIOD, çekirdeğe translokasyon yapar ve protein-protein etkileşimi ile CLOCK-BMAL1 aktivitesini inhibe eder. Bu aktivasyon ve inhibisyon, CCG'lerde ikincil döngüler ve post-translasyonel modifikasyonlarla daha da ayarlanan salınımlı bir model oluşturur. Saat genlerindeki varyasyonların etkilerini incelemek, insan sağlığı ile ilişkisi nedeniyle sıcak bir konudur. Bununla birlikte, SNP'ler gibi küçük değişikliklerin varyasyonunun etkisini vahşi tip genlerin varlığında gözlemlemek zor olabilir. Bu nedenle, nakavt hücrelerin üretilmesi ve daha sonra vahşi tip proteinden herhangi bir müdahale olmaksızın doğrudan etkilerini incelemek için hedef genlerin varyantlarının geri eklenmesi umut verici bir stratejidir.

Bu çalışmada, BMAL1'in sirkadiyen saatteki işlevini incelemek için bir sistem geliştirdik. Bu amaçla, CRISPR/Cas9 gen düzenleme sistemini kullanarak U2OS hücrelerinde BMAL1 genini devre dışı bıraktık ve sirkadiyen salınının bozulduğunu doğruladık. Daha sonra, BMAL1 kodlama dizisi, salınının geri kazanılmasını görmek için nakavt edilmiş hücre hattına geri eklendi. Bu hücre hattının oluşturulmasının temel amacı, SNP'ler gibi BMAL1 varyasyonlarının sirkadiyen salınım üzerindeki etkilerini incelemektir. Bu strateji artık BMAL1 genindeki genetik değişikliklerin işlevsel uygunluğunu incelemek için kullanılabilir ve diğer sirkadiyen saat genleri veya hatta herhangi bir gen için uygulanabilir.

Anahtar kelimeler: Gen düzenleme, CRISPR/Cas9, sirkadiyen saat, BMAL1, SNP'ler.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	: Centigrade Celsius
µg	: Microgram
µL	: Microliter
µM	: Micromolar
Amp	: Ampicillin
APS	: Ammonium persulfate
ATP	: Adenosine triphosphate
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle Arnt-like 1
Bsd	: Blasticidin
Bp	: Base pair
CLOCK	: Circadian locomotor output cycles protein kaput
CO ₂	: Carbon dioxide
CoA	: Coenzyme A
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
CRY	: Cryptochrome
CT	: Circadian time
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
FBS	: Fetal Bovine Serum
DMEM	: Dulbecco's Modified Eagle Medium
DNA	: Deoxyribonucleic acid
EDTA	: Ethylenediaminetetraacetic Acid
ECL	: Enhanced chemiluminescence
HCl	: Hydrochloric Acid
HEK293T	: Human embryonic kidney 293T cells
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h-CTZ	: Coelenterazine H
IGEPAL	: Octylphenoxy poly(ethyleneoxy)ethanol
Kan	: Kanamycin
KCl	: Potassium chloride
KH ₂ PO ₄	: Potassium dihydrogen phosphate
LB Broth	: Luria-Bertani Broth
M	: Molar
mM	: Millimolar
ms	: Millisecond
NaCl	: Sodium chloride

Na ₂ EDTA	: Ethylenediaminetetraacetic Acid, Disodium Salt
Na ₂ HPO ₄	: Dibasic sodium phosphate
NaOH	: Sodium Hydroxide
nM	: Nanomolar
Na ₄ P ₂ O ₇	: Tetrasodium pyrophosphate
NEAA	: Non-essential amino acid
NP-40	: nonyl phenoxypolyethoxylethanol
MgCl ₂	: Magnesium chloride
mL	: Millilitre
PBS	: Phosphate buffered saline
Pen/strep	: Penicillin/streptomycin
PEI	: Polyethylenimine
PER	: Period gene
pH	: potential of hydrogen
PI	: Protease inhibitor
Puro	: Puromycin
PTC-124	: Ataluren
PTM	: Post-translational modification
RNA	: Ribonucleic acid
REV-ERB	: Nuclear receptor subfamily 1 group D
RIPA	: Radio Immuno Precipitation Assay
ROR	: Retinoid-related orphan receptor
rpm	: Revolutions per minute
RT	: Room temperature
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sgRNA	: Single guide ribonucleic acid
TBE	: Tris-Borate-EDTA
TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline-Tween 20
TEMED	: Tetramethylethylenediamine
TRIS	: Hydroxymethylaminomethane
TTFL	: Transcription translation feedback loop
V	: Volt
V:V	: Volume to volume
U	: Unit
U2OS	: Human Bone Osteosarcoma Epithelial Cells
Zeo	: Zeocin

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1. INTRODUCTION

The mammalian circadian clock is a cell-autonomous oscillation mechanism that regulates daily physiologic and metabolic activities. In mammals, the master clock in suprachiasmatic nucleus (SCN) controls the peripheral clocks of organ and tissues to generate these 24-hr oscillations. At the molecular level, the circadian clock in mammals is generated by a transcription-translation feedback loop (TTFL) (Dunlap, 1998). In TTFL model, positive elements activate the genes of negative repressors, while the negative components inhibit the transcriptional activators after accumulating in the cytoplasm at the cost of stopping their own activation. This cyclic process generates a harmony in the organism and each element of this cyclic process is of particular importance. The positive elements are transcription factors, *Bmal1* and *Clock* while the negative components are *Cryptochrome (Cry)* and *Period (Per)* genes. It is known that the absence of *Bmal1* which regulates negative elements *Per* and *Cry* expressions causes lack of circadian rhythmicity (Kim and Forger, 2012). Disrupted circadian oscillation has known and unknown consequences for the organism. For example, >90% deletion of *Bmal1* in the SCN leads to complete loss of circadian rhythm (Izumo et al., 2014) so that, any uncharacterised mutation in *BMAL1* could cause this loss of oscillation. For this reason, we designed a biological tool to investigate the effect of any mutation or variation in *Bmal1* gene. In this system, U2OS cells, in which circadian oscillation is best recorded, were selected. In these cells, the human *BMAL1* gene was knocked out using CRISPR/Cas9 technology. A system expressing luciferase under the *Bmal1* promoter was added to the same cells for circadian oscillation monitoring. After the circadian oscillation was shown to be disrupted, the mouse *Bmal1* gene, which is not targeted by the designed Cas9/sgRNA was added back into the cells. The rescue of oscillation in *Bmal1* added-back in cells was tested using the luciferase system based on *Bmal1* promoter activity. The observation of the oscillation when variants of *Bmal1* gene are added back into this cell line it will give clues about the effect of the variations on the properties of the oscillation such as amplitude and period. This tool has great potential as a tool for the diagnosis and treatment of physiological or psychological diseases caused by circadian dysfunctions.

2. PURPOSE OF THE STUDY

The overarching objective of this study is to delete *BMAL1* gene, a key player in the circadian clock mechanism (Figure 2.1. Step 1). Subsequently, the disruption of circadian oscillation is observed to confirm the knockout process (Figure 2.1. Step 2). Following this, *Bmal1* coding sequence is added back to the knockout cell line to demonstrate the re-gain of circadian oscillation (Figure 2.1. Step 3). Thus, any mutations or variations in the *Bmal1* gene sequence can be investigated for its effects on circadian oscillation. In this context, circadian oscillation with an approximate 25-hour period was recorded in U2OS-pLV7-Bsd-p(Bmal1)-*dluc* cells by monitoring *Bmal1* promoter activity. Cells with the luciferase reporter controlled by *Bmal1* promoter were subjected to genome edition process using Cas9-sgRNA combinations targeting different gene regions to knockout *BMAL1* gene which disrupt the circadian oscillation. Subsequently, attempts were made to obtain *Bmal1* add back-in clones through both transfection and viral transduction processes. Furthermore, monoclonal, and polyclonal cell lines were generated from these systems, and oscillation rescue was examined. The overall aim of the methodology in this study is to improve the system and enhance the ease of use of the designed tool in terms of its application.

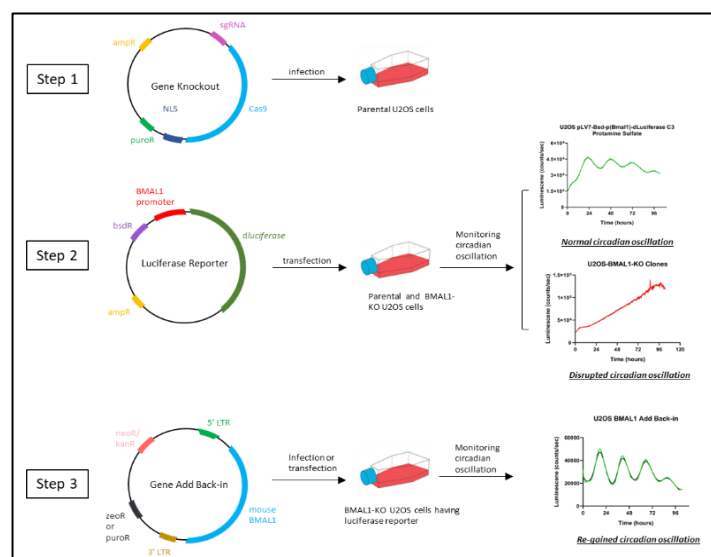


Figure 2.1: Illustration of the overall procedure designing of this thesis. Step 1 represents *BMAL1* gene knockout via CRISPR-Cas9 system. Step 2 shows the bioluminescence recordings of regular and disrupted circadian oscillation. Step 3 represents circadian oscillation rescued by *Bmal1* add back into the genome.

2.1. Literature Review

2.1.1. Circadian Clock in Mammals

In mammals, circadian rhythms are controlled by the master clock in the suprachiasmatic nucleus (SCN), the pacemaker of the circadian clock, located in a specific region of the hypothalamus (Stetson and Watson-Whitmyre, 1976). Although the SCN is in full control, tissues and organs also have their own timekeepers, called *peripheral clocks* (Sinturel et al., 2021). Environmental cues, referred to as Zeitgebers (German for 'time-givers'), have the capability to reset both the central and peripheral clocks. Light serves as the predominant Zeitgeber for the central clock, with the retina sensing it and conveying signals directly to the SCN. Moreover, cellular molecular clocks are the protagonists in the arising of peripheral clocks. The molecular clock is generated by a transcription translation feedback loop (TTFL). There are 4 main proteins in the core of the molecular clock, of which BMAL1 (Brain and Muscle ARNT-Like 1, or ARNT-Like), and CLOCK (circadian locomotor out-put cycles kaput) act as transcriptional activators and regulate the expression of clock-controlled genes (ccgs). The products of *PER* (*PERIOD*) and *CRY* (*CRYPTOCHROME*) genes, the negative elements of TTFL then inhibit their transcription by blocking the trans-activity of BMAL1 and CLOCK (Figure 2.2). Firstly, BMAL1 heterodimerizes with CLOCK. This heterodimer complex then binds to the E-box sequences of *PERIOD* (*PER1* and *PER2*) and *CRYPTOCHROME* (*CRY1* and *CRY2*) genes to induce their transcription. Translational products of *Per* and *Cry* genes recruit CASEIN KINASE 1 δ (CK1 δ) then translocate to the nucleus and binds to BMAL1:CLOCK heterodimer structure for repressing their own transcription by inhibiting transcriptional activator BMAL1:CLOCK complex.

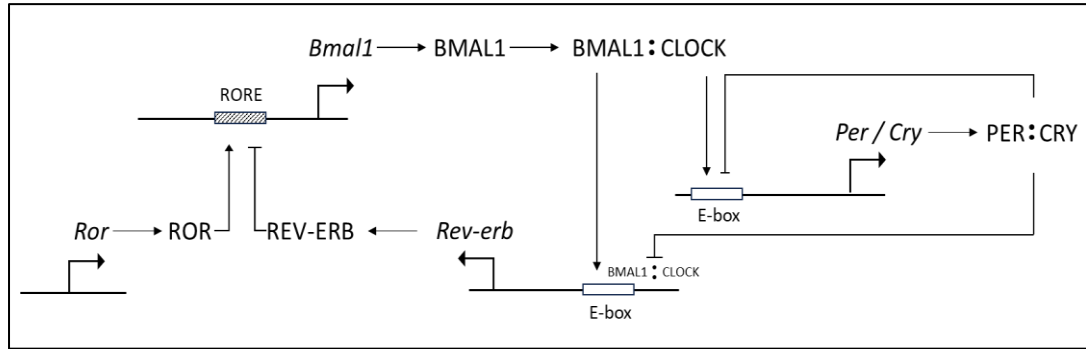


Figure 2.2: Schematic view of the mammalian circadian core clock TTFL model.

The other loop which regulates *BMAL1* expression consists of retinoic acid receptor-related orphan receptors (RORs), nuclear receptor subfamily 1 group D (REV-ERBs) and *BMAL1*. In this secondary loop, *BMAL1* transcription is regulated by RORs and REV-ERBs. Among these pairs that compete for the regulatory element ROR response elements (ROREs), which is located at the promoter region of *BMAL1*, RORs function as transcriptional activators, while REV-ERBs are participated in this system as inhibitors. This competition generates a transcriptional oscillation in *BMAL1* gene (Kim et al., 2022).

2.1.2. A Closer Look at BMAL1 Structure

The functionality of the core proteins involved in the circadian clock mechanism mentioned in the previous section for circadian rhythm is indisputable. *BMAL1* gene is located at q arm of chromosome 11 (11p15.3). At the protein level, BMAL1, is a transcription factor that functions the CLOCK:BMAL1 heterodimer to induce the transcription of CCGs including *CRY* and *PER*. The molecular structure of BMAL1 includes a PAS (Per-Arnt-Sim) domain at its C-terminus, crucial for interaction with the CLOCK protein to form the heterodimer complex (Figure 2.3). The transcription activation domain of BMAL1 is instrumental in regulating gene expression. The other part of BMAL1 structure, bHLH domain provides the binding domain to a motif on DNA. The examination of the crystal structure when the bHLH domain of CLOCK:BMAL1 heterodimer binds to an E-box on DNA reveals that CLOCK and BMAL1 bHLH domains can mutually selected for DNA binding, and their E-box recognition is mediated by hydrogen-bonding networks (Wang et al., 2013). Also, it was observed that the efficient blocking of DNA binding and the abolishment of

normal circadian oscillation in cells were achieved through the phospho-mimicking mutation on *BMAL1* Ser78 (Wang et al., 2013).

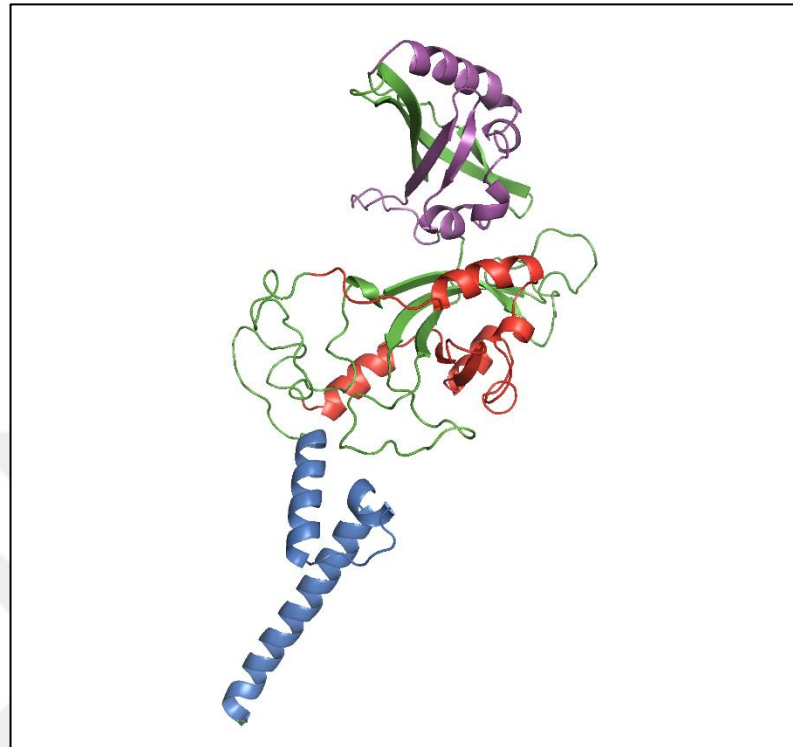


Figure 2.3: The structure of BMAL1, generated through the Swiss Model homology modelling approach with the 4f3l.1.A template, was visualized using PyMOL (github.com/schrodinger/pymol-open-source). Cyan: bHLH the DNA binding domain, Red: PAS1 domain, Magenta: PAS2 domain in magenta.

2.1.3. Consequences of Circadian Clock Disruption

Every part of the systematic order in circadian clock mechanism is obliged to oscillate in harmony for the sake of circadian balance. For example, the fact that overexpression of CRY causes an increase in BMAL1 expression (Shearman et al., 2000) or deficiency of PER causes a decrease in BMAL1 expression (Fan et al., 2007) are evidence for this phenomenon. Also, it was observed that the knockout of the *BMAL1* gene sensitized healthy breast cells and breast cancer cells to apoptosis induced by cisplatin and doxorubicin, which are commonly used in specific cancer treatments (Korkmaz et al., 2018). On the other hand, in a study investigating the effects of circadian components on oscillation, the period was shortened or prolonged in *Cry1* or *Cry2* knockdown cells respectively. In *Cry1/Cry2* knockdown

cells, circadian oscillation was completely lost (Baggs et al., 2009). In the same study, other clock elements were also knockdown by 60-80% and it was found that only 3 genes, *Bmal1*, *Clock* and *Per1*, disrupted the robustness of circadian oscillation (Baggs et al., 2009). Most importantly, one of the foci of this study is that *BMAL1* is the only clock gene for which a single inactivation leads to the loss of circadian rhythmicity (Figure 2.4) (Bunger et al., 2000). Furthermore, *BMAL1* expressed from a constitutive promoter can restore circadian oscillations monitored with *Per2* promoter activity in *BMAL1*^{-/-} fibroblast cells (Liu et al., 2008). Another important finding is that oscillation rescue was successfully achieved in mouse fibroblast cells obtained from *Bmal1*^{-/-} mice, where oscillation was monitored with *Per2:Luc*, through BMAL1 complementation under a constitutive promoter UBC (Huang et al., 2012). In the same study, it was emphasized that although *Bmal2* exhibits similar rhythmic transcript and protein level expressions to *Bmal1*, it is not sufficient for oscillation rescue.

2.1.3.1. General Effects of *BMAL1* Deficiency on Human Health

The *BMAL1* gene is known as a gene that plays an important role in the regulation of the biological clock, so molecular studies such as knockdown, knockout or knock in of *BMAL1* are important for understanding the basic mechanisms of circadian rhythms.

Several studies have shown that knockout of *BMAL1* leads to impairments in DNA repair or cell proliferation processes, thus preventing cells from dividing in an orderly manner and preventing the efficient repair of DNA damage. For instance, it has revealed that BMAL1 is up-regulated in response to double-strand break (DSB) inducing agents such as zeocin in a dose-dependent manner (Zhang et al., 2023). This discovery suggests the involvement of BMAL1 in the DNA damage response (DDR) mechanism, particularly in the response to DSBs. In one of the studies, it was discovered that BMAL1 regulates the G2/M phase of the cell cycle, thereby impacting the proliferation and differentiation of tumor cells (Zeng et al., 2014).

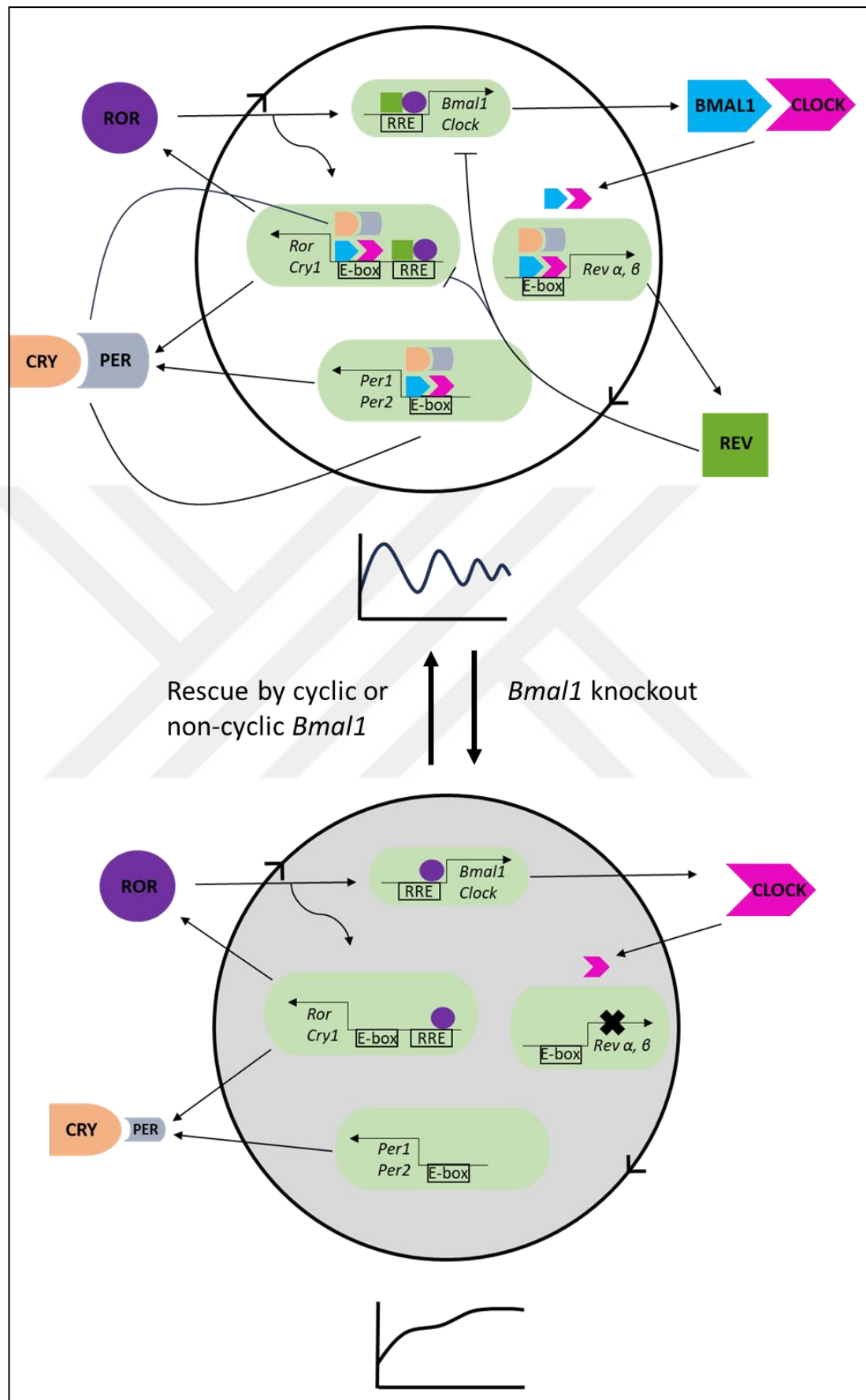


Figure 2.4: Molecular mechanism of circadian clock (TTFL) and the effect of *BMAL1* knockout on the core clock system.

As mentioned in the previous section, when the circadian clock mechanism is investigated through the immune system, down-regulation of *Bmal1* in mouse macrophages has been observed to result in a reduction in cytokine expression and a concomitant decrease in the activity of NF- κ B, which is involved in the activation of inflammation-related genes response to infection (Shen et al., 2009).

One of the most significant health issues threatening human health today is cancer. When the relationship between the circadian clock and cancer, specifically focusing on *BMAL1* SNPs, is examined at the molecular level, the finding that the rs7950226 SNP in *BMAL1* is associated with the risk of less aggressivity in prostate cancer (Gršković and Korać, 2023) emphasizes once again the significance of investigating mutations in circadian clock genes in terms of human health. Furthermore, carriers of the T allele in the *BMAL1* rs2279287 SNP have been found to exhibit a decrease in the risk of developing breast cancer (Horita and Kaneko, 2015), and the A allele in the *BMAL1* rs3816358 SNP has also been associated with a reduction in the risk of breast cancer (Rabstein et al., 2014).

More examples can be added to the given research samples, and ultimately, emphasizing the inclusion of the circadian clock, specifically *BMAL1*, in research is crucial for future investigations into the relevant stages of diseases. For example, it has been demonstrated that *BMAL1* inhibits tumor formation and proliferation in nasopharyngeal carcinoma (Zhao et al., 2022), while loss of *BMAL1* promotes pancreatic cancer proliferation (Jiang et al., 2016). Conversely, the CLOCK:*BMAL1* heterodimer has been shown to promote cell proliferation in hepatocellular carcinoma (Qu et al., 2023) and cell metastasis in breast cancer (Wang et al., 2019). Indeed, *BMAL1* has been implicated in inducing colorectal cancer metastasis (Jiang et al., 2016).

The comprehensive investigation of disruptions in core clock components holds immense significance for the diagnosis and treatment of certain diseases. The utilization of clock elements for therapeutic or prognostic studies holds promising prospects for various diseases, particularly cancer, based on this information.

2.1.4. Core Clock SNPs

Disruptions in the circadian system can adversely affect overall health and lead to problems in the functioning of organs or cellular processes. These disruptions arise from environmental such as night-shift work, wrong feeding habits and or cellular factors such as gene mutations and polymorphisms (Baris et al., 2023). While some diseases have been reported to be associated with pathogenic variants resulting from mutations in core clock genes in humans (which are only *Cry1*, *Per2*, and *Per3*), unfortunately, the number of these diseases is limited. Variants in the *Cry1* gene have been associated with delayed sleep phase disorder and, variants in *Per2*, and *Per3* have been linked to disturbances in sleep patterns (Patke et al., 2017). Findings of numerous studies suggesting the association of pathogenic variants of circadian clock genes with obesity, psychiatric disorders, and cancer. However, some research indicates that the majority of variations in clock genes have no significant effect on phenotype. For instance, while certain SNPs on *CLOCK* (rs12649507/rs11932595) are thought to be associated with sleep disorders (Allebrandt et al., 2010), other studies have failed to establish this link. Similarly, a SNP (rs11022775) on *BMAL1* increases the risk of Type 2 diabetes in Asian populations but shows the opposite effect among Europeans (Pappa et al., 2013). Furthermore, the majority of identified variations in clock genes have been localized in noncoding regions. It has yet to be demonstrated whether these variations affect circadian rhythms and whether they are associated with numerous behavioural and physiological diseases, including cancer, metabolic disorders, and others. Determining the protein structure and amino acid sequence of clock gene variants resulting from genetic mutations, investigating their effects on cellular processes, and ultimately associating them with life-threatening diseases at the molecular level contribute to the prognosis or prevention of diseases associated with chronic circadian disruption. Additionally, this facilitates the development of circadian clock modulating molecules and other strategies aimed at addressing such conditions. Considering all these findings, there is a need for further research to characterize SNPs in clock genes and investigate their effects and variations comprehensively.

2.1.5. Advantages of Circadian Oscillation Monitoring

In circadian rhythm monitoring, promoter activity of certain circadian genes is usually measured using luciferase reporter system. BMAL1 is one of the key regulators of the circadian rhythm and plays a crucial role as a component of the CLOCK-BMAL1 heterodimer, initiating the transcription of other circadian genes. Consequently, monitoring *BMAL1* promoter activity provides an opportunity to scrutinize the fundamental regulators of the biological clock more closely.

In many studies, the promoter activity of these circadian genes is measured by putting the relevant promoter in front of a *luciferase* gene. For circadian clock research *Bmal1* or *Per2* promoter is preferred in luciferase reporters. Measuring promoter activity is important for understanding how activity in the regulatory regions of specific genes changes over time. Such studies are used to understand the molecular mechanisms of circadian rhythm and how genetic activities at the cellular level are regulated (Padlom et al., 2022).

By conducting circadian oscillation studies, the effects of small molecules which are suggested to be candidates affecting to the circadian systems, changes in clock elements (e.g., knockouts or knockdowns), or mutations in these elements on the circadian rhythm can be determined. Deletion or significant reduction in the levels of the *BMAL1* gene leads to the disruption of circadian oscillation because BMAL1 is one of the key regulatory elements of the biological clock. Collaborating with the CLOCK to form the CLOCK:BMAL1 heterodimer, the BMAL1 initiates the transcription of other circadian genes *PER*, *CRY*, and other CCGs. The dysfunction of *BMAL1* at the gene level prevents the formation of the CLOCK:BMAL1 heterodimer, consequently halting the transcription of circadian genes. This situation leads to the disruption of circadian oscillation and the disturbance of normal functions of the biological clock. Therefore, the function of BMAL1 plays a critical role in regulating the circadian rhythm, and irregularities at the gene level result in disruptions within the circadian system.

2.1.7. Gene Knockout Studies with CRISPR/Cas9

The CRISPR/Cas9 gene editing technology represents a revolutionary breakthrough in molecular biology (Jinek et al., 2012). Originally discovered as a natural immune

mechanism in prokaryotes, this system has been adapted as a tool for gene editing. CRISPR/Cas9 operates within an RNA-guided system capable of recognizing and cleaving a targeted DNA sequence, enabling precise genetic modifications. Its flexibility, efficiency, and cost-effectiveness have revolutionized gene editing studies, offering researchers extensive applications. In this system, the target region guided by a single guide-RNA, a double-stranded DNA break (DSB) is induced by Cas9. Subsequently, one of the two major endogenous DNA repair pathways is utilized. Nonhomologous end-joining (NHEJ) is error-prone and typically results in short insertions or deletions (indels) at the target site, often causing frame shifts and premature STOP codons; therefore, NHEJ is appropriate when achieving a gene knockout is intended. However, for a knock-in system, the advantage of homologous recombination (HR)-mediated repair is preferred, where an externally introduced repair template is necessary.

This technology has been widely used for loss-of-function studies in cell culture or in animal models. Today, advancements in circadian clock mechanisms have been achieved through previous studies employing various technologies, including the CRISPR/Cas9 system, within the circadian clock system (DeBruyne et al., 2006; Korge et al., 2015; Trebucq et al., 2024). Extensive studies have revealed that deletion of *BMAL1* results in the loss of circadian oscillations. Indeed, studies have revealed that *BMAL1* is the only clock gene for which a single inactivation leads to the loss of circadian rhythmicity (Bunger et al., 2000). In line with this finding, following the deletion of endogenous *BMAL1* using CRISPR/Cas9 technology, exogenous *BMAL1* was reintroduced under the control of an inducible DOX promoter, leading to dose-dependent re-expression of *BMAL1* and restoration of oscillations (Padlom et al., 2022).

3. MATERIALS

3.1. Kits and Commercial Supplies

BCA Protein Assay Kit (Thermo Scientific, cat. no: 23227), Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, cat. no: S8830-2TAB), Protein Marker (Intron Biotech., GangNam-Stain:Prestained Protein Ladder, cat. no:S24052), 1 kb DNA marker (New England Biolabs cat. no: N3232S), 100 bp DNA marker (Enzymomics, cat. no: DM001), Chemiluminescent HRP Substrate (Advansta, WesternBright-Sirius, cat. no: K-12043-D10), RedSafe-Nucleic Acid Staining Solution (IntronBio, cat. no: 21141), RNase A (Sigma Aldrich, cat no: R4642), , 100X Tris-EDTA buffer solution (Sigma-Aldrich, cat. no: T9285), Beetle Luciferin (Promega, cat. no: E1602), Coenzyme A (Nanolight, cat. no: 309-205).

3.2. Chemicals

Dimethyl sulfoxide (Merc, cat. no: 1167431000), Methanol (Sigma-Aldrich, cat. no: 32213-2, 2-Propanol (Merc, cat. no: 109634), 2-mercaptoethanol (Sigma-Aldrich cat. no: M6250-1L), Acrylamide (Sigma-Aldrich cat. no: A8887), Glycine (ISOLAB, cat no: 9.270.321.000) Zeocin (Thermo Fisher Scientific, cat. no: R25001), Puromycin dihydrochloride (Sigma-Aldrich, cat. no: P8833), Blasticidin S, Hydrochloride (Merck, cat. no: 203350), h-Coelenterazine (Nanolight, cat. no: 301-1), IGEPAL® CA-630 (Sigma-Aldrich, cat. no: I8896), Dexamethasone (Sigma-Aldrich, cat. no: D4902), DMEM powder (Sigma-Aldrich cat. no: D-2902), Dimethylsulfoxide (DMSO) (Merck cat. no: 1167431000), Sodium Bicarbonate (Sigma-Aldrich cat. no: S5761), D-(+)-glucose powder (Sigma-Aldrich cat. no: G7021), HEPES buffer (Gibco™, Thermo Fisher Scientific, cat. no: 15140-122), Polybrene (Santa Cruz Biotechnology, cat. no: 134220), Boric acid (Biofrox, cat. no: 1252K600), Tris base (Fisher Bioreagents, cat. no: BP152-1), Tween-20 (Sigma-Aldrich cat. no: P1379), TEMED (Sigma-Aldrich cat. no: T7024), Sodium chloride (Sigma-Aldrich cat. no: 31434), DTT (Fisher Scientific, cat. no: BP172-5), EDTA (Fisher Scientific, cat. no: BP2482-100), ATP Disodium Salt (WISSENT Multicell cat. no: 802-033 UG), LB broth (Formedium, cat. no: LBX0104), Hydrochloric acid (Merck cat. no:100317.2500), D-Luciferin Potassium Salt (GOLD BIO cat. no: LUCK 100),

Thiazolyl blaue tetrazolium bromide (MTT) (BioFroxx cat. no: 1334GR001), LB Agar (Caisson cat. no: LBP02), Agarose (Thermo Fisher Scientific, cat. no: 16500500), Sodium Dodecyl Sulfate (Thermo Fisher Scientific, cat. no: BP166-500), Ampicillin (Fisher Scientific, cat. no: BP1760-25), Bromophenol Blue (Thermo Fisher Scientific, cat. no: BP115-25), Sodium Hydroxide (Merc, cat. no: 1.06498.1000), IGEPAL CA-630 (Sigma-Aldrich, cat. no: 18896-100 ML), Potassium chloride (Sigma-Aldrich, cat. no: 12636), Potassium dihydrogen phosphate (Merc, cat. no: 1048731000), di-Sodium hydrogen phosphate Na_2HPO_4 (Merc, cat. no: 106586.1000), Dynabeads Protein G (Invitrogen, cat. no: 10004DMgCl₂ (Acros Organics, cat. no: 223.210.010), Triton-X-100 (Fisher Scientific, cat. no: BP151-100).

3.3. Ingredients of Buffers

Table 3.1: Contents and preparation of buffers.

Solution Name	Ingredient and Molarities
Running Buffer (100X, 2 L)	2.5 M Tris Base 12.2 M Glycine 340 mM SDS
Transfer Buffer (1X, 1 L)	24.8 mM Tris Base 122 mM Glycine 3.4 mM SDS 20% Methanol
TBE Buffer (100X, 1L)	890 mM Tris Base 890 mM Boric Acid 40% 0.5 M EDTA
TBS Buffer (100X, 2 L)	15 M NaCl 2 M Tris Base-HCl (pH: 7.9)
TBS-T	1X TBS 0.5% Tween-20
PBS (100X, 1 L)	13.6 M NaCl 270 mM KCl 200 mM KH_2PO_4 17.5 mM Na_2HPO_4
Laemmli Buffer (10X, 100 mL)	694.5 mM Tris Base-HCL (pH: 6.8) 44.4% Glycerol (v:v) 2.7 M SDS 0.02% Bromophenol blue

Table 3.1: Continued.

ECL Solution A	100 mM Tris Base pH: 8.8 90 mM Coumeric acid 250 mM Luminol
ECL Solution B	100 mM Tris Base pH: 8.8 50% H ₂ O ₂
DNA Loading Dye (6X)	1X Tris Base-EDTA 30% Glycerol 0.25% Bromophenol blue
RIPA Buffer (10X)	0.5 M Tris base-HCl (pH:8 8.0) 1.5 M NaCl 1% SDS 16% IGEPAL 2% NP-40
Triton-X Lysis Buffer	150 mM Tris Base-HCl (pH: 7) 75 mM NaCl 3 mM MgCl ₂ 0.25% Triton-X
Firefly-Luciferase Buffer (3X)	15 mM DTT 0.6 mM CoA 0.45 mM ATP 0.4 mM Luciferin Prepare in Triton-X Lysis Buffer
Renilla Salt Buffer	45 mM Na ₂ EDTA 30 mM Na ₄ P ₂ O ₇ 1.425 M NaCl 0.06 mM PTC124 0.01 mM h-CTZ
Blocking Buffer	5% non-fat dry milk powder In 1X-TBS-T
Miniprep Resuspension Buffer	50 mM Tris Base-HCL (pH: 8.0) 10 mM EDTA 100 µg/mL RNase A
Miniprep Lysis Buffer	200 mM NaOH 1% SDS
Miniprep Neutralization Buffer	3 M CH ₃ CO ₂ K (pH: 5.5)
Miniprep Wash Buffer	10 mM Tris Base-HCL (pH: 7.5) 80% Ethanol
Bioluminescence Recording Medium (for 1 L)	10 g DMEM powder 0.35 g Sodium Bicarbonate 3.5 g D (+)-glucose powder 1 M HEPES buffer 1% Pen/strep 5% FBS 0.1 mM luciferin
Transfection Buffer	150 mM NaCl In 1X Tris-EDTA solution

Table 3.2: Recipe for SDS-PAGE preparation.

Solution Name	Ingredient and usage amounts
Separating Gel (10%, 10 mL)	4.8 mL of ddH ₂ O 2.5 mL of 40% Ac:Bis 2.5 mL of 1.5 M Tris-HCl pH:8.8 0.1 mL of 10% SDS 0.1 mL of 10% APS 0.004 mL of TEMED
Separating Gel (8%, 10 mL)	5.3 mL of ddH ₂ O 2 mL of 40% Ac:Bis 2.5 mL of 1.5 M Tris-HCl pH:8.8 0.1 mL of 10% SDS 0.1 mL of 10% APS 0.006 mL of TEMED
Stacking Gel (5%, 4 mL)	2.92 mL of ddH ₂ O 0.5 mL of 40% Ac:Bis 0.5 mL of 1 M Tris-HCl pH:6.8 0.04 mL of 10% SDS 0.04 mL of 10% APS 0.004 mL of TEMED

3.4. Enzymes

T4 DNA ligase (Thermo Fisher Scientific cat. no: 15224017), *KpnI* (Thermo Fisher Scientific, cat. no:ER0521), *BamHI* (New England Biolabs, cat. no: R0136S), *MssI* (*PmeI*) (Thermo Fisher Scientific, cat. no: ER1341), *Eco147I* (*StuI*) (Thermo Fisher Scientific, cat. no: 10359889) *Esp3I* (*BsmBI*) (Thermo Fisher Scientific, cat. no: ER0451).

3.5. General Cell Culture Supplies and Buffers

Dulbecco's Modified Eagle Medium high glucose, pyruvate (Gibco™, Thermo Fisher Scientific, cat. no: 41966029), MEM Nonessential Amino Acids (EuroClone, cat. no: ECB3054D), Trypsin-EDTA (0.05%), phenol red (Gibco™, Thermo Fisher Scientific, cat. no: 25300054), Penicillin/Streptomycin (10,000 U/mL) (Gibco™, Thermo Fisher Scientific, cat. no: 25300054), Fetal Bovine Serum, Qualified (Gibco™, Thermo Fisher Scientific, cat. no: 10270106), 10X, Phospahe Buffer Saline (Gibco™, Thermo Fisher Scientific, cat. no: 70011036).

3.6. Antibodies

Goat-anti mouse IgG (Advansta, cat. no: R-05071-500), Goat-anti-rabbit HRP secondary antibody (Advansta, cat. no: R-05072-500), Anti-GAPDH-(14C10)-Rabbit (Cell Signalling Technologies, cat. no: 2118S), Anti-Flag Antibody (Sigma-Aldrich cat. no: F1804), β -Actin Antibody (Cell Signalling Technologies, cat. no:4967S), GAPDH Antibody (Cell Signalling Technologies, cat. no:2118S), BMAL1 Antibody (Cell Signalling Technologies, cat. no:14020, BMAL1 Antibody (Bethyllabs, cat. no: A302616A), BMAL1 Antibody (SantaCruz, cat. no:SC365645)

3.6. Vectors

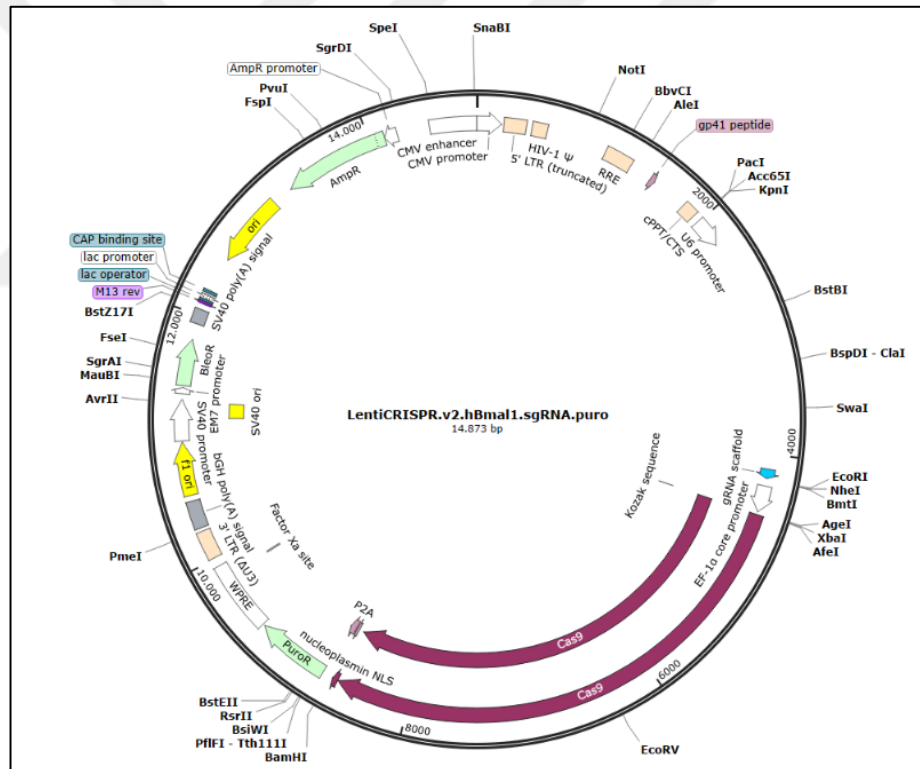


Figure 3.1: Map of LentiCRISPR-v2-BMAL1-sgRNA-puro sequence. This map was created by SnapGene Viewer version 6.2.2.

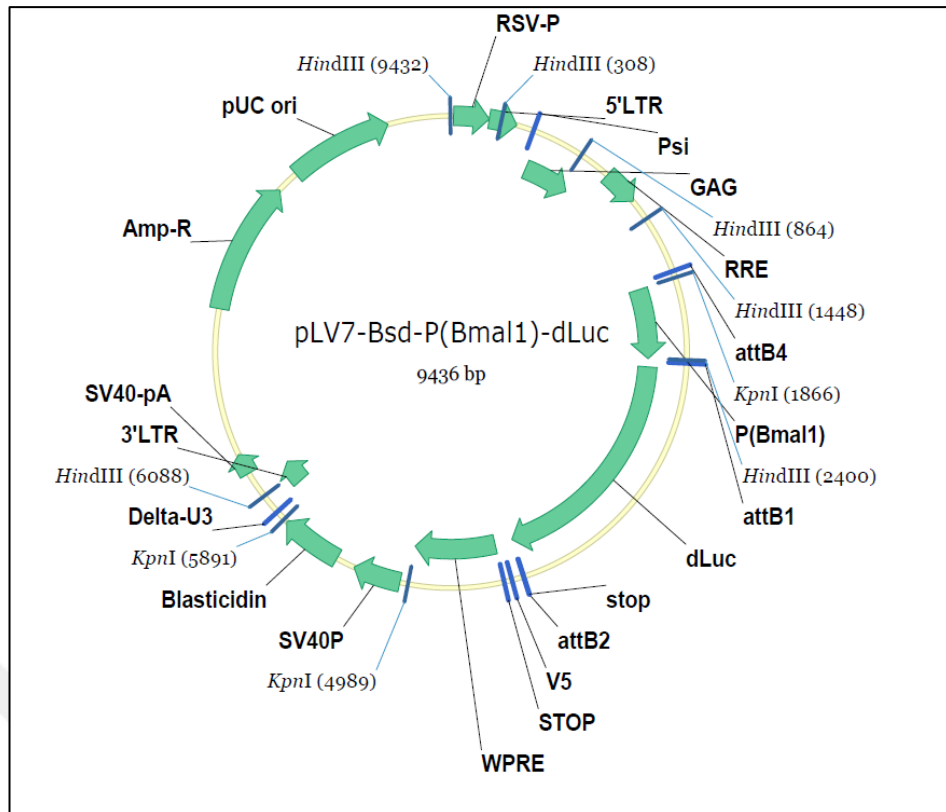


Figure 3.2: Map of pLV7-Bsd-p(*Bmal1*)-d*Luciferase* construct a gift from Dr Liu (Ramanathan et al., 2012).

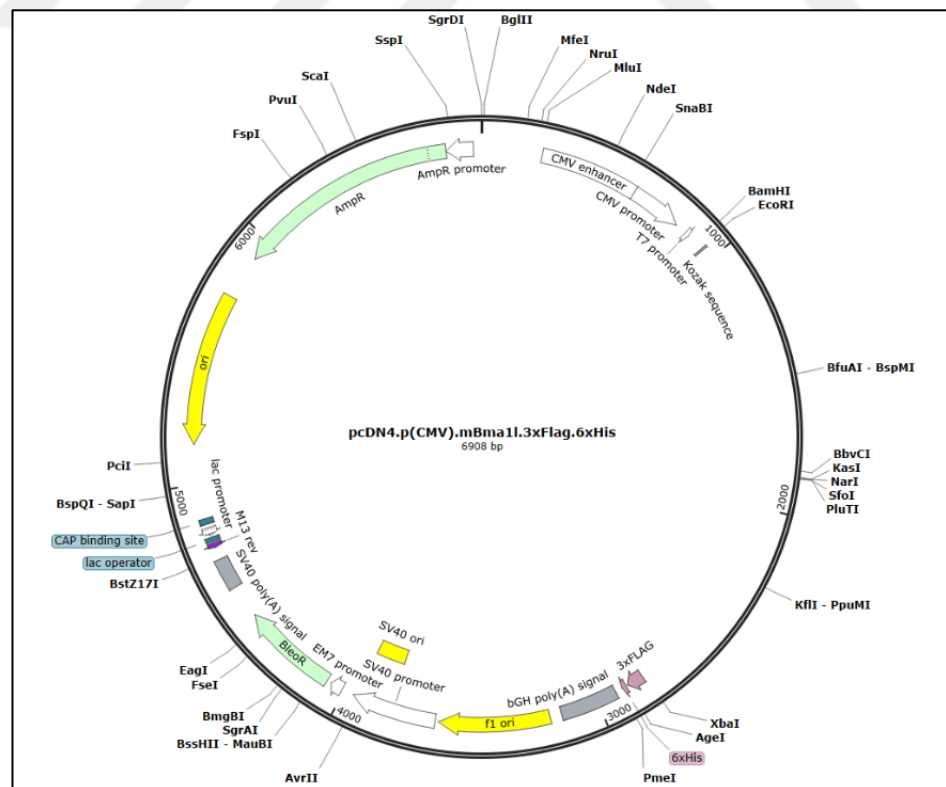


Figure 3.3: Map of pcDNA4-p(CMV)-m*Bmal1*-3xFlag-6xHis sequence. This map was created by SnapGene Viewer version 6.2.2.

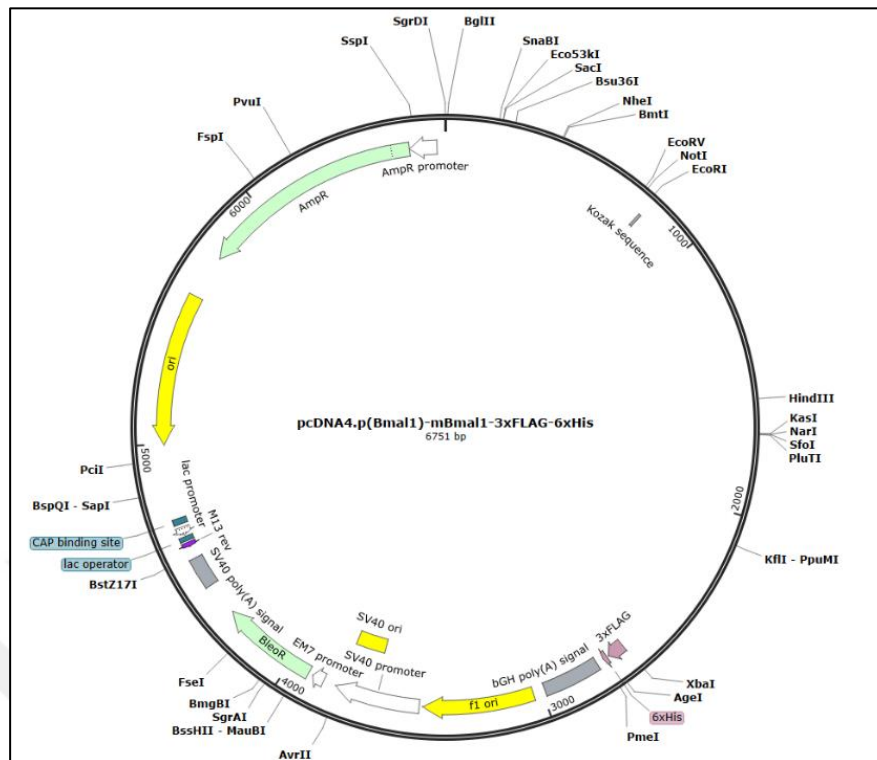


Figure 3.4: Map of pcDNA.4-p(Bmal1)-mBmal1-3xFlag-6xHis sequence. This map was created by SnapGene Viewer version 6.2.2.

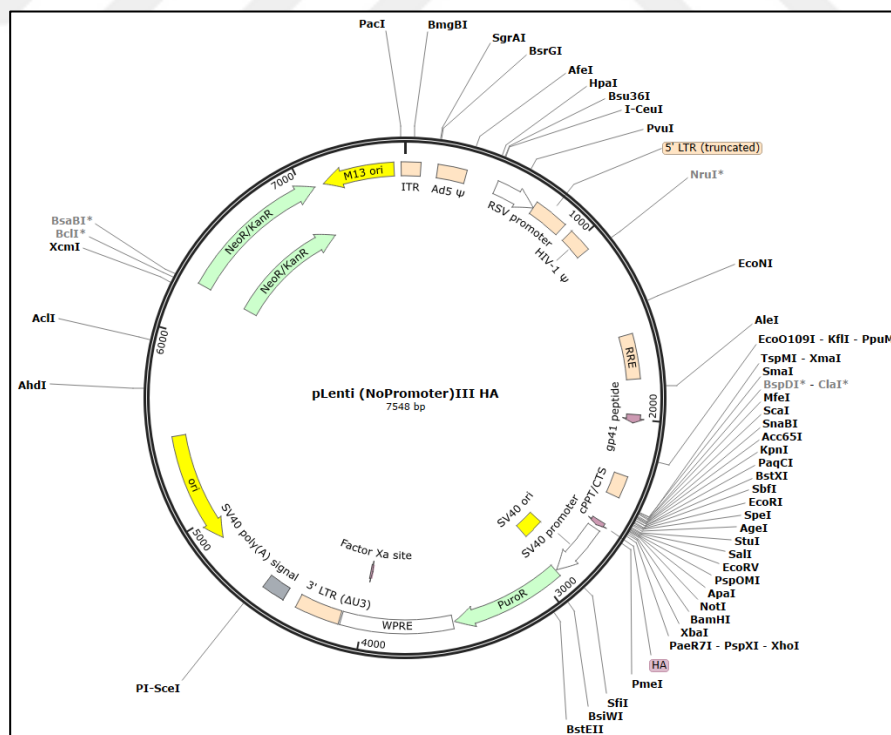


Figure 3.5: Map of pLenti III (no promoter)-HA sequence. This map was created by SnapGene Viewer version 6.2.2.

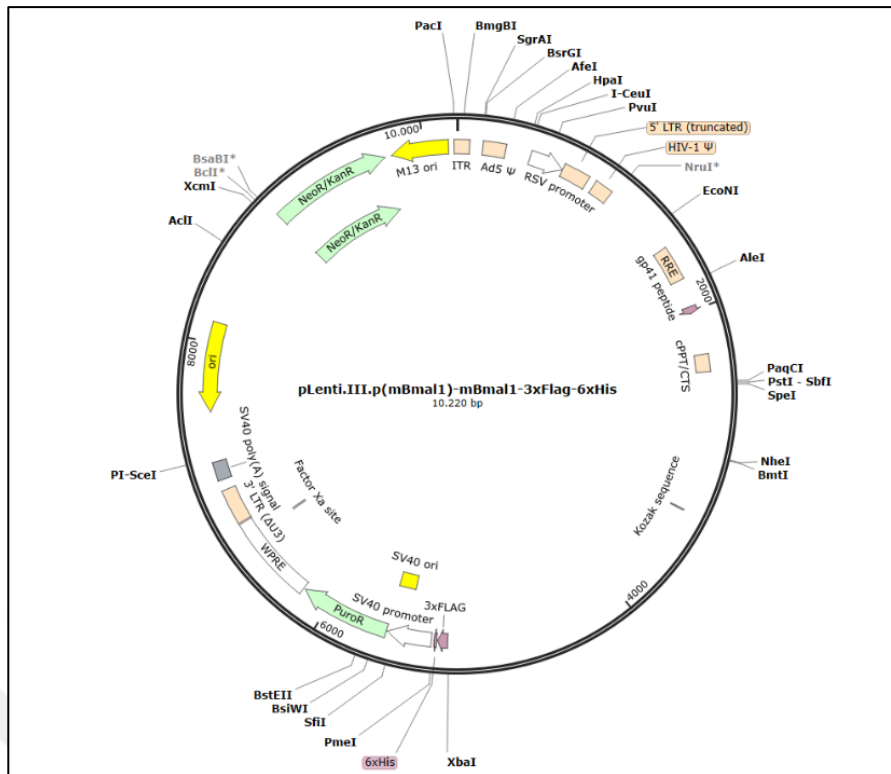


Figure 3.6: Map of pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis sequence. This map was created by SnapGene Viewer version 6.2.2.

3.7. Oligonucleotides

Double-stranded sgRNAs targeted to BMAL1 were purchased from MacroGen, Inc.

3.8. Equipment and Devices

Laminar Flow Cabinet (Thermofisher Scientific MN 09/120 ClassII Cabinet,), CO₂ incubator (NUVE EC 160), Synergy™ H1 hybrid multi-mode microplate reader, inverted premium phase contrast microscope (Soptop, ICX41), refrigerator (Vestel, V10757442), refrigerated centrifuge (Micro Star 17R, 521-1647, VWR), centrifuge (Micro Star 17, 521-1646, VWR), centrifuge NF400 (Nuve, 02-1827), chemiluminescence imaging device (ChemiDoc™ XRS+ System, Bio-Rad), Western blotting system (Bio-rad), transblot transfer system (Bio-rad), agarose gel electrophoresis system (Bio-Rad), NanoDrop device (Shimadzu Biotech), shaker (VWR), waterbath (Wisd), magnetic rack (DynaMag™-2 Invitrogen), -80°C ULT Freezer (Thermo Fischer Electron Corporation), Varioskan Microplate Reader (Thermo Fischer Scientific).

4. METHODS

4.1. Cell Culture

4.1.1. Maintenance of Cell Lines

U2OS cells were maintained at 37°C humidified incubator containing 5% CO₂. Cells were cultured within the high glucose DMEM containing 10% FBS, 1% NEAA and 1% Pen/Strep (100 U/mL of penicillin and 100 µg/mL of streptomycin). Cells were passaged with trypsinization into a new sterile culture flask when they reached at about 90% confluency.

4.1.2. Stocking of Cell Lines

Cells were stocked in the cryotubes with the freezing medium containing 80% cell suspension 10% FBS and 10% DMSO. The medium of the cells was aspirated and washed with 1X sterile PBS. Appropriate volume of trypsin was added into the cells and incubated at 37°C for 4-5 minutes. DMEM containing 10% FBS at least 3-fold the volume of trypsin was added to the culture flasks for trypsin inhibition and physically pipetted. Detached cells were collected in a sterile 15-mL conical tubes and centrifuged at 4000 rpm for 5 minutes. After the supernatant was aspirated, the cell pellet was dissolved with 1 mL of DMEM and 800 µL of cell suspension (80%) was transferred to the cryotube. It was immediately brought to -20°C after adding 100 µL of FBS (10%) and 100 µL of DMSO (10%) and stored at -80°C on the very next day.

4.1.3. Thawing of Cell Lines

To re-culture frozen cells, cryotubes were removed from liquid nitrogen tank and placed on ice. The thawed cells in 1 mL of freezing medium were added to 5 mL of DMEM containing 10% FBS before cell suspensions were completely dissolved. When the solid state was completely dissolved, the cell mixture was centrifuged at 4000 rpm for 5 minutes. The supernatant, including DMSO, was aspirated and the

cell pellet was dissolved with fresh DMEM and finally cells were seeded into new culture flasks.

4.1.4. Cell Viability Assay for Mammalian Antibiotics

5×10^3 U2OS cells/well were seeded into the 96-well tissue culture plate. Antibiotic treatments were started after 16 hours of cell seeding. Concentrations of mammalian culture antibiotics were determined according to manufacturer instructions. Cells were selected with puromycin, blasticidin and zeocin for 2 times with 2-day freshening intervals. MTT assay was performed at the end of the selection. 5 mg/mL MTT powder was dissolved in PBS (12mM) and protected from light. Stocks were stored at -20 °C. At 1:10 ratio, MTT:DMEM (10 μ L MTT and 90 μ L DMEM) mix was prepared (100 μ L/well). Antibiotics-DMEM mixes were aspirated, and cells were washed with 1X sterile PBS. 100 μ L of MTT-DMEM mix were pipetted to each well and plates were incubated at 37 °C incubator for 4 hours. MTT mix was aspirated and 100 μ L of DMSO were pipetted to each well and plates were incubated on shaker at RT for 5-15 minutes. Colorimetric measurement at 570 nm and data analysis were carried out with GraphPad Prism 8.0 software. Each experimental group was seeded with 3 replicates. Data represents of 3 independent experimental result.

4.2. Establishment of *BMAL1* Knockout Stable U2OS Cell Line

4.2.1. Designing of Oligos

sgRNAs were designed to target the genomic regions corresponding to sequences that encodes before ATG and just after stop codons (Table 4.1). When they are used together whole *BMAL1* coding sequence is either removed or they cause gross changes which disrupt the *BMAL1* coding sequence.

Table 4.1: Sense and antisense sgRNA oligonucleotide sequences targeted to human *BMAL1* gene.

Name	Sequence
hB1-T6-Sen	CACCGTCTGTGAAGCTTACTGGATA
hB1-T6-Ant	AAACTATCCAGTAAGCTTCACAGAC
hB1-T7-Sen	CACCGTAAGATTGACTTGTATCAA
hB1-T7-Ant	AAACTTGATACAAGTCAATCTTAC
hB1-T8-Sen	CACCGCATGCCTGTTCTGGGTCCGG
hB1-T8-Ant	AAACCCGGACCCAGAACAGGCATGC

4.2.2. Cloning of *BMAL1* sgRNA into LentiCRISPR.v2-Puro

4.2.2.1. Annealing of sgRNA Oligos

To anneal sgRNA oligo nucleotides, each sgRNA were diluted with 1X NEBuffer 2 (New England Biolabs, cat. no: B7002S) to a final concentration 50 ng in a 100 μ L total reaction volume. sgRNAs were heated at 95°C for 5 minutes and allowed to cool down to temperature after turning the heater off. After annealing the oligos generates overhangs to facilitate cloning into *Bsm*BI-generated overhangs.

4.2.2.2. Digestion of Vector and sgRNA

Approximately 1000 ng of LentiCRISPR.v2 was digested with *Esp*3I (*Bsm*BI) enzyme (Thermo Fisher Scientific, cat. no: ER0451) in 50 μ L total reaction volume for 4 hours at 37°C.

4.2.2.3. Gel Clean-up

6X DNA loading dye was added to the restriction enzyme digested vector at 1X final concentration. Digested LentiCRISPR.v2 plasmid were run on agarose gel and visualized with a 366-nm UV-light. The large fragment of the digestion product was isolated from agarose gel using NucleoSpin Gel and PCR Clean-up mini kit (Macherey-Nagel, cat. no: 740609.50) to clone the annealed sgRNA oligos. For this, the larger fragment was excised from agarose gel and transferred to a microcentrifuge tube. Approximately, 3-4-fold volume of NT Buffer was added to

the gel pieces, and the tube was placed on to a heat block at 55°C for 10 minutes. Following the gel melting in the NT buffer, then 200 µL of isopropanol was added to the samples and loaded into the gel clean-up column. Column was centrifugated at 13.000 rpm for 30 seconds. Flowthrough was discarded and column was washed with NT Wash Buffer twice. Finally, column was centrifuges at 13,000 rpm to remove all liquid residues, and DNA was eluted with 50 µL of nuclease free H₂O.

4.2.2.4. Ligation of Plasmid and sgRNAs

50 ng of annealed sgRNA oligos and 50 ng of digested LentiCRISPR.v2 were ligated with 0,5 µL (5 units) of T4 DNA ligase in 1x T4 DNA ligation buffer to a 10 µL total reaction volume. Ligation reactions were incubated at +4-8°C overnight.

4.2.3. Transformation of sgRNA-Cas9 Expression Constructs into Competent Cells

NEB stable competent cells were used to amplify the generated LentiCRISPR-v2-*BMAL1*-sgRNA-puro constructs. All bacterial culture procedures were carried out under sterile conditions. LentiCRISPR-V2-*BMAL1*-T6, LentiCRISPR-V2-*BMAL1*-T7 and LentiCRISPR-V2-*BMAL1*-T8 constructs were added to 100 µL of NEB stable competent cell separately. pLenti-P2A and pLenti-P2B also, transformed to NEB stable bacteria (they were grown at large scale). The bacterial DNA mixture was incubated on ice for 24 minutes and flicked the bottom of the tube every 6-7 minutes. Competent bacteria-DNA mixture was placed on heater at 42°C for 1 minute. The tubes were then placed back on ice immediately and incubated for 2 minutes. 950 µL of Luria broth (LB) broth was added to each tube without any antibiotics. The tubes were incubated on a shaker at 37°C 200 rpm for 50 minutes. At the end of 50 minutes, the bacteria were centrifuged at 13.000 rpm for 1 minute. The bacteria were dissolved with 50 µL of LB remaining in the tube after removing the supernatant and spread on LB agar containing 100 µg/mL ampicillin. Plates were incubated upside down at 30°C overnight. The next day, 2 of the single colonies formed on each plate were picked to 3.5 mL of LB broth containing 100 µg/mL ampicillin (each of them was named T6.1, T6.2, T7.1, T7.2, T8.1, and T8.2). The inoculated bacteria were incubated on a shaker at 37°C 200 rpm for 22 hours.

4.2.4. Isolation of LentiCRISPR.v2-*BMAL1*-sgRNA-puro Expression Constructs

Bacteria cultured for 22 hours were centrifuged at 4000 rpm for 10 minutes. LB was removed, and pellets were dissolved with 230 μ L of resuspension buffer (50 mM Tris-HCl pH: 8.0, 10 mM EDTA, 100 μ g/mL RNase A, stored at 4°C). 250 μ L of bacteria cell suspensions were transferred to the test tubes and 250 μ L (V:V) of cell lysis buffer (200 mM NaOH, 1% SDS) were added onto the bacterial cell suspension. The tubes were upside down for 6-8 times and incubated at RT for exactly 4 minutes. 350 μ L of neutralization buffer (3 M potassium acetate pH:5.5) was added to the tubes and the tubes were gently inverted. The phase-separated mixtures were centrifuged at 13.000 rpm for 10 minutes at RT. 650 μ L of the supernatant was loaded onto the miniprep columns and the columns were centrifuged at 13.000 rpm for 1 minute. Flow through was discarded and 650 μ L wash buffer (10 mM Tris-HCl pH:7.5, 80% ethanol) was loaded onto the columns and they were centrifuged at 13.000 rpm for 1 minute. Flow through was discarded. The column systems were centrifuged empty at 13.000 rpm for 1 minute to dry the silica-based membrane with the collection tube changed. 50-100 μ L of elution buffer (1X Tris-EDTA Sigma-Aldrich, Cat. No: T9285) were dropped on the column membrane and columns were incubated at RT for 2 minutes. DNA concentrations were measured with NanoDrop spectrophotometer after the columns were centrifuged at 13.000 rpm for 2 minutes.

4.2.5. Production of LentiCRISPR.v2-*BMAL1*-sgRNA-puro Containing Lentivirus Particles

HEK-293T cells were seeded on sterile 6 cm² tissue culture dishes at 80% confluency. Transfection was started at least 12-16 hours after cell seeding. Vectors were prepared as pLenti-P2A, pLenti-P2B and target vector (2:1:3) 2,66 μ g of P2A, 1,33 μ g of P2B and 4 μ g of LentiCRISPR-V2-*BMAL1*-gRNA.puro (Figure 3.1) (gRNA means T6.1, T6.2, T7.1, T7.2, T8.1, T8.2). PEI (polyethylene imide) was used in the HEK-293T transfection system. First, 150 μ L of transfection buffer (1x TE, 150 mM NaCl) was pipetted in the tubes. 2,66 μ g of P2A, 1,33 μ g of P2B and 4 μ g of target vector were added and vortexed gently. 3 times the volume of μ g DNA used (2,66 μ g P2A + 1,33 μ g P2B + 4 μ g target vector = 8 μ g DNA then, 24 μ L of PEI should be used for 8 μ g of DNA) was added to PEI tubes and mixed well. The mixture was incubated at RT for 15 minutes to form DNA:PEI complex. At the end

of this period, it was carefully and gently mixed with the medium and slowly dripped onto the HEK-293T cells. Exactly 12 hours after the transfection time, the medium was replaced with fresh medium. The transfected HEK-293T cells were kept at 37°C humidified incubator containing 5% CO₂ for 72 hours to obtain virus particles containing the target vector to be released into the medium in the lytic cycle of the cells. Virus-containing mediums were harvested after 72 hours incubation. These mediums were centrifuged at 4000 rpm for 5 minutes at RT and filtered with 0.22 µm syringe filter and finally, collected in a falcon tube. Lentiviral stocks were kept at -80°C for a long-term storage.

4.2.6. Transduction of U2OS Cells with LentiCRISPR-v2-*BMAL1*-sgRNA-puro

U2OS-wt cells were seeded into 12-well tissue culture plates at 70-80% confluency 24 hours prior to viral infection. Viral particles containing the appropriate sgRNA combination (T6 and T7 cut after stop codon and T8 cuts before the first ATG) were mixed in test tubes in a 1:1 ratio (V:V). 500 µL of T6 or T7 and 500 µL of T8 were used for 12-well tissue culture dishes. 10 µg/mL polybrene was added into the combined virus mixture in the tube and mixed well. The medium containing polybrene:virus complexes were dripped onto the U2OS-wt cells. The medium was replaced with selective medium containing 2 µg/mL puromycin after 24 hours post-infection to obtain a stable expression. The selective medium was renewed every 2 days. Cells were passaged sparsely (approximately 30-40%) when the cell density increased above 60%.

4.2.7. Generation of LentiCRISPR-v2-*BMAL1*-sgRNA-puro Stably Expressed Single Cell Clones

Heterogeneous cells that were infected and treated with selective medium (2 µg/mL puromycin) at least 4 times were detach with trypsin and counted using a hemacytometer. 5000 cells from each counted cell pool (sgRNA combinations of the cell pools: T6.1-T8.1, T6.1-T8.2, T6.2-T8.1, T6.2-T8. 2, T7.1-T8.1, T7.1-T8.2, T7.2-T8.1, T7.2-T8.2) were seeded into well A1 (left, top) of the 96-well tissue culture plate at a volume of 200 µL separately. Selective media with puromycin were still used while generating single cell clones for stably expression. 100 µL of DMEM was added to all remaining wells of consecutive column (B1-H1). Cells in the first well

were pipetted well and serially diluted 1:2 downwards. The remaining 100 μ L cell mixture in the last well (H1) was discarded. Volumes of all column-1 wells were completed to 200 μ L. 100 μ L of DMEM was added to all the remaining wells (from top to bottom B to H, from left to right 2-12) then all wells in the 1st column were serially diluted 1:2, this time to the right, by a multichannel pipette. 100 μ L of cell mixtures remaining in consecutive column 12 were discarded. Ultimately, each well volume in the 96 well was completed to 200 μ L. All 96 well tissue culture plates were placed at 37°C humidified incubator containing 5% CO₂ for 4 days without any action. The medium was renewed every 3 days. Wells with single, 2 and 3 cell colonies were noted after day 4 of plating. These cell colonies were followed for 10 days. If single-cell derived colonies reached sufficient size, then they were picked with trypsinization and transferred to 24-well tissue culture plate (single U2OS cell clone growth takes approximately 20-25 days). Antibiotics were not treated to monoclonal cell lines in a 24 well plate hence they have already become stable. Clones of sufficient cell density in 24-well tissue culture plate were passaged in duplicate into another 24-well tissue culture plate and they were grown to test for *Bmal1* deletion with immunoblotting.

4.2.8. Protein Level Analysis of Candidate *BMAL1* Knockouts

When U2OS *BMAL1*^{-/-} candidates were confluent then, they were removed from the incubator for protein detection by Western blotting. The medium was removed, and the cells were washed with 1X PBS. Considering the cell density, attached cells were lysed with 1.2X Laemmli buffer (85 mM Tris-HCl pH 6.8, 37% glycerol, 6.6% SDS, 0.02% bromophenol blue, 10% β -mercaptoethanol) directly, and the cells lysates were transferred to the test tubes. *BMAL1*^{-/-} candidates were subjected to Western blot analysis.

4.3. Establishment of p(Bmal1)-*dluc* Stable Cell Lines

4.3.1. Transformation of p(Bmal1)-*dluc* Vector into Competent Cells

DH5 α were used to amplify the generated pLV7-Bsd-p(Bmal1)-*dLuciferase* construct a gift from Dr Liu (Ramanathan et al., 2012). Plasmid DNAs were

transferred to 50 μ L of DH5 α cells. The bacterial DNA mix was incubated on ice for 24 minutes and flicked the bottom of the tube every 6-7 minutes. Competent bacteria-DNA mixture was placed on heater at 42°C for 1 minute. The tubes were then placed back on ice immediately and incubated for 2 minutes. 950 μ L of Luria broth (LB) broth was added to each tube without any antibiotics. The tubes were incubated on a shaker at 37°C 200 rpm for 50 minutes. 50 μ L of transformed bacteria were spread on LB agar containing 100 μ g/mL ampicillin. Plates were incubated upside down at 37°C overnight. The next day, a single colony was picked to 10 mL of LB broth containing 100 μ g/mL ampicillin. The inoculated bacteria were incubated on a shaker at 37°C 200 rpm for 22 hours.

4.3.2. Isolation of pLV7-Bsd-p(Bmal1)-dLuciferase Construct

Plasmid isolation was performed as described in section 4.2.4. Transformed bacteria grown at 10 mL were centrifuged at 4000 rpm for 10 minutes and LB was removed. Differently, according to the bacterial density, the pellet was dissolved at 3-fold normal volume used and taken into 3 separate tubes. The supernatant, which was centrifuged for 10 minutes at 13,000 rpm, was passed through the same miniprep column in 3 times after the lysis process and neutralization were completed in separate tubes.

4.3.3. Production of pLV7-Bsd-p(Bmal1)-dLuciferase Lentivirus Particles

Section 4.2.5 was followed for production of pLV7-Bsd-p(Bmal1)-dLuciferase virus particles. 2,66 μ g of pLenti-P2A, 1,33 μ g of pLenti-P2B and 4 μ g of pLV7-Bsd-p(Bmal1)-dLuciferase vector were combined at 2:1:3 ratio.

4.3.4. Transduction of Cells with pLV7-Bsd-p(Bmal1)-dLuciferase

Transduction of cells with pLV7-Bsd-p(Bmal1)-dLuciferase was carried out with the same procedure in section 4.2.6. U2OS-wt and U2OS-LentiCRISPR-v2-*BMAL1*-sgRNA-puro cell clones (U2OS-*BMAL1*^{-/-}) were seeded into 12-well tissue culture plates at 80% confluency. 10 μ g/mL protamine sulfate was added into the pLV7-Bsd-p(Bmal1)-dLuciferase virus mixture in the tube and mixed gently. The medium

containing protamine sulfate:virus complexes were pipetted onto the U2OS-wt cells and U2OS-*BMAL1*^{-/-} cell clones. The medium was replaced with selective medium containing 8 µg/mL blasticidin after 24 hours post-infection to obtain a stable expression. The selective medium was renewed every 2-3 days. Cells were passaged when the cell confluency increased above 60-70%.

4.3.5. Monoclonal Isolation of pLV7-Bsd-p(Bmal1)-dLuc Clones

Isolating single cell clones were carried out as the same with the section 4.2.7. 8 µg/mL blasticidin was used in the selective medium used to obtain pLV7-Bsd-p(Bmal1)-dLuc stable cell lines. All Bmal1-dLuc were subjected to the luciferase assay for confirmation of luciferase expression and for clone selection.

4.3.6. Determination of Luciferase Activity of p(Bmal1)-dLuc Clones

Single clones were grown and expression of "pLV7-Bsd-p(Bmal1)-dLuciferase" was tested with the luciferase assay. Each clone was seeded on 96-well tissue culture dishes at full confluency. After the cells attached to the surface, (approximately 16-20 hours), then, 1X luciferase lysis buffer (50 mM Tris pH:6.8, 25 mM NaCl, 1 mM MgCl₂, 0.8% Triton X-100, 5 mM DTT, 0.6 mM CoA, 0.45 mM ATP and 0.4 mM potassium luciferin) were prepared from 3X stock. Potassium luciferin was freshly added to the lysis buffer. The medium was discarded, and the cells were washed with 1x PBS. 200 µL of 1x luciferase lysis buffer was added to each well. It was incubated on a shaker for 10 minutes at RT in the dark conditions. Lysate in each well was transferred to a white optic 96-well tissue culture plate at the same location with a multichannel pipette. The 96-well plate was placed into the Varioscan optic plate reader and measured 10.000 ms (millisecond) luminometrically. Candidates with high luciferase activity were identified from each U2O2-pLV7-Bsd-p(Bmal1)-dLuc and U2OS- *BMAL1*^{-/-}-p(Bmal1)-dLuc clone.

4.4. Complementation of *BMAL1* Gene at U2OS-*BMAL1*^{-/-} Cells

4.4.1. Analysis of Dose-Dependent *BMAL1* or *CLOCK* Increase with Dual Luciferase-Renilla Reporter System

1×10⁴ U2OS-*BMAL1*^{-/-} clones were seeded on a 96-well plate in 3 replicates. 10 ng of pGL3-m*Per1:Luc*, 1 ng of pGL4-*Renilla* (normalisation control) were transiently transfected to U2OS-*BMAL1*^{-/-} clones. 25 ng of pCMV-Sport6-*Clock* and pCMV-Sport6 empty vector were transiently transfected to control group. 25 ng of pCMV-Sport6-*Clock* and 2,5 ng 5 ng and 10 ng of pcDNA4-m*Bmal1*-3xFlag-His were transiently transfected to experimental cell groups. Dual-Luciferase-Renilla Reporter assay was carried out 24 hours after transfection. 1X luciferase lysis buffer (50 mM Tris pH:6.8, 25 mM NaCl, 1 mM MgCl₂, 0.8% Triton X-100, 5 mM DTT, 0.6 mM CoA, 0.45 mM ATP and 0.4 mM potassium luciferin) were prepared from 3X stock. Potassium luciferin was freshly added to the lysis buffer. The medium was discarded, and the cells were washed with 1x PBS. 200 µL of 1x luciferase lysis buffer was added to each well. It was incubated on a shaker for 10 minutes at RT in the dark conditions. Then, the cell lysate in each well was transferred to a white optic 96-well tissue culture plate at the same location with a multichannel pipette. The 96-well plate was placed into the Varioscan optic plate reader and measured 10.000 ms luminometrically. 1X Renilla salts buffer (15 mM Na₂EDTA, 10 mM Na₄P₂O₇, 475 mM NaCl) was prepared and 0.06 mM PTC-124 and 0.01 mM h-CTZ were freshly added to 1X Renilla salts buffer. Renilla buffer was added to all wells and plate were incubated at RT on a shaker for 10 minutes. The plate then, was placed into the Varioscan optic plate reader and measured 10.000 ms luminometrically again. Data were analysed with GraphPad Prism 8 (GraphPad Software, Inc.). Dose dependent *CLOCK* increase in U2OS-*BMAL1*^{-/-} cells was analysed with two-way ANOVA method with Sidak's multiple comparison test ($p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)). Dose-dependent *BMAL1* increase in U2OS-*BMAL1*^{-/-} cells was analysed with one-way ANOVA method with Tukey's multiple comparison test ($p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)), $p \leq 0.0001$ (****)).

4.4.2. Transformation of pcDNA.4-p(CMV)-mBmal1-3xFlag-6xHis into Competent Cells

pcDNA.4-p(CMV)-mBmal1-3xFlag-6xHis was added onto 100 μ L of DH5 α competent cells. The DNA bacteria mix was incubated on ice for 24 min and gently flicked every 5 minutes. Bacteria-DNA mixture was placed on a water bath at 42°C for 1 minute. The tube was put on ice immediately and incubated for about 2 minutes. 950 μ L of Luria broth (LB) broth was added to the tube without any antibiotics. The tube was incubated on a shaker at 37°C, 200 rpm for 50 minutes. 50 μ L of growth bacteria and spread on LB agar containing 100 μ g/mL ampicillin. Plates were incubated upside down at 37°C overnight. The next day, 1 of the single colony were picked to 10 mL of LB broth containing 100 μ g/mL ampicillin. The inoculated bacteria were incubated on a shaker at 37°C 200 rpm for 22 hours.

4.4.3. Isolation of pcDNA.4-p(CMV)-mBmal1-3xFlag-6xHis

Plasmid isolation was carried out as described in section 4.2.4. Transformants grown at 10 mL were centrifuged at 4000 rpm for 10 minutes and LB broth was discarded. Bacteria pellets were dissolved with 690 μ L of resuspension buffer and these resuspended bacteria were separated. 250 μ L of bacterial cell suspensions were pipetted to 3 test tubes. 250 μ L (V:V) of cell lysis buffer were added onto the bacterial cell suspension. The tubes were upside down for 6-8 times and incubated at RT for exactly 4 minutes. 350 μ L of neutralization buffer was put in the tubes and tubes were gently inverted. The phase-separated mixtures were centrifuged at 13,000 rpm for 10 minutes at RT. 650 μ L of the supernatant was loaded onto the miniprep columns and the columns were centrifuged at 13,000 rpm for 60 seconds. Flow through was discarded. This step was repeated for 3 times to collect large scale DNA. 650 μ L wash buffer was pipetted into the columns and they were centrifuged at 13,000 rpm for 60 seconds. The column systems were centrifuged empty at 13,000 rpm for 60 seconds to dry the silica-based membrane with the collection tube changed. 70 μ L of elution buffer were dripped into the column membrane and columns were incubated at RT for 2 minutes. Columns were centrifuged at 13,000 rpm for 2 minutes and DNA concentrations were measured with NanoDrop spectrophotometer.

4.4.4. Transfection of pcDNA.4-p(CMV)-m*Bmal1*-3xFlag-6xHis into U2OS-*BMAL1*^{-/-}-Cells

U2OS-*BMAL1*^{-/-}-p(*Bmal1*)-d*Luc* cells were seeded on 12-well culture plate at 70-80% confluency. Cells were transfected with 500 ng of pcDNA.4-p(CMV)-m*Bmal1*-3xFlag-6xHis 12-16 hours after cell seeding. First, 500 ng of DNA was added to 300 μ L of transfection buffer, then 1,5 μ L of PEI was added and the mixture was gently vortexed and spin down. This mixture was incubated at room temperature for 15 minutes. Finally, the mixture was added to the seeded cells and the cells were placed on the incubator. After 24 hours, 100 μ g/mL of Zeocin selection was started. Antibiotic selection was carried out 2-day intervals for 1-2 weeks. Cells were passaged when they reached about 70% confluency. After non-transfected cells were eliminated and transfected construct were stably expressed, then cells were subjected to monoclonal isolation by serial dilution.

4.4.5. Isolation of Complemented p(CMV)-m*Bmal1* Single Cell Clones by Serial Dilution

Isolating single cell clones were carried out as the same with the section 4.2.7. 100 μ g/mL zeocin was used in the selective medium used to obtain pcDNA.4-p(CMV)-m*Bmal1*-3xFlag-6xHis stable cell lines. All add back in clones were subjected to Western blot experiment for protein level analysis.

4.4.6. Confirmation of *Bmal1* Complementation at Protein Level

pcDNA.4-p(CMV)-m*Bmal1*-3xFlag-6xHis stably expressed cells were cultured for Western blotting procedure. When cells were at 90% confluency, the medium was removed, and the cells were washed with 1X PBS. Cells were lysed with 1.2X Laemmli buffer (85 mM Tris-HCl pH 6.8, 37% glycerol, 6.6% SDS, 0.02% bromophenol blue, 10% β -mercaptoethanol) directly, and the cells lysates were transferred to the test tubes. Add back in candidates were subjected to Western blot analysis.

4.4.7. Construction of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis

Vector and insert were digested as followed, 1 µg of pcDNA4-mBmal1-3xFlag-6xHis was digested with *SpeI* and filled with Q5 polymerase to obtain blunt ended DNA and then digested with *MfeI*. 3 µg of pLenti-plus III-p(Bmal1)- HA sequence was digested with *XbaI* and *ScaI*. Vector and insert were run on an agarose gel for gel clean-up procedure.

Vector and insert were then isolated from agarose gel. 6X DNA loading dye was added to the restriction enzyme digested vector and insert reaction mixtures at a final concentration of 1X. Samples were run on agarose gel and visualized with a UV light. Vector and insert DNA fragments were cut from agarose gel and placed in a clean Eppendorf. 3-fold of gel weight NT-I buffer were added on gel pieces and they were placed on a heat block at 55°C for 10 minutes. Following the gel melting in the NT-I buffer, then 200 µL of isopropanol were pipetted and this mixture containing DNAs of interest were loaded on a gel clean-up column. Columns were centrifugated at 13.000 rpm for 30 seconds. Flowthrough was discarded and column were washed with NT-III buffer twice. Finally, columns were dried, and DNAs were eluted with 50 µL of nuclease free H₂O.

Finally 150 ng of p(Bmal1) insert and 50 ng of pcDNA4-mBmal1-3xFLAG-6xHis vector (3:1) ligated with 0,5 µL of T4 DNA ligase in 1x T4 DNA ligation buffer to a 10 µL total reaction volume. Ligation reactions were incubated at +4°C overnight.

4.4.8. Transformation of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis into Competent Cells

DH5α competent cells were used to amplify the generated pcDNA.4-p(Bmal1)-mBmal1-3xFlag-His construct. Construct was dripped onto 100 µL of DH5α competent cells. The mixture was incubated on ice for 24 min and gently flicked every 5 minutes. Competent bacteria-DNA mixture was submerged into water bath at 42°C for 1 minute. The tube was put on ice immediately and incubated for about 2 minutes. 950 µL of LB broth was pipetted to the tube without any antibiotics. The tubes were incubated on a shaker at 37°C 200 rpm for 50 minutes. The tubes were centrifuged at 13.000 rpm for 1 minute. The bacterial pellet was dissolved with 50 µL of LB and spread on LB agar containing 100 µg/mL ampicillin. Plates were

incubated upside down at 37°C overnight. The next day, 5 of the single colonies were picked to 3.5 mL of LB broth containing 100 µg/mL ampicillin. The inoculated bacteria were incubated on a shaker at 37°C 200 rpm for 22 hours.

4.4.9. Isolation of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis

Plasmid isolation was carried out as described in section 4.2.4. Transformants grown at 3.5 mL were centrifuged at 4000 rpm for 10 minutes and LB broth was discarded. Bacteria pellets were dissolved with 230 µL of resuspension buffer and 250 µL of bacterial cell suspensions were pipetted to the test tubes. 250 µL (V:V) of cell lysis buffer were added onto the bacterial cell suspension. The tubes were upside down for 6-8 times and incubated at RT for exactly 4 minutes. 350 µL of neutralization buffer was put in the tubes and tubes were gently inverted. The phase-separated mixtures were centrifuged at 13.000 rpm for 10 minutes at RT. 650 µL of the supernatant was loaded onto the miniprep columns and the columns were centrifuged at 13.000 rpm for 60 seconds. Flow through was discarded. 650 µL wash buffer was pipetted into the columns and they were centrifuged at 13.000 rpm for 60 seconds. Flow through was discarded. The column systems were centrifuged empty at 13.000 rpm for 60 seconds to dry the silica-based membrane with the collection tube changed. 50 µL of elution buffer were dripped into the column membrane and columns were incubated at RT for 2 minutes. Columns were centrifuged at 13.000 rpm for 2 minutes and DNA concentrations were measured with NanoDrop spectrophotometer.

4.4.10. Confirmation of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis Construction with Restriction Analysis

The total reaction volume was set to 20 µL. 1 µg each of the DNAs was pipetted into tubes for restriction endonuclease cutting. 2 µL of NEBuffer™ r1.1 (10X, New England Biolabs, Cat. No: B7030S) was added to each tube with 1X in the final concentration. 0,1 µL of *KpnI* (10 U/µL, New England Biolabs, Cat. No: R0142) was added to the reaction tubes. The reaction volume was completed to 20 µL with nuclease free sterile water. The tubes were incubated at 37°C for 1.5 hours. After the incubation period, 6X DNA loading dye was added to the tubes with 1X in the final concentration.

According to DNA fragments consisted after restriction, 1% agarose gel was prepared, and it was ready for loading with the addition of 1X TBE buffer (90 mM of Tris base, 88.95 mM of Boric acid, 0.4% of 0.5 M EDTA pH:8.0), samples were loaded onto agarose gel and run at 150 V. Once the appropriate DNA fragments were appeared, the agarose gel was visualized on the chemiluminescence visualization device ChemiDoc XRS+ (Bio-Rad) instrument under Trans-UV light (Figure 5.12).

4.4.11. Reverse Transfection of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis into U2OS-*BMAL1*^{-/-} Cells

U2OS-LentiCRISPR-v2-*BMAL1*-sgRNA-puro/pLV7-Bsd-p(Bmal1)-dLuc cell clones (U2OS-*BMAL1*^{-/-} p(Bmal1)-dLuc) were detached from the culture plate and centrifugated at 4000 rpm for 5 minutes. pcDNA.4-p(Bmal1)-mBmal1-3xFlag-His C1, C2 and C3 DNAs were mixed with PEI (volume equal to 3-fold of amount of DNA µg) included transfection buffer (1x TE, 150 mM NaCl) and the mixtures were incubated at RT for 15 min. Pellets were resuspended with 10 mL of DMEM. Transfection mixtures were added into the cell suspension directly and transfection buffer:DNA:PEI:cell mixtures were seeded into a 12-well culture plate. 100 µg/mL of Zeocin (Thermo Fisher Scientific, cat. no: R25001) selection was started after 24 hours of cell attachment to the plate surface. Zeocin selection was carried out 2-day intervals for 1-2 weeks. Cells were passaged when they reached about 70% confluency. After non-transfected cells were eliminated and transfected construct were stably expressed, then cells were subjected to serial single clonally expansion with serial dilution.

4.4.12. Isolation of p(mBmal1)-mBmal1 Complemented Single Cell Clones by Serial Dilution

Isolating single cell clones were carried out as the same with the section 4.2.7. 100 µg/mL zeocin was used in the selective medium used to obtain . pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis stable cell lines. All add back in clones were subjected to Western blot experiment for protein level analysis.

4.4.13. Confirmation of Bmal1 Complementation at Protein Level

pcDNA.4-p(mBmal1)-mBmal1-3xFlag-His stable cell clones were cultured for Western blotting procedure. When cells were at 90% confluency, the medium was removed, and the cells were washed with 1X PBS. Cells were lysed with 1.2X Laemmli buffer (85 mM Tris-HCl pH 6.8, 37% glycerol, 6.6% SDS, 0.02% bromophenol blue, 10% β -mercaptoethanol) directly, and the cells lysates were transferred to the test tubes. Add back in candidates were subjected to Western blot analysis.

4.5. Complementation of *Bmal1* Gene at U2OS- *BMAL1*^{-/-} Cells by Viral Infection

4.5.1. Construction of pLenti III-p(mBmal1)-mBmal1-3xFlag-His

Vector and insert were restricted as follow, 1 μ g of pcDNA4-p(mBmal1)-mBmal1-3xFLAG-His was digested with *Bam*HI in a 200 μ L of reaction volume and cleaned-up then digested with *Pme*I in a 100 μ L of reaction volume. 3 μ g of pLenti-plus III-no promoter-HA sequence was digested with *Bgl*II in a 200 μ L of reaction volume and cleaned-up then digested with *Pme*I in a 100 μ L of reaction volume. Vector and insert were run on an agarose gel for gel clean-up procedure.

Agarose gel clean-up procedure was carried out as section Methods 4.4.2.2. Unlike the gel clean-up procedure, when performing RE digestion clean-up, 800 μ L of NT-I buffer and 200 μ L of isopropanol were first added to the 200 μ L reaction volume and a heat block was not used. After this mixture is flowed through the gel-clean up column, the next part is the same as the gel-clean up protocol.

150 ng of p(mBmal1)-mBmal1-3xFlag-6xHis insert and 50 ng of pLenti-plus III-no promoter vector (3:1) ligated with 0,5 μ L of T4 DNA ligase in 1x T4 DNA ligation buffer to a 10 μ L total reaction volume. Ligation reactions were incubated at +4°C overnight.

4.5.2. Transformation of pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis into Competent Cells

STBL3 competent cells were used to amplify the generated pLenti III-p(mBmal1)-mBmal1-3xFlag-His construct. Construct was dripped onto 80 µL of STBL3 competent cells. The mixture was incubated on ice for 24 min and gently flicked every 5 minutes. Competent bacteria-DNA mixture was submerged into water bath at 42°C for 1 minute. The tube was put on ice immediately and incubated for about 2 minutes. 980 µL of Luria broth (LB) broth was pipetted to the tube without any antibiotics. The tubes were incubated on a shaker at 37°C 200 rpm for 50 minutes. The tubes were centrifuged at 13.000 rpm for 1 minute. The bacterial pellet was dissolved with 50 µL of LB and spread on LB agar containing 100 µg/mL kanamycin. Plates were incubated upside down at 37°C overnight. The next day, 6 of the single colonies were picked to 3.5 mL of LB broth containing 100 µg/mL kanamycin. The inoculated bacteria were incubated on a shaker at 37°C 200 rpm for 22 hours.

4.5.3. Isolation of pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis

Plasmid isolation was carried out as described in section 4.2.4. Transformants grown at 3.5 mL were centrifugated at 4000 rpm for 10 minutes and LB broth was discarded. Bacteria pellets were dissolved with 230 µL of resuspension buffer and 250 µL of bacterial cell suspensions were pipetted to the test tubes. 250 µL (V:V) of cell lysis buffer were added onto the bacterial cell suspension. The tubes were upside down for 6-8 times and incubated at RT for exactly 4 minutes. 350 µL of neutralization buffer was put in the tubes and tubes were gently inverted. The phase-separated mixtures were centrifuged at 13.000 rpm for 10 minutes at RT. 650 µL of the supernatant was loaded onto the miniprep columns and the columns were centrifuged at 13.000 rpm for 60 seconds. Flow through was discarded. 650 µL AW wash buffer (containing guanidine hydrochloride at 36 - <50 % and isopropanol at 20 - <35 %) was pipetted into the columns and they were centrifuged at 13.000 rpm for 60 seconds. Flow through was discarded. 650 µL wash buffer (containing ethanol) was pipetted into the columns and they were centrifuged at 13.000 rpm for 60 seconds. Flow through was discarded. The column systems were centrifuged empty at 13.000 rpm for 60 seconds to dry the silica-based membrane with the collection

tube changed. 50 μ L of elution buffer were dripped into the column membrane and columns were incubated at RT for 2 minutes. Columns were centrifuged at 13.000 rpm for 2 minutes and DNA concentrations were measured with NanoDrop spectrophotometer.

4.5.4. Confirmation of pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis Construction with Restriction Analysis

The total reaction volume was set to 20 μ L. 300 ng each of the DNAs was pipetted into tubes for restriction endonuclease cutting. 2 μ L of Buffer B (10X, Thermo Scientific, cat. no:BB5) was added to each tube with 1X in the final concentration. 0,1 μ L of *Eco147I* (*StuI*) (10 U/ μ L, Thermo Scientific, Cat. No: ER0421) was added to the reaction tubes. The reaction volume was completed to 20 μ L with nuclease free sterile water. The tubes were incubated at 37C for 1.5 hours. After the incubation period, 6X DNA loading dye was added to the tubes with 1X in the final concentration.

According to DNA fragments consisted after restriction, 1% agarose gel was prepared, and it was ready for loading with the addition of 1X TBE buffer (90 mM of Tris base, 88.95 mM of Boric acid, 0,4% of 0.5 M EDTA pH:8.0), samples were loaded onto agarose gel and run at 150 V. Once the appropriate DNA fragments were appeared, the agarose gel was visualized on the chemiluminescence visualization device ChemiDoc XRS+ (Bio-Rad) instrument under Trans-UV light (Figure 5.21).

4.5.5. Transduction of Cells with pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis

U2OS- *BMAL1*^{-/-} p(Bmal1)-dLuc cells were seeded into 12-well tissue culture plates at 30-40% confluency 24 hours prior to viral infection. Viral particles containing the pLenti III-p(mBmal1)-mBmal1-3xFlag-6xHis were combine with 10 μ g/mL polybrene in the tube and mixed well. The medium containing polybrene:virus complexes were dripped onto the U2OS-*BMAL1*^{-/-} p(Bmal1)-dLuc cells. The medium was replaced with selective medium containing 3 μ g/mL puromycin after 24 hours post-infection to obtain a stable expression. The selective medium was renewed every 2 days. Cells were selected for 4 weeks and were passaged sparsely (approximately 10-20%) when the cells were divided for 2-3 times. The rescue in

knockout cells after adding back in of *mBmal1* under native promoter in polyclonal state were analysed by real time bioluminescence assay.

4.6. Monitoring the Real-Time Circadian Oscillation

5×10^4 U2OS- pLV7-Bsd-p(Bmal1)-dLuc (for monitoring normal *BMAL1* oscillation), U2OS-LentiCRISPR-v2-*BMAL1*-sgRNA / pLV7-Bsd-p(Bmal1)-dLuc (for monitoring oscillation whose *BMAL1*^{-/-} cells) and finally, U2OS-LentiCRISPR-v2-*BMAL1*-sgRNA / pLV7-Bsd-p(Bmal1)-dLuc/pcDNA.4-p(Bmal1)-m*Bmal1*-3xFlag-His (for monitoring rescued *Bmal1* oscillation) cells were seeded in opaque white 96-well plate. Cells were reset with DMEM containing 0,1 μ M dexamethasone for 2 hours. 0.1 mM beetle luciferin (Promega, cat. no: E1602) was freshly added to the ATM medium (DMEM powder (Sigma cat. no: D-2902), Sodium Bicarbonate (Sigma cat. no: S5761), D-(+)-glucose powder (Sigma cat. no: G7021), 1 M HEPES buffer (Gibco cat. no: 15140-122), 1% Pen/strep, 5% FBS, 1% NEAA, Sterile milliQ water) when the last 15 minutes remain. The medium contained dexamethasone was aspirated, and the cells were washed with 1X PBS. Finally, recording medium was added into each well and the plate was placed on Synergy™ H1 Hybrid Multi-Mode Microplate Reader. Device settings were adjusted as manufacturer's instructions. Data recording was continued for 5 days (approximately 103 hours) and analysed with GraphPad Prism 8.0.

4.7. Immunoprecipitation

The interaction of CLOCK and BMAL1 was investigated to test the functionality of BMAL1 which was re-introduced with the add back in system. U2OS-LentiCRISPR-v2-*BMAL1*-sgRNA/pLV7-Bsd-p(Bmal1)-dLuc/pcDNA.4-p(Bmal1)-m*Bmal1*-3xFlag-His C37.1.D1.B1 and C39.2.D1.B1 BMAL1 add back in clones were plated at 90% confluency on a 10 cm² cell culture plate. The next day, 20 μ L/sample of Dynabeads Protein G (Invitrogen, cat. no: 10004D) was equilibrated with NP-40 lysis buffer (50 mM Tris-HCl pH: 7.4, 5mM EDTA, 1% NP-40, 250 mM NaCl, 1x PI). The beads were placed on the magnetic rack after spin down and the supernatant was removed during each washing step. 200 μ L/sample NP-40 lysis buffer was added to the beads in 2 tubes and 1 μ L/sample anti-FLAG antibody was added to one of them and 1

μL/sample IgG antibody was added to the other. The tubes were rotated for 1 hour at +4°C. During the 1-hour incubation, cells were collected by scraping and washed with 1X PBS. Cells were lysed with 1 mL of NP-40 lysis buffer. Tubes were incubated on ice for 20 minutes with vortexing every 5 minutes. Samples were centrifuged at 13,300 rpm for 20 minutes to obtain protein lysate. 50 μL of each of the total protein lysates was left for input samples and stored at -20°C. 450 μL of the remaining protein lysate was added onto Flag antibody-coated beads and another 450 μL onto Mouse IgG-coated beads. Tubes were then rotated overnight at +4°C. The tubes containing the bead-antibody-protein complexes were placed in the magnetic rack and beads were collected, clarified supernatant was removed. Beads were then washed 4-5 times with 1X NP-40 lysis buffer. 1.5X of Laemmli lysis buffer (85 mM Tris-HCl pH 6.8, 37% glycerol, 6.6% SDS, 0.02% bromophenol blue, 10% β-mercaptoethanol) was added to the beads subsequent to the final washing step. Samples were then subjected to Western blot analysis to evaluate the physical interaction between the endogenous CLOCK and exogenous mBmal1 which was added back in.

4.8. Western Blotting

4.8.1. Cell Lysis and Sample Preparation

The medium was removed and U2OS cells were scraped off in 1X PBS. Cells were then centrifuged at 4000 rpm for 5 min at +4°C. PBS was removed and cells were lysed with 1.2X Laemmli buffer (85 mM Tris-HCl pH 6.8, 37% glycerol, 6.6% SDS, 0.02% bromophenol blue 10% β-mercaptoethanol). Samples were boiled at 95°C for 10 min.

4.8.2. Gel Electrophoresis

SDS-PAGE was prepared according to protein weight. Isopropanol was dropped on the surface of the separating gel to obtain a smooth surface. Finally resolving gel was poured and 15-wells comb was placed carefully. Samples were loaded after polymerisation step was completed and run at 70 volts until the proteins passed through the stacking gel, and at 120 volts in the separating gel.

4.8.3. Protein Transfer

The gel was transferred to a nitrocellulose membrane when protein running process was completed. Gel was removed from the gel glasses and placed on a tank filled with transfer buffer. The membrane and 4 pieces of Whatman papers were soaked in the same buffer. 2 pieces of Whatman papers, membrane gel and finally 2 pieces of Whatman papers were placed on the transfer cassette respectively. The lid of the transfer cassette was locked, and system was run at 12 V for 60 min.

4.8.4. Immunoblotting and Chemiluminescence Visualization

After the transfer, the membrane was incubated with 5% skimmed milk powder solution on a shaker at RT for 1 hour. The membranes were incubated with appropriate primary antibodies at +4°C overnight with rotating. The next day, the membranes were from washed with 1X TBS-T for 5, 15, 5 and 5 minutes. The membranes were incubated for 1 hour on a shaker at room temperature with the appropriate secondary antibodies after washing step with 1X TBS-T. Finally, the membranes were washed again with 1X TBS-T for 5, 15, 5 and 5 minutes and visualized on the ChemiDoc XRS+ (Bio-Rad) instrument with ECL for imaging.

5. RESULTS

In the Results Section, the results of the demonstration of the disruption of the circadian oscillation by knocking out the *BMAL1* gene, then rescuing the oscillation because of the *Bmal1* gene add back in by different ways and obtaining cell lines which *BMAL1*^{-/-} and *BMAL1*^{WT} containing destabilized luciferase (*dLuc*) expression under control the native *BMAL1* promoter to monitor the oscillation for both U2OS *BMAL1*^{-/-} and *BMAL1*^{WT} cell lines.

5.1. Knockout of *BMAL1* Gene at U2OS Cell Line

Establishment of *BMAL1*^{-/-} U2OS cell line was achieved as described in Methods Section 4.2 using sgRNA combinations targeting different regions cloned into the LentiCRISPR.v2-*BMAL1*-sgRNA-puro plasmid.

5.1.1. Determination of Antibiotic Concentrations for Selection

In the cell culture studies within the scope of this thesis, antibiotic resistance genes which inserted into the plasmid backbones were used for the selection of the genetic modifications. The appropriate concentrations to be used for antibiotic selections were calculated on the kill curve in accordance with the data obtained by treated these antibiotics to wt-U2OS cells.

The kill curve was created with a cell viability assay based on the reduction of tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) in living cells.

The appropriate antibiotic concentrations were applied to cells for making kill curves following the literature (Oseni et al., 2021; Limoni et al., 2019). Appropriate antibiotic concentrations were determined by evaluating the IC₅₀ values (Figure 5.1).

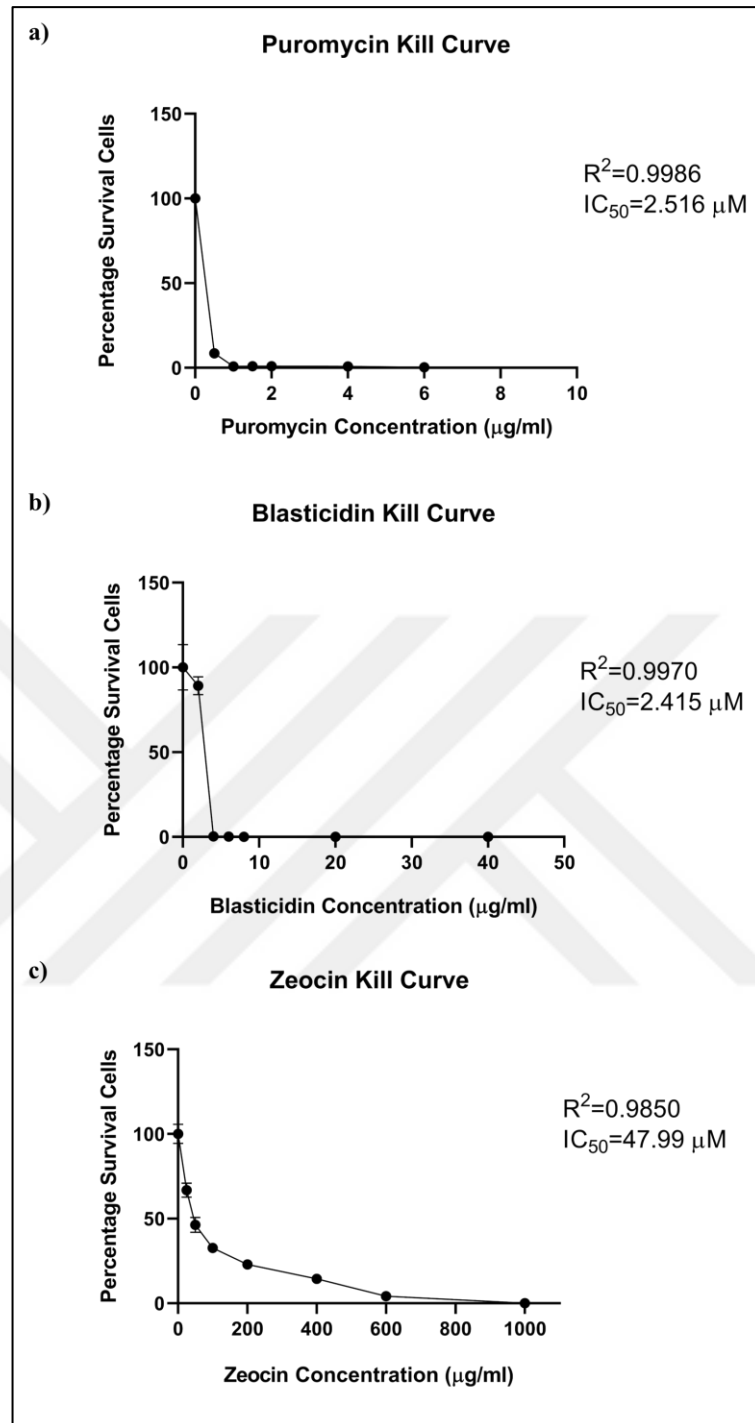


Figure 5.1: Kill curves of antibiotics. Each used antibiotic for selection was treated to wt-U2OS cells. Cells were selected with appropriate antibiotics for 2 times. The antibiotic was renewed 48 hours after the cells were treated with antibiotics.

The LentiCRISPR.v2-*BMAL1*-sgRNA plasmid used to knockout the *BMAL1* gene in wt-U2OS cells contains the puromycin resistance gene. Considering the IC_{50} values obtained, 2 $\mu\text{g/mL}$ puromycin was preferred for polyclonal and monoclonal selection (Figure 5.1 part “a”).

In the first part of the BMAL1 add back in system, the *Bmal1* gene under the CMV promoter was tried to be transferred to the cells. Zeocin resistance gene was used to select pcDNA4-p(CMV)-m*Bmal1*-3xFlag-6xHis C2 plasmid. Cells were treated with 100 µg/mL Zeocin (Figure 5.1 part “c”).

In the 2nd strategy, *Bmal1* coding sequence under its native promoter was transfected into *BMAL1*^{-/-} cells using pcDNA4-p(Bmal1)-m*Bmal1*-3xFlag-6xHis C3 plasmid. 100 µg/mL zeocin as applied to select the transfected cells.

Finally, a *Bmal1* add back in system was tested by viral transduction without monoclonal selection. The pLenti-III-p(Bmal1)-m*Bmal1*-3xFlag-6xHis C3 plasmid used here contained the puromycin resistance gene that was used in the obtaining *BMAL1*^{-/-} system already. Therefore, considering the difficulty of a second selection, the concentration of puromycin was increased up to 3 µg/mL.

Following the generation of both cell lines, cells were transfected with pLV7-Bsd-p(Bmal1)-d*Luc* plasmid and then selected with blasticidin at 8 µg/mL concentration to obtain the p(Bmal1)-d*Luc* stable cell line (Figure 5.1 part “b”).

5.1.2. Determination of the Strategy to Obtain *BMAL1*^{-/-} U2OS Cell Line

The first step of this thesis was to knockout the *BMAL1* gene in the U2OS cell line with a guide RNA-Cas9 complex to disrupt the circadian oscillation. As mentioned in the previous sections, the LentiCRISPR.v2-*BMAL1*-gRNA construct targeting *BMAL1* was used for this purpose. At 3 different location targeted gRNA sequences were cloned into LentiCRISPR.v2 plasmid, of which T8 targets before the first ATG exon sequence, T6 and T7 targets after stop codon of the gene. This was tested with two different DNA delivery processes, a non-viral method transfection and viral transduction. The transduction method, in which we used lentivirus, which has the feature of integration of DNA into the target cell genome, gave much more effective results than the transfection method, which usually provides transient gene expression. When polyclonal stable cells were analysed, western blotting also revealed that the transduced cells contained more *BMAL1*^{-/-} than the transfected cells with PEI agent (Figure 5.2).

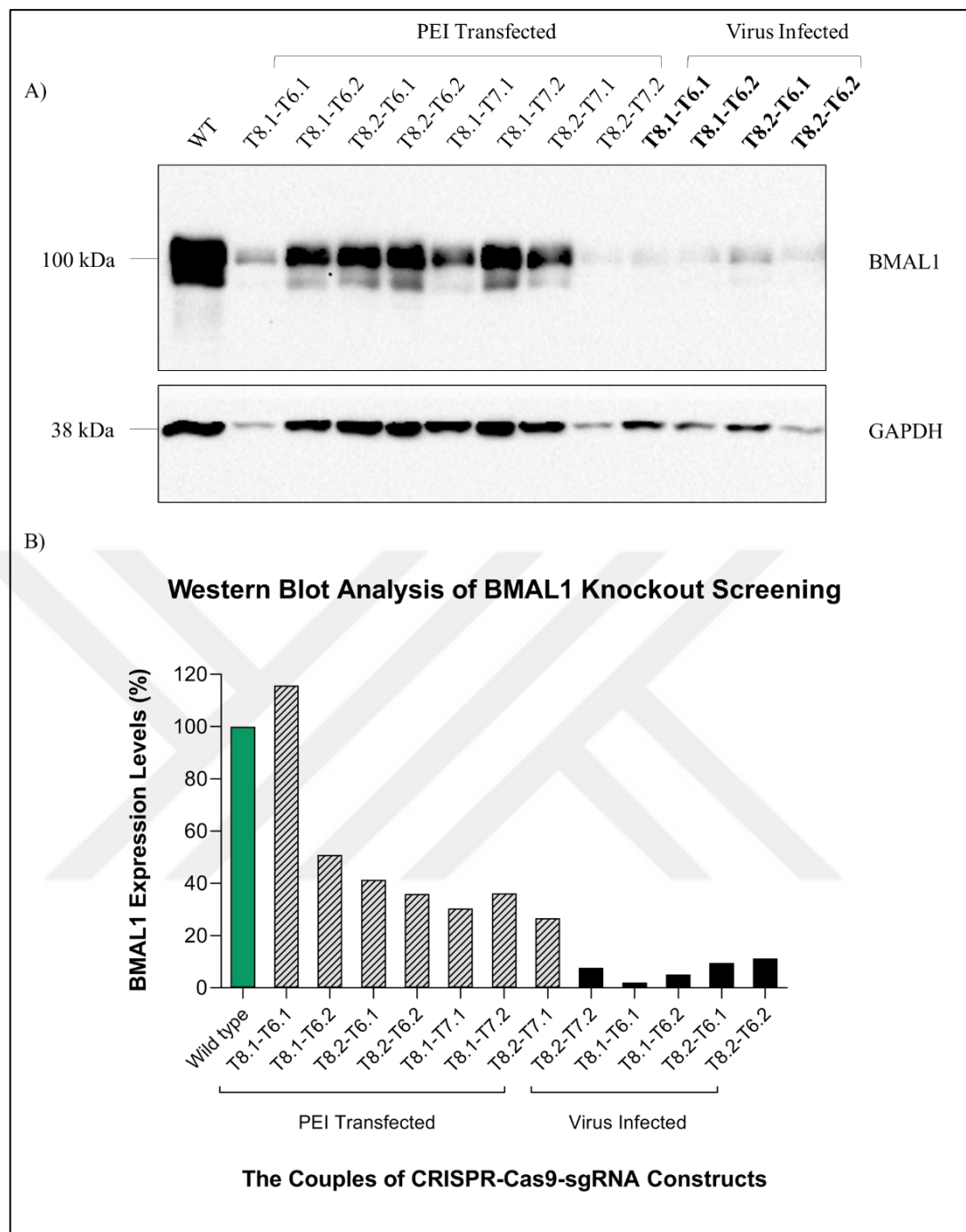


Figure 5.2: Comparison of success rates of two DNA delivery processes “transfection with PEI” and “viral transduction” in polyclonally screened cells to establish *BMAL1*^{-/-} cells in U2OS cell line at the protein level. A) Result of BMAL1 and GAPDH immunoblot, B) Analysis of Western blot result. GAPDH was used for normalisation and loading control. WT: wild type, kDa: kilodaltons, T: Target of sgRNA.

The T8-T6 pair was first used during the viral DNA delivery trial. T8.1/T8.2 and T6.1/T6.2 refer to the selection of 2 different transformant single bacterial colonies obtained during the DNA amplification phase.

5.1.3. Confirmation of Monoclonal *BMAL1*^{-/-} U2OS Cell Lines

Monoclonal cell lines were isolated by serial dilution from virally transduced cells showing a significant decrease in BMAL1 protein level in polyclonal screening. Monoclonal cell lines were generated (Figure 5.3) from each of the gRNA combinations, all of which showed lentivirus-mediated decreases in BMAL1 protein levels, and from the T8.2/T7.1 pair, which showed the greatest decrease in BMAL1 protein levels among the combinations in which DNA was transfected into the cell (Figure 5.2). Whether the monoclonal cells obtained were *BMAL1*^{-/-} or not was screened with immunoblotting using anti-BMAL1 antibody at protein level by Western blot (Figure 5.3).

As a result, a total of 6 *BMAL1*^{-/-} U2OS cell lines were obtained from different sgRNAs targeting different locations. C6, C15, C36, C37 and C39 indicates the clone number of single cell-derived *BMAL1*^{-/-} U2OS cell lines. WT refers to the parental cell line and was used as a positive control. GAPDH with small kDa was used as a loading control in immunoblotting to determine if truncated proteins were formed.

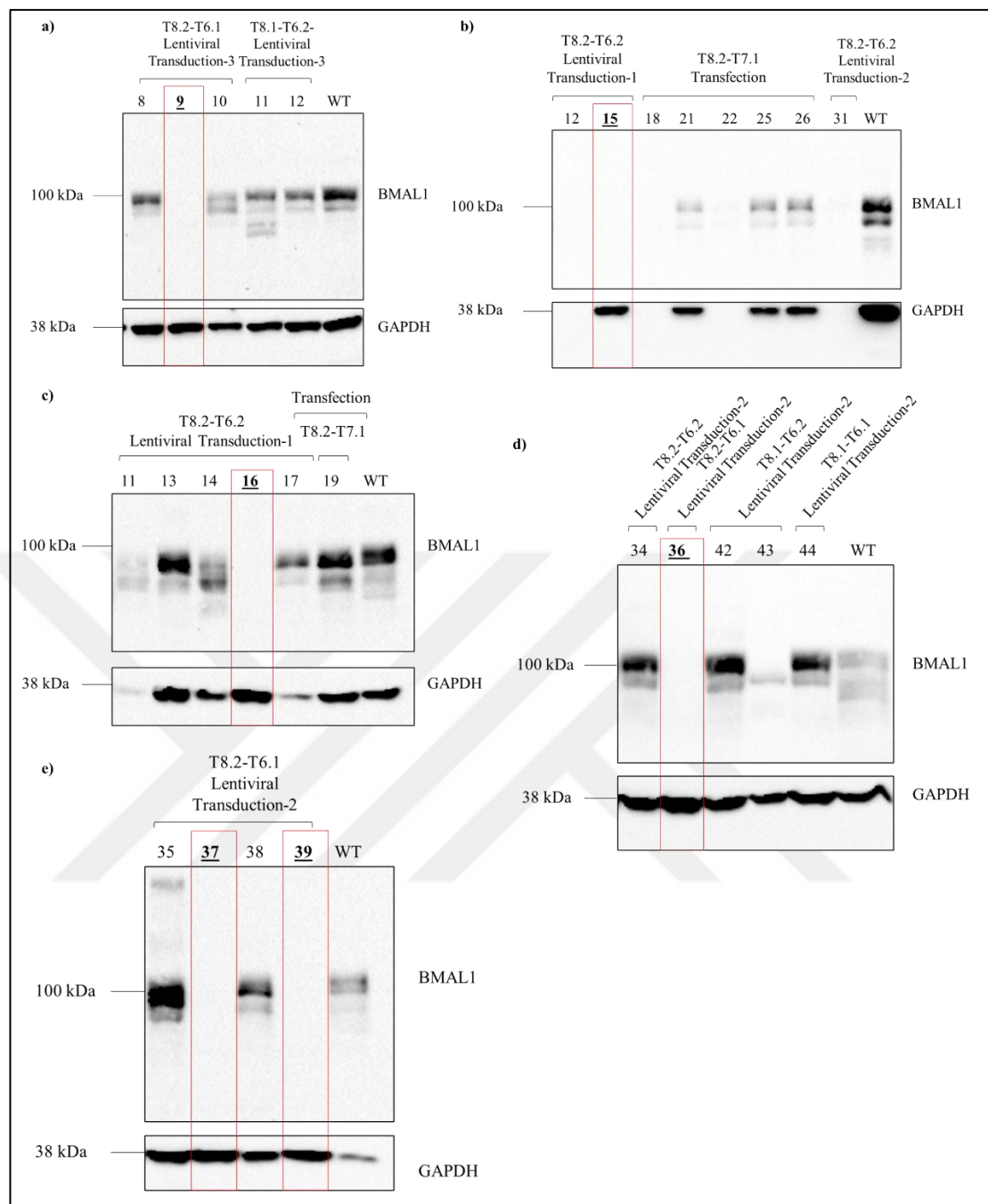


Figure 5.3: Screening of CRISPR-Cas9 mediated U2OS *BMAL1* knockout (*BMAL1*^{-/-}) candidates with Western blotting system. T letter and number represents different location targeted sgRNA. “a-e” parts of figure belong to different Western blot screenings which resulted with 6 different U2OS *BMAL1*^{-/-} clones represented with red boxes. WT: wild type, kDa: kilodaltons, T: Target of sgRNA.

5.1.4. Confirmation of *BMAL1*^{-/-} U2OS Subclones

To test the accuracy of the isolated monoclonal, a second single cell isolation was performed and new monoclonal were obtained from each main single-cell derived *BMAL1*^{-/-} clone and screened by immunoblotting (Figure 5.4). Screening of subclones derived from each main clone revealed that each subclone was *BMAL1*^{-/-}.

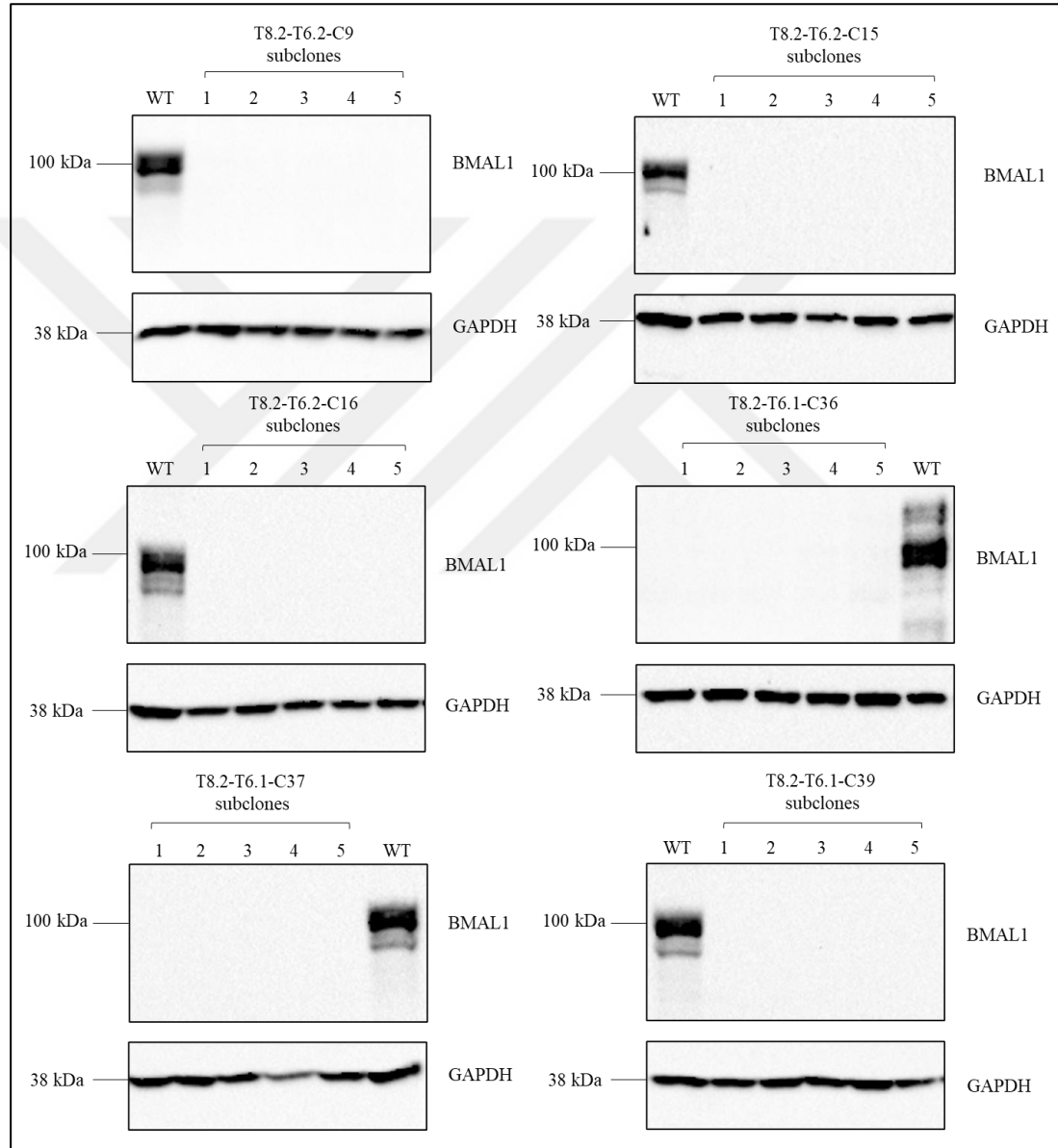


Figure 5.4: Confirmation of U2OS *BMAL1*^{-/-} subclones with Western blotting system. WT: wild type, kDa: kilodaltons, C: clone, T: Target of sgRNA.

5.2. Generation of Stable Cell Lines with p(Bmal1)-dLuc Reporter System

To monitor circadian oscillation in *BMAL1*^{WT}, *BMAL1*^{-/-} and *Bmal1*⁺ cell lines by p(Bmal1) activity, these cell lines were stably transfected with pLV7-Bsd-p(Bmal1)-dLuc lentiviral reporter plasmid. The p(Bmal1)-dLuc reporter plasmid was first transduced into parental U2OS cells to monitor the usual circadian oscillation. The reporter system was then embedded in the *BMAL1*^{-/-} main clones and subclones. All reporter system transduced cells were subjected to monoclonal isolation. These cells were then used to monitor normal, disrupted and finally re-gained circadian rhythm.

5.2.1. Introduction of p(Bmal1)-dLuc System into Parental U2OS Cells to be used as Control Cells

Control cells with p(Bmal1)-dLuc reporter were generated from parental U2OS cells to observe normal circadian rhythm. pLV7-p(Bmal1)-dLuc plasmid was transduced into parental U2OS cells via lentiviruses. This process was carried out with two different infection agents as Polybrene (Figure 5.5 “a and b”) and Protamine sulfate (Figure 5.5 “c and d”). Monoclonal cells were then isolated for both. Unlike the parental cells, the single cell-derived cells which stably expressed the reporter system were produced luciferase due to *BMAL1* promoter activity. Before real time bioluminescence, these cells were lysed with dual luciferase firefly lysis buffer containing luciferin and the presence of enzymatic activity was investigated (Figure 5.5 “a and c”). It was also questioned which of the two different infection agents used would give better results. Afterwards, clones with better enzymatic activity were selected from the experimental groups in which both infection agents were used. Selected clones were subjected to real time bioluminescence assay to demonstrate normal circadian oscillation (Figure 5.5 “b and d”). According to the results of real time bioluminescence assay and period analysis resulted approximately 25 hours (Figure 5.5 “e”), it was found that the clone C3 from the transduced cells using protamine sulfate had a better pattern and it was decided to use it as a control cell in the next experiments.

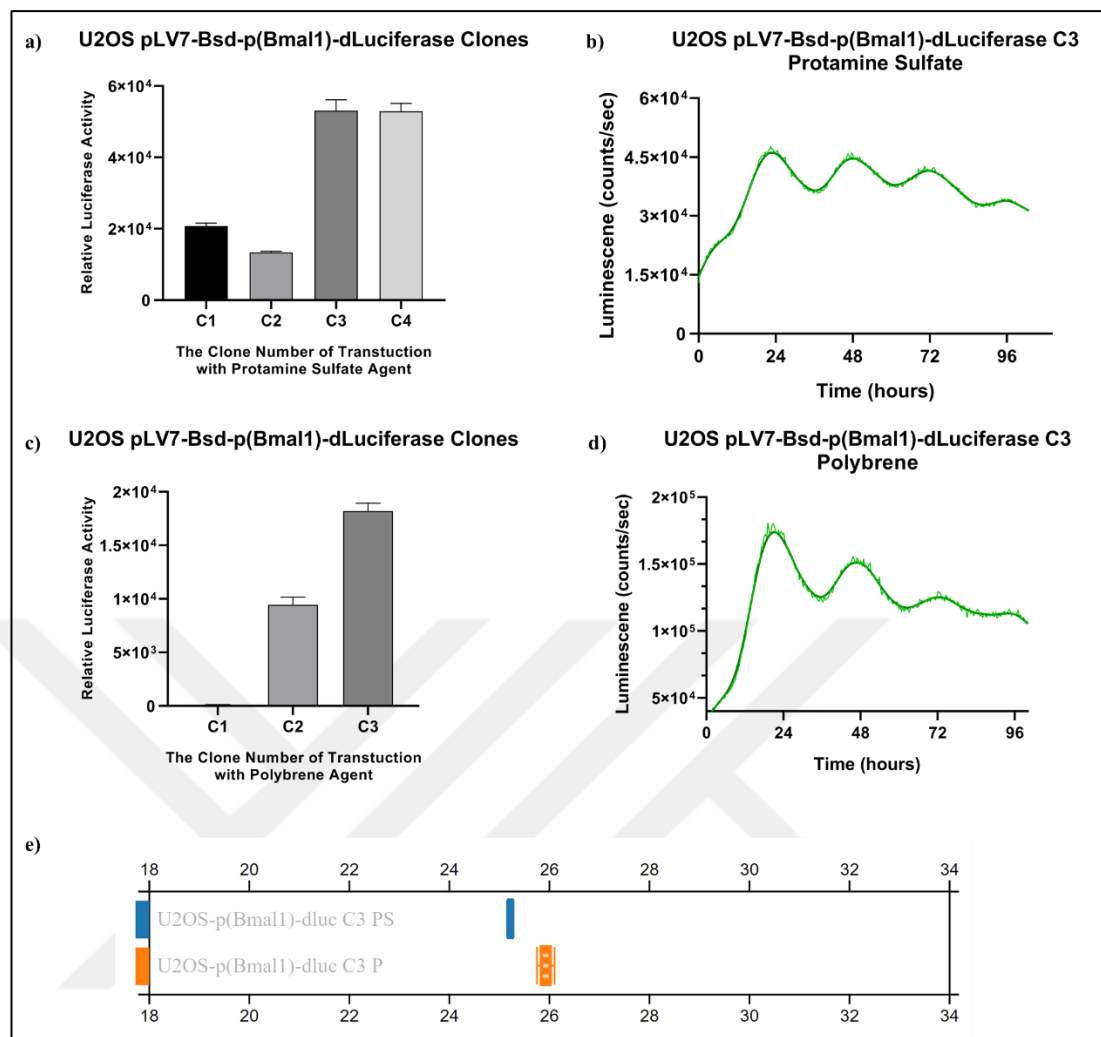


Figure 5.5: Comparison of polybrene and Protamine sulfate transduction agents to obtain U2OS-p(Bmal1):dLuc stable cell line. a) luciferase activities of different U2OS-p(Bmal1):dLuc clones generated with protamine sulfate measured by Dual-Luciferase Assay. b) Real time bioluminescence result of the C3 clone generated with protamine sulfate which gives the best luciferase activity according to the “a” part. c) luciferase activities of different U2OS-p(Bmal1):dLuc clones generated with polybrene measured by Dual-Luciferase Assay. d) Real time bioluminescence result of the C3 clone generated with polybrene which gives the best luciferase activity according to the “c” part. e) Period analysis with Biodare2 webtool. Input data were analysed with Amp&baseline detrending method. Period analysis method was adjusted to FFT NLLS option. Period values were adjusted to 18.0-34.0 (min-max). C: clone, PS: protamine sulfate, P: polybrene. (Data are mean $\pm$ s.e.m.; n=3 replicated in the experiments).

5.2.2. Introduction of p(Bmal1)-dLuc System into *BMAL1*^{-/-} U2OS Cells

The next step was to monitor the disruption of circadian oscillation in the resulting *BMAL1*^{-/-} U2OS cells by obtaining U2OS- *BMAL1*^{-/-}-p(Bmal1)-dLuc stably expressed cells. Single-cell derived cells were again isolated from pLV7-Bsd-p(Bmal1)-dLuc transduced all *BMAL1*^{-/-} clones and subclones (C9, C15, C16, C36, C37, C39 and C15.2, C16.1, C36.1, C37.1 and C39.2) and antibiotic-selected cells by serial dilution. Since the luciferase enzyme will also be produced in these cells depending on p(Bmal1) activity, these cells (both main and subclones) were lysed with dual luciferase firefly lysis buffer containing luciferin and enzymatic activity was analysed using luminometric measurement data (Figure 5.6 “a and c”). After this process, depending on the luciferase activity, one of the reporter-containing monoclonal cells obtained from each *BMAL1*^{-/-} main clone and subclone with luciferase activity (i.e., monoclonal cells with successful reporter insertion) was selected and subjected to real time bioluminescence assay comparing with U2OS-*BMAL1*^{WT}-p(Bmal1)-dLuc cells (Figure 5.6 “b and d”). When *BMAL1*^{WT} and *BMAL1*^{-/-} cells with reporter system inserts were compared, it was observed that *BMAL1*^{WT} cells exhibited a circadian oscillation with a period of approximately 25 hours (Figure 5.6 “e”), whereas the circadian oscillation was disrupted in *BMAL1*^{-/-} cells (Figure 5.6 “b and d”).

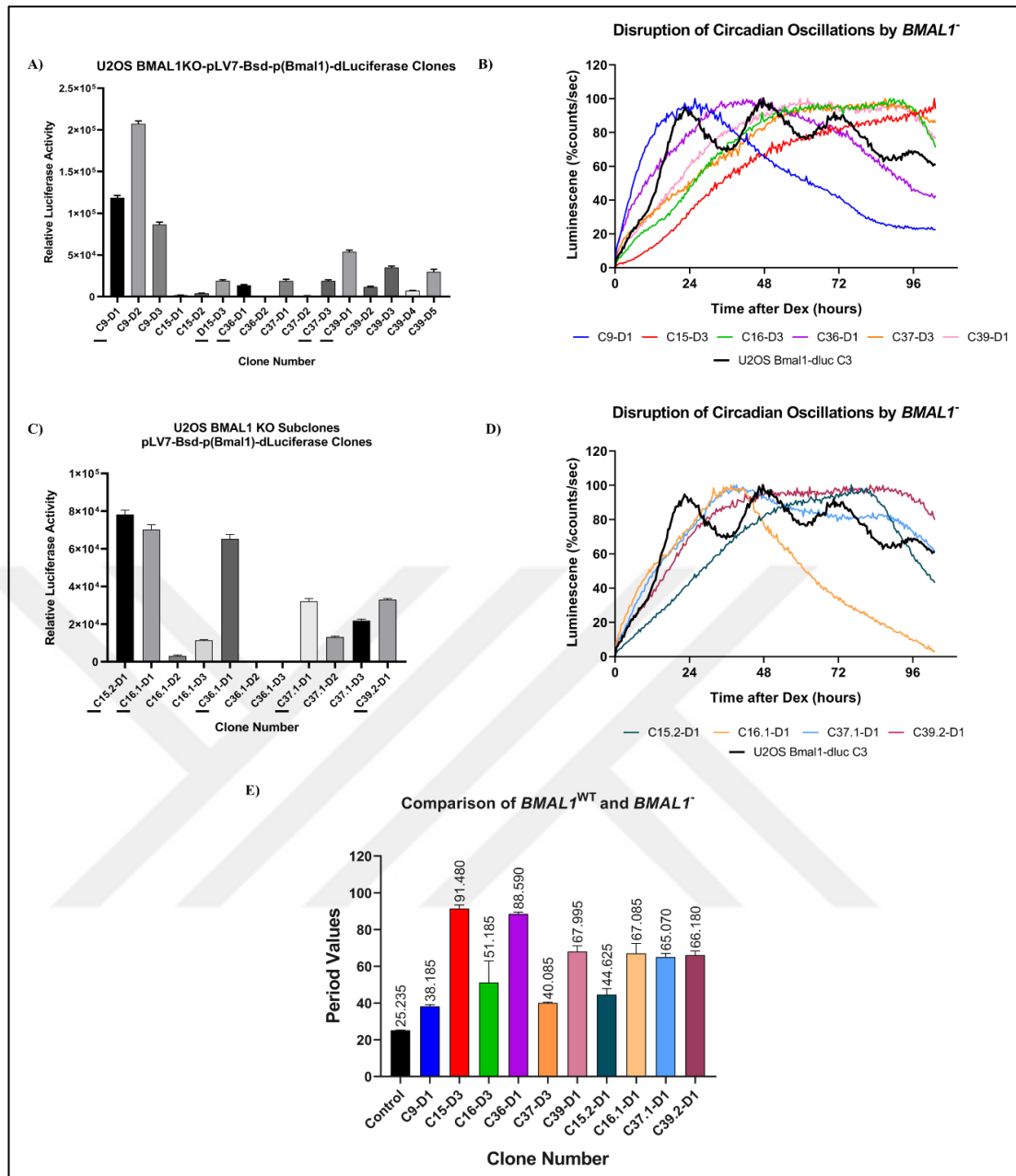


Figure 5.6: Comparison of generated U2OS- *BMAL1*^{-/-} clones which expressed p(Bmal1):*dluc* system and U2OS-p(Bmal1):*dluc* control cells by real time bioluminescence. a) The result of measuring luciferase activities after stabilization of the p(Bmal1):*dluc* system into the generated U2OS- *BMAL1*^{-/-} clones. b) Comparison the *BMAL1* oscillation of *BMAL1*^{WT} and *BMAL1*^{-/-} U2OS clones with real time bioluminescence assay and data were normalized with GraphPad Prism 8. c) The result of measuring luciferase activities after stabilization of the p(Bmal1):*dluc* system into the generated U2OS- *BMAL1*^{-/-} subclones. d) Comparison the *BMAL1* oscillation of *BMAL1*^{WT} and *BMAL1*^{-/-} U2OS subclones with real time bioluminescence assay and data were normalized with GraphPad Prism 8. e) Period analysis with Biodare2 webtool. Input data were analysed with Amp&baseline detrending method. Period analysis method was adjusted to FFT NLLS option. (Data are mean ± s.e.m.; n=2 replicated in the experiments).

5.3. Investigation of *Bmal1* Add Back-in on U2OS- *BMAL1*^{-/-} Cells

It was shown in the previous section that circadian oscillation is disrupted in *BMAL1*^{-/-} cells. In this part, oscillation was somehow restored by reinsertion of *Bmal1* coding sequence. Circadian TTFL rescue was first performed transiently and measured by *Per1* promoter activity via DLR assay.

Subsequently, *mBmal1* under the CMV promoter was stably expressed in *BMAL1*^{-/-} cells. This system was carried out by transfection, a non-viral method, followed by monoclonal isolation. Only one clone achieved success with this system.

The CMV promoter was then replaced with the native *BMAL1* promoter and p(Bmal1)-*mBmal1* construct on the same backbone was transfected into *BMAL1*^{-/-} cells and monoclonal isolation was performed in the same way. In contrast to 1 positive clone obtained from the *Bmal1* add back-in under the CMV promoter, 8 positive clones were obtained from this system.

Finally, to improve and facilitate this biological tool designed to be used in subsequent circadian research, the monoclonal isolation step was tried to be eliminated. In this case, it was decided to work with the viral method for efficient genome insertion. The p(Bmal1)-*mBmal1*-3xFlag-6xHis sequence was cloned into the pLenti III-3xHA-MiniTurbo plasmid and the 3xHA-Miniturbo sequence was removed. Cells were infected with virus particles containing the pLentiIII-p(Bmal1)-*mBmal1*-3xFlag-6xHis C3 construct and then subjected to antibiotic selection for 3 weeks. The resulting *mBmal1* expressed under native promoter add backed-in polyclonal cells were directly checked for rescue of circadian oscillation by real time bioluminescence assay.

5.3.1. Effect of Dose-Dependent CLOCK Increase on *Per1* Promoter Activity in U2OS- *BMAL1*^{-/-} Cells

A comparison was made between U2OS-*BMAL1*^{WT} and U2OS- *BMAL1*^{-/-} cells about the effects on *Per1* promoter activity after transfection of increasing concentrations of CLOCK, which is an interaction partner of BMAL1 and co-functioning in TTFL with BMAL1. No matter how much the interaction partner CLOCK is increased in *BMAL1*^{-/-} cells, the system that activates its transcription by binding to e-boxes in the

regulatory region of PER, CRY, and other CCGs together under normal conditions must be disrupted. While no change in *Per1* promoter activity is expected in U2OS-*BMAL1*^{-/-} cells, an increase in *Per1* promoter activity is expected at increasing CLOCK transfection concentrations, although BMAL1 in U2OS-*BMAL1*^{WT} cells is not stoichiometrically the same as the amount of CLOCK after transfection. As expected, it was found that *Per1* promoter activity increased at increasing doses of CLOCK in U2OS-*BMAL1*^{WT} cells, while no significant increase or change was detected in U2OS-*BMAL1*^{-/-} cells according to the experiment using the DLR system (Figure 5.7, Figure 5.8).

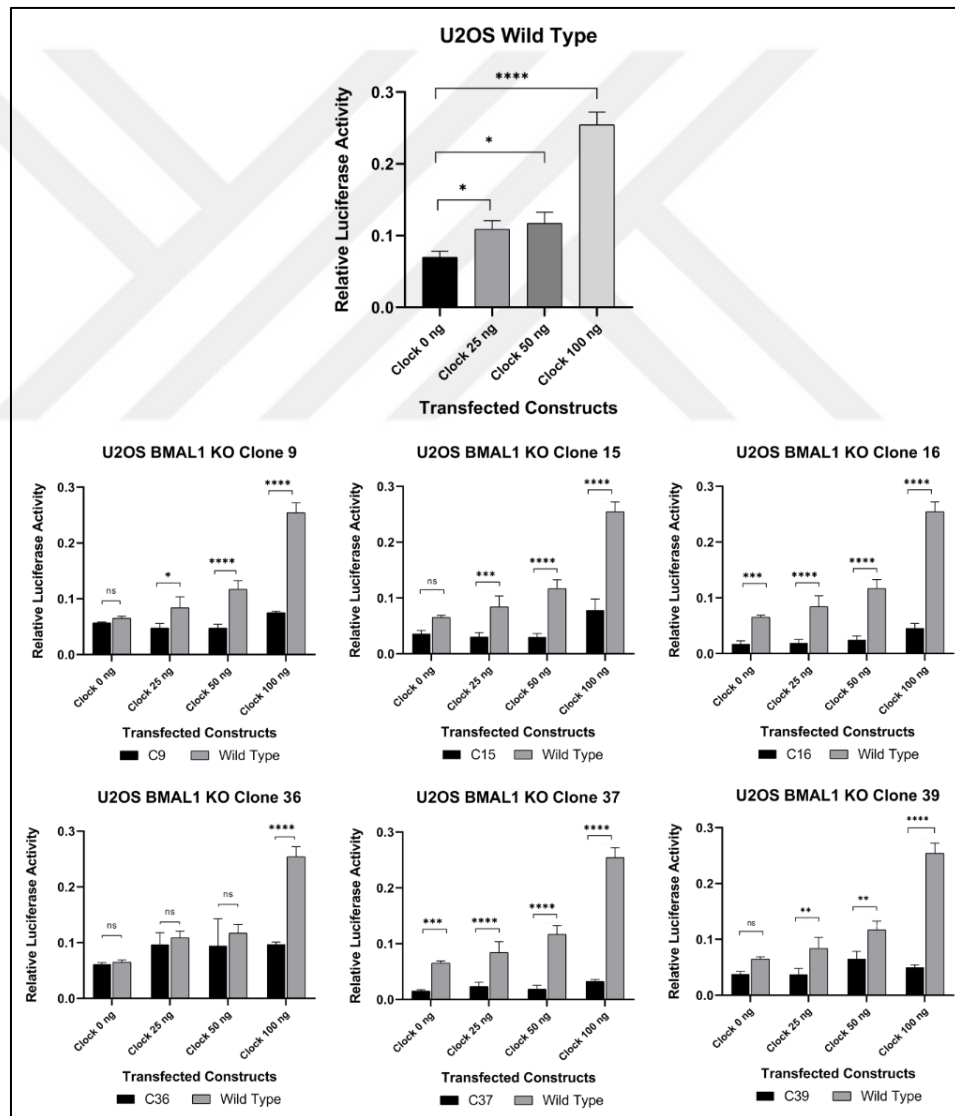


Figure 5.7: Comparison of the change in *Per1* activity in parental U2OS-*BMAL1*^{WT} and U2OS-*BMAL1*^{-/-} main clones by CLOCK increase. Data were analysed using two-way ANOVA method. (p > 0.05 (ns, non-significant), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***), p ≤ 0.0001 (****)). (Data are mean ± s.e.m.; n=4 replicated in the experiments).

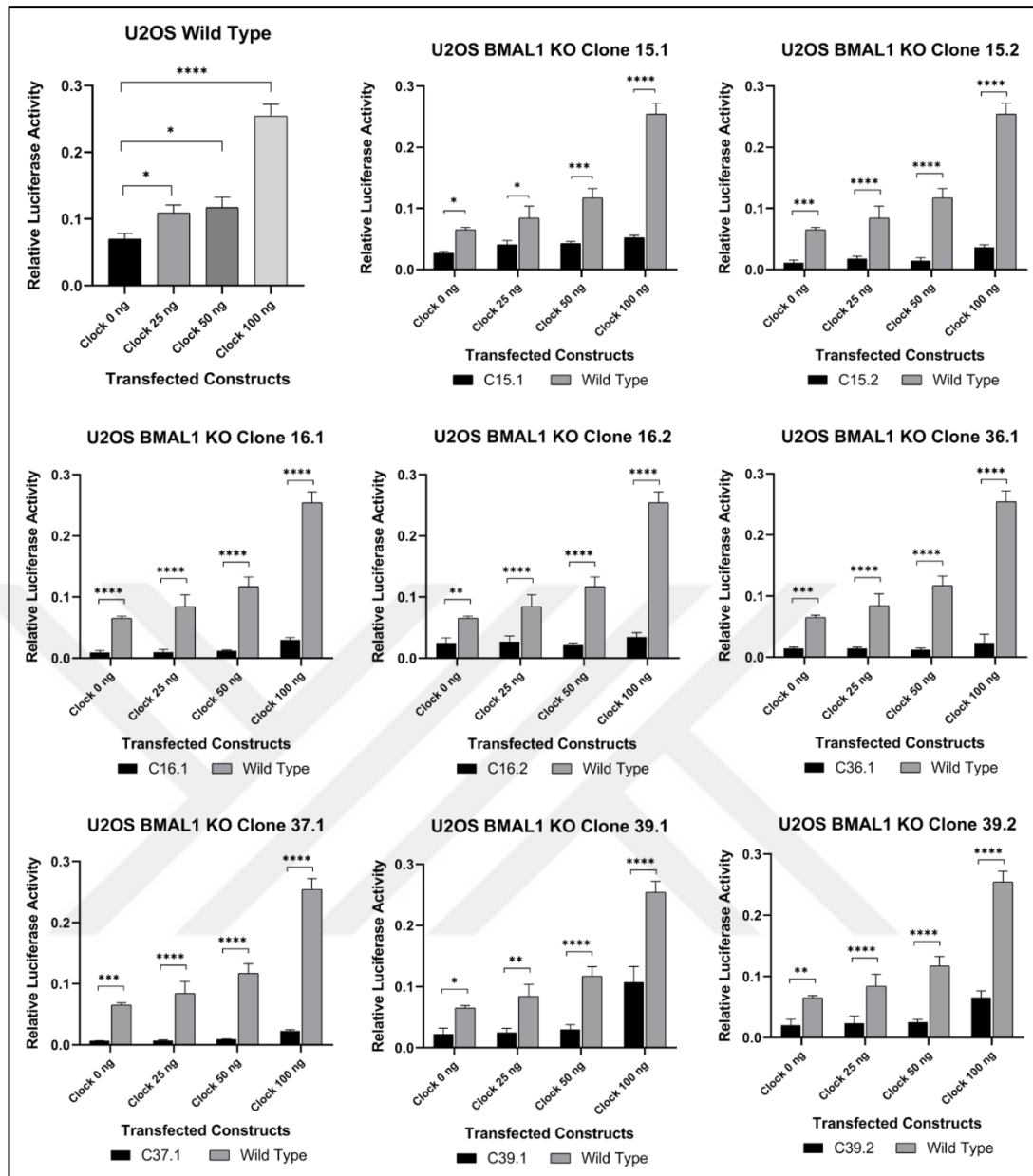


Figure 5.8: Comparison of the change in *Per1* activity in parental U2OS-*BMAL1*^{WT} and U2OS- *BMAL1*^{-/-} subclones by *CLOCK* increase. Data were analysed using two-way ANOVA method. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Data are mean $\pm$ s.e.m.; n=4 replicated in the experiments.

5.3.2. Effect of Dose-Dependent *BMAL1* Increase with *CLOCK* on *Per1* Promoter Activity in U2OS- *BMAL1*^{-/-} Cells

Using U2OS- *BMAL1*^{-/-} cells, the effects on *Per1* promoter activity of *Bmal1* add back in transiently, after transfection of increasing concentrations of *BMAL1* with the constant amount of *CLOCK* cloned into an expression plasmid into these cells were examined with the p(m*Per1*)-*dluc* construct. In a previous experiment, no significant

change or increase in *Per1* promoter activity was observed when increasing concentrations of *CLOCK*, -the interaction partner of BMAL1 in TTFL-, were transiently transfected to *BMAL1*^{-/-} cells. This time, *BMAL1*^{-/-} cells were transiently transfected with increasing concentrations of *BMAL1* together with a constant concentration of *CLOCK*. As a result of this experiment, a U2OS-*BMAL1*^{WT}-like increase in m*Per1* promoter activity was detected in *BMAL1*^{-/-} cells depending on the presence and increase of *Bmal1* (Figure 5.9, Figure 5.10).

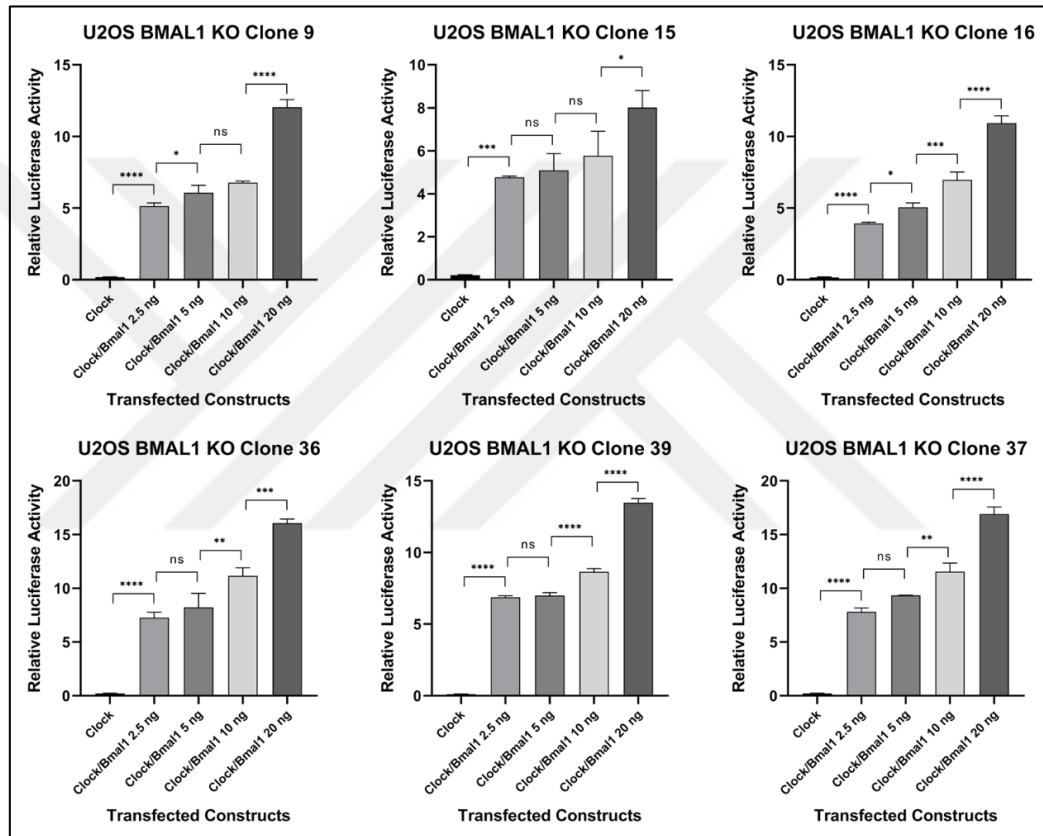


Figure 5.9: Investigation of the change in *Per1* activity in U2OS- *BMAL1*^{-/-} “main clones” by *BMAL1* increase. Data were analysed using one-way ANOVA. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Data are mean $\pm$ s.e.m.; n=4 replicated in the experiments.

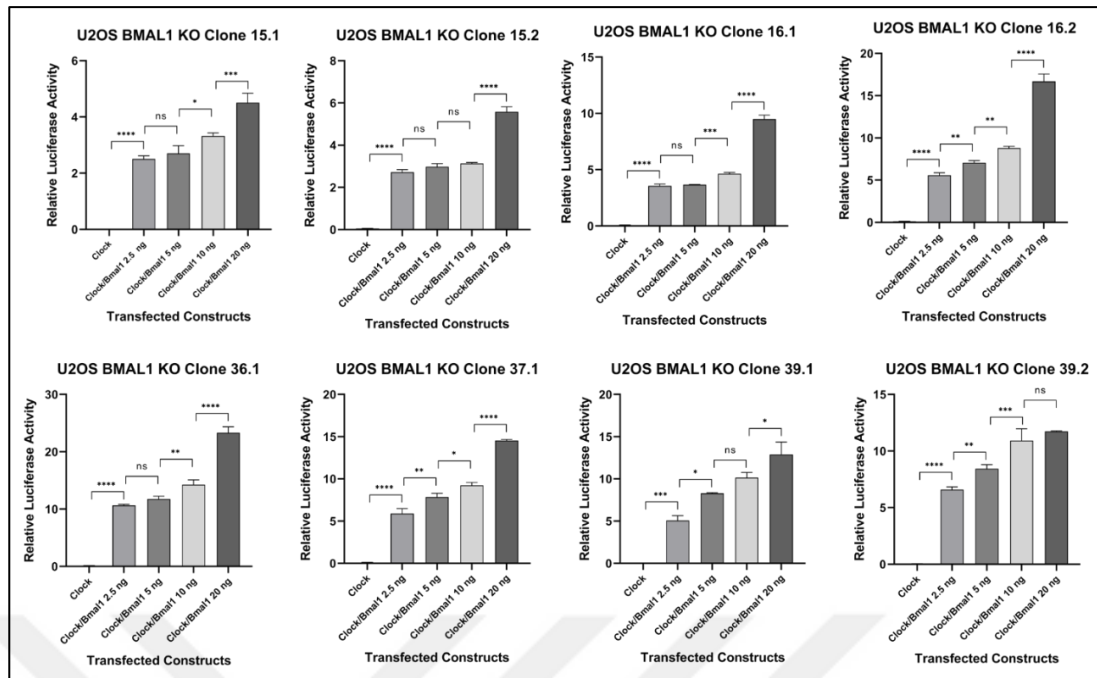


Figure 5.10: Investigation of the change in *Per1* activity in U2OS- *BMAL1*^{-/-} “subclones” by *BMAL1* increase. Data were analysed using one-way ANOVA. $p > 0.05$ (ns, non-significant), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Data are mean $\pm$ s.e.m.; $n=4$ replicated in the experiments.

5.3.3. Rescue of Circadian Oscillation through *Bmal1* Add Back-in under the CMV Promoter

Within the overall aim of this thesis, the circadian oscillation lost in *BMAL1*^{-/-} cells is expected to be restored through the add back-in of *Bmal1*. This has been achieved in different ways to improve the methodology. First, *BMAL1*^{-/-} cells were transfected with m*Bmal1* expressed under the CMV promoter via transfection which is a non-viral method followed by monoclonal isolation. Although this was attempted in all *BMAL1*^{-/-} clones, only in clone C16 did circadian oscillation exhibit a pattern like control cells (Figure 5.11). This clone was labelled C16-D3-CB3.

These cells were also investigated at the protein level by immunoblotting with anti-BMAL1 antibody (b part of Figure 5.11). In the first line, proteins from parental U2OS cells, in the second line proteins only from reporter inserted U2OS cells, in the third line proteins only from *BMAL1*^{-/-} U2OS cells, in the fourth line from *BMAL1*^{-/-} and reporter inserted U2OS cells, and finally in the fifth line from stably expressed m*Bmal1*-3xHis-6xFlag add back-in cells under the CMV promoter were loaded (b part of Figure 5.11). On the other hand, these cells were cultured for 6 months,

passed once a week and then subjected to real time bioluminescence assay (c part of Figure 5.11). This data proved that cells do not lose circadian oscillation. Finally, the data were analysed, and the period of circadian oscillation is calculated using Biodare2 webtool (d part of Figure 5.11). While the C16-D3-CB3 clone exhibited a similar period with control cells when first obtained and even 6 months later, the deviation in the period in the *BMAL*⁻ state of these cells was shown on the graph.

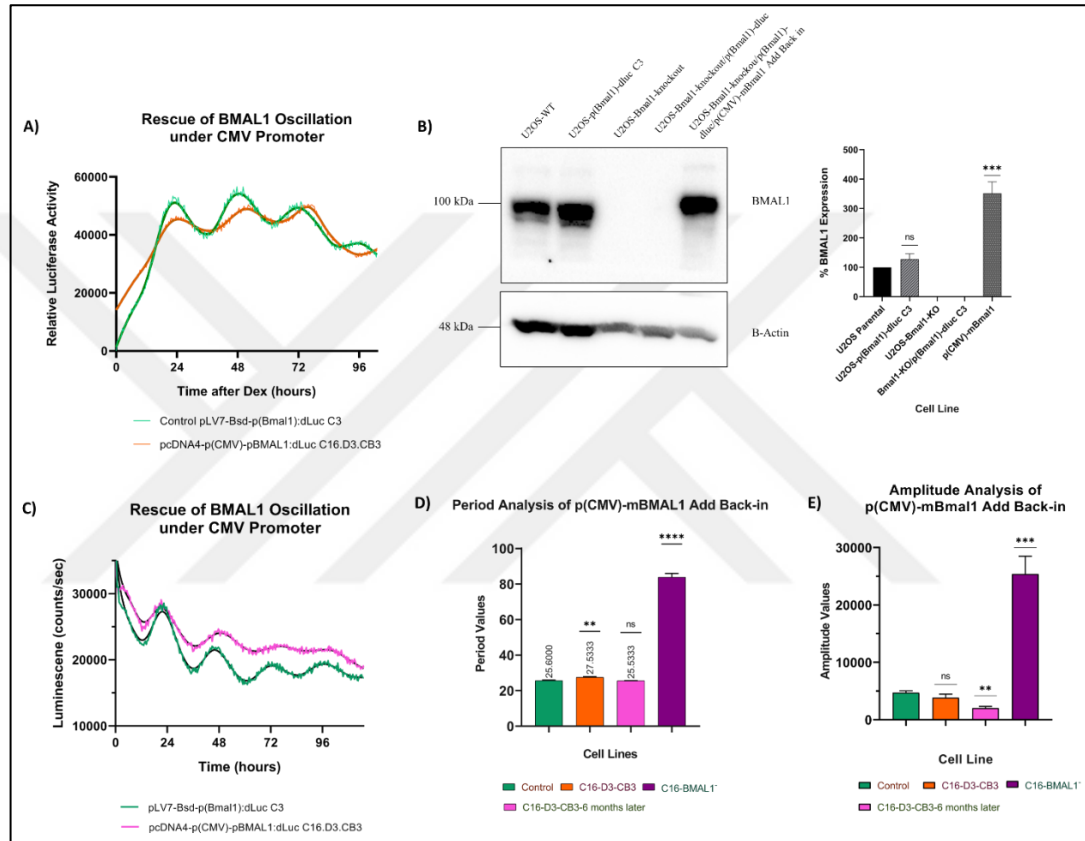


Figure 5.11: Rescue of circadian oscillation by *Bmal1* add back-in expressed under the CMV Promoter. A) Bioluminescence data of rescued oscillation. B) Immunoblotting of *Bmal1* add back-in expressed under the CMV Promoter. C) 6 months after rescue of circadian oscillation under CMV promoter. D) Period analysis and E) amplitude analysis with Biodare2 webtool. Data were baseline normalized in bioluminescence data representation. Input data were analysed with Amp&baseline detrending method. Period analysis method was adjusted to FFT NLLS option. Period values were compared with control cell line by unpaired student's *t*-test (two-tailed, * $p < 0.01$). Data are mean $\pm$ s.e.m.; $n=3$ replicated in the experiments. kDa: kilodalton, C: clone, B: *Bmal1*⁺ add back-in clone number.

5.3.4. Rescue of Circadian Oscillation through *Bmal1* Add Back-in under the Native Promoter

Circadian oscillation recovery with *Bmal1* expressed under the CMV promoter was a step to be improved due to the scarcity of positive clones obtained. Instead of rescuing with a promoter that is constantly active in the TTFL mechanism, the use of a native promoter would have allowed this system to work more smoothly. The CMV promoter sequence in the pcDNA.4-p(CMV)-m*Bmal1*-3xFlag-6xHis C3 construct was removed and the native *Bmal1* promoter was cloned instead. The recombinant constructs obtained were subjected to RE analysis (Figure 5.12) and then screened at protein level by immunoblotting in HEK-293T cells (Figure 5.13).

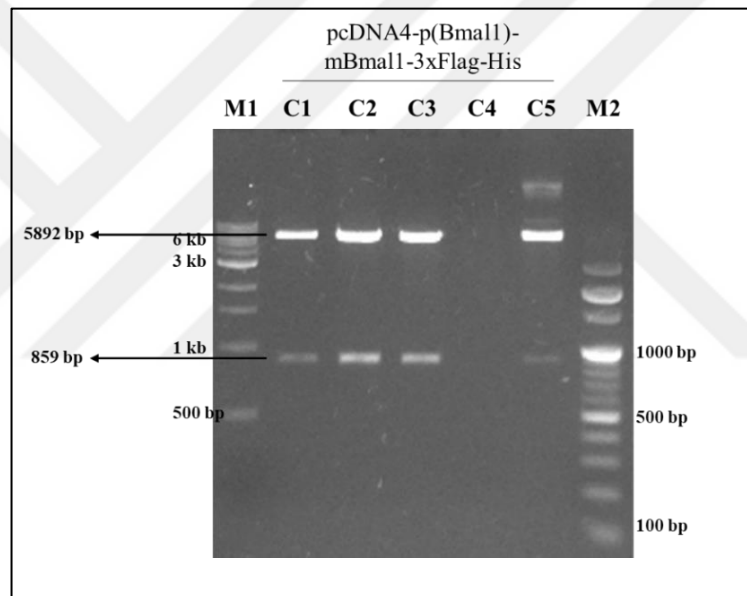


Figure 5.12: Restriction endonuclease analysis of construction pcDNA4-p(m*Bmal1*)-m*Bmal1*-3xFlag-6xHis with *Kpn*I. M: marker (M1 is 1 kb, M2 is 100 bp DNA markers), C: clone, bp: base pair.

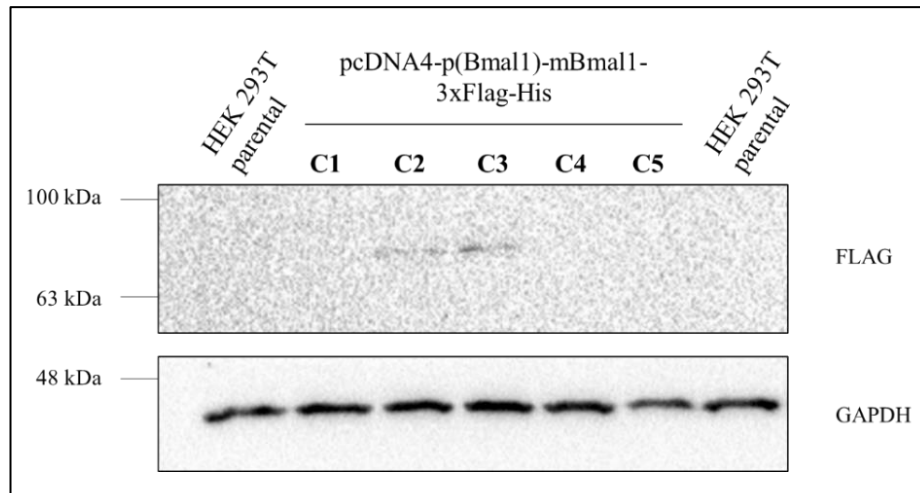


Figure 5.13: Immunoblotting of pcDNA.4-p(mBmal1)-mBmal1-3xFlag-6xHis constructs with anti-FLAG antibody. kDa: kilodalton, C: clone.

Since the constructs were transfected into HEK-293T cells 24 hours after the cells were harvested and proteins were isolated, weak expressions were detected. However, better expression was detected in the C3 clone compared to the others. For this reason, the C3 clone was selected for further studies.

The selected pcDNA.4-p(Bmal1)-mBmal1-3xFlag-6xHis C3 was transfected into U2OS-BMAL1^{-/-}-p(Bmal1)-dluc cells and polyclonally selected with antibiotic for 1 week followed by monoclonal isolation. The resulting Bmal1 stably expressed cells were confirmed by immunoblotting in the presence of negative controls (Figure 5.14).

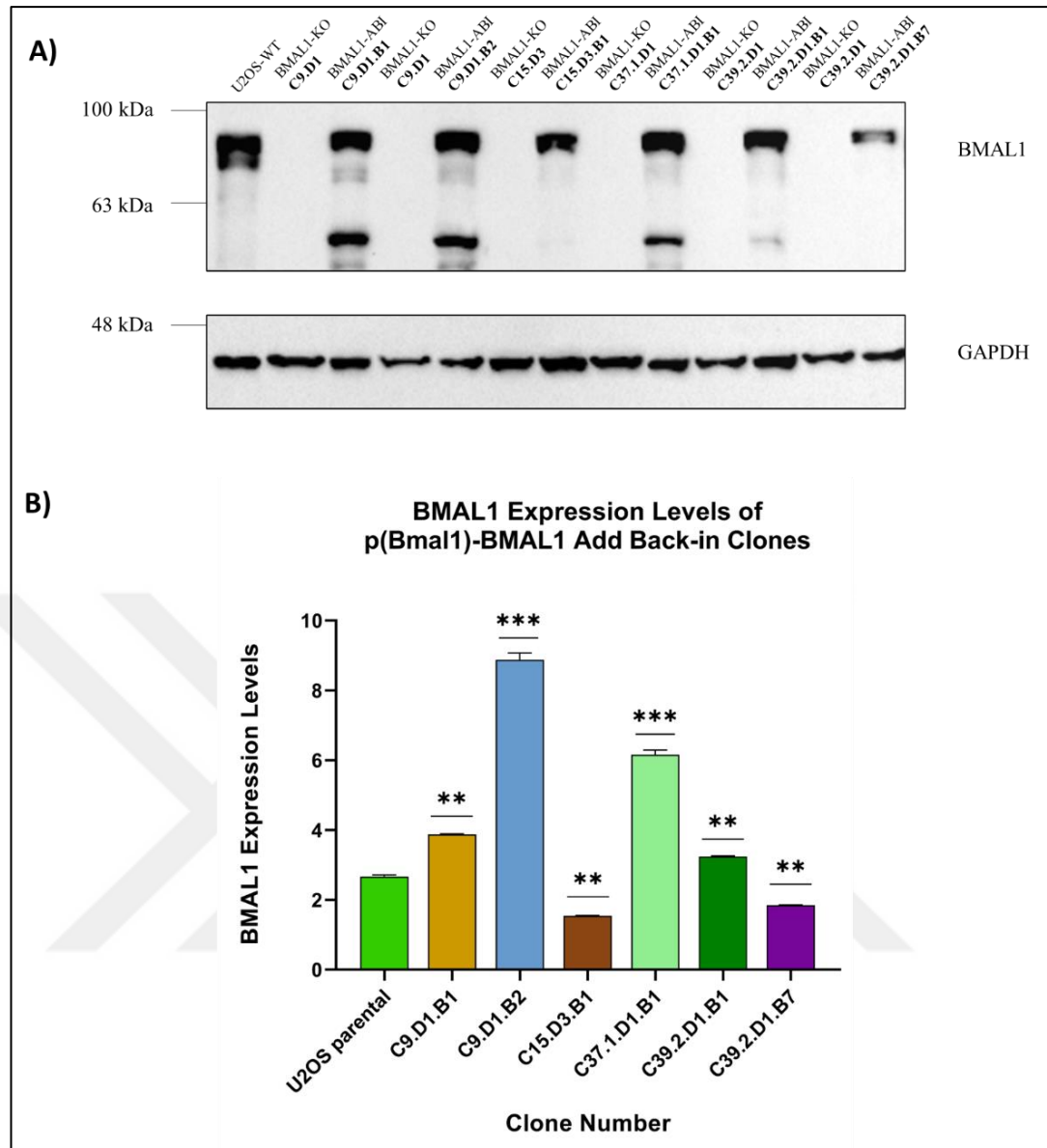


Figure 5.14: A) Immunoblotting of BMAL1 whose U2OS *Bmal1* add back-in expressed under the native promoter. B) Analysis of immunoblotted BMAL1 at p(Bmal1)-U2OS-*Bmal1*⁺ add backed-in cells. KO: knockout, WT: wild type, C: clone, ABI: *Bmal1*⁺ add back-in clones, D: *dluc* reporter clones, kDa: kilodalton.

Circadian oscillation rescue was monitored in *Bmal1*⁺ clones whose presence was detected at protein level by Western blot analysis. In the real time bioluminescence experiment, the number of repetitions was increased to maximize reliability. In this experiment, the control cell line U2OS-pLV7-Bsd-p(Bmal1)-*dluc* cells were seeded in 10 wells of an opaque white luminometric 96-well cell plate and each was included in the analysis (Figure 5.15).

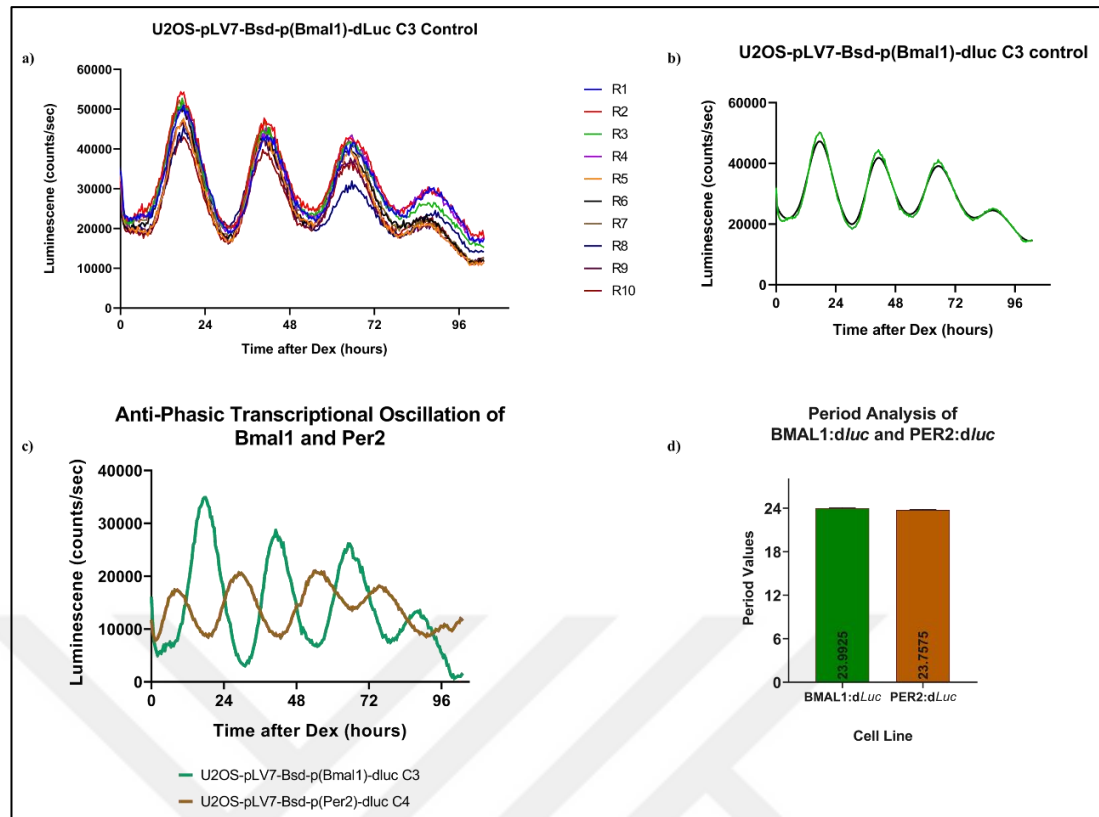


Figure 5.15: Circadian oscillation belongs to control cell line U2OS-pLV7-Bsd-p(Bmal1)-dLuc cells. a) represents each replicate individually. b) The overall circadian oscillation of the control cells, where each oscillation obtained from 10 different wells is evaluated as one repetition and combined into a whole. c) Normalized data of anti-phasic transcriptional oscillation of *BMAL1* and *PER2*. d) Period analysis of control cell line U2OS-pLV7-Bsd-p(Bmal1)-dLuc cells and U2OS-pLV7-Bsd-p(Per2)-dLuc, with Biodare2 webtool. Input data were analysed with Amp&baseline detrending method. Period analysis method was adjusted to FFT NLLS option. R: replicate, C: clone, p: promoter. (Data are mean $\pm$ s.e.m.; n=10 replicated in the experiments).

On the other hand, circadian oscillation recovery was investigated in U2OS-*Bmal1*⁺ cells. U2OS *BMAL1*^{WT}-dLuc, U2OS-*BMAL1*^{-/-}-dLuc and U2OS-*Bmal1*⁺-dLuc cells (Figure 5.16, Figure 5.17). The raw data from the Synergy H1 microplate reader of the control group and the clone from which the oscillation was rescued were written on the different two columns together with the replicates. These two data sets were averaged separately, and the mean was subtracted from each specific column value. The smallest value in the new data set (both of control and experimented) was determined and the absolute value of this value was added to each data set to normalize data according to baseline normalization. Period analysis show that all positive U2OS-*Bmal1*⁺-dLuc clones have the closely similar period values to the

control cell line (“a” part of Figure 5.18). However, the amplitude values of each clone are quite different from each other and from the control cell (“b” part of Figure 5.18). The highest amplitude values belong to C9-D1-B1 and C9-D1-B2. Even after 24 hours, the luminescence in C9-D1-B1 was damped due to the lack of luciferin in the medium. The most damped ones are C15-D3-B1 and C36-D1-B4. Clones C37.1-D1-B1 and C39.2-D1-B1 exhibit oscillations (at amplitude and period level) closest to the control cell (Figure 5.18). As expected, the add back-in system of *Bmal1* expressed under the native promoter was detected to be stronger than the add back-in system of *Bmal1* expressed under the CMV promoter.

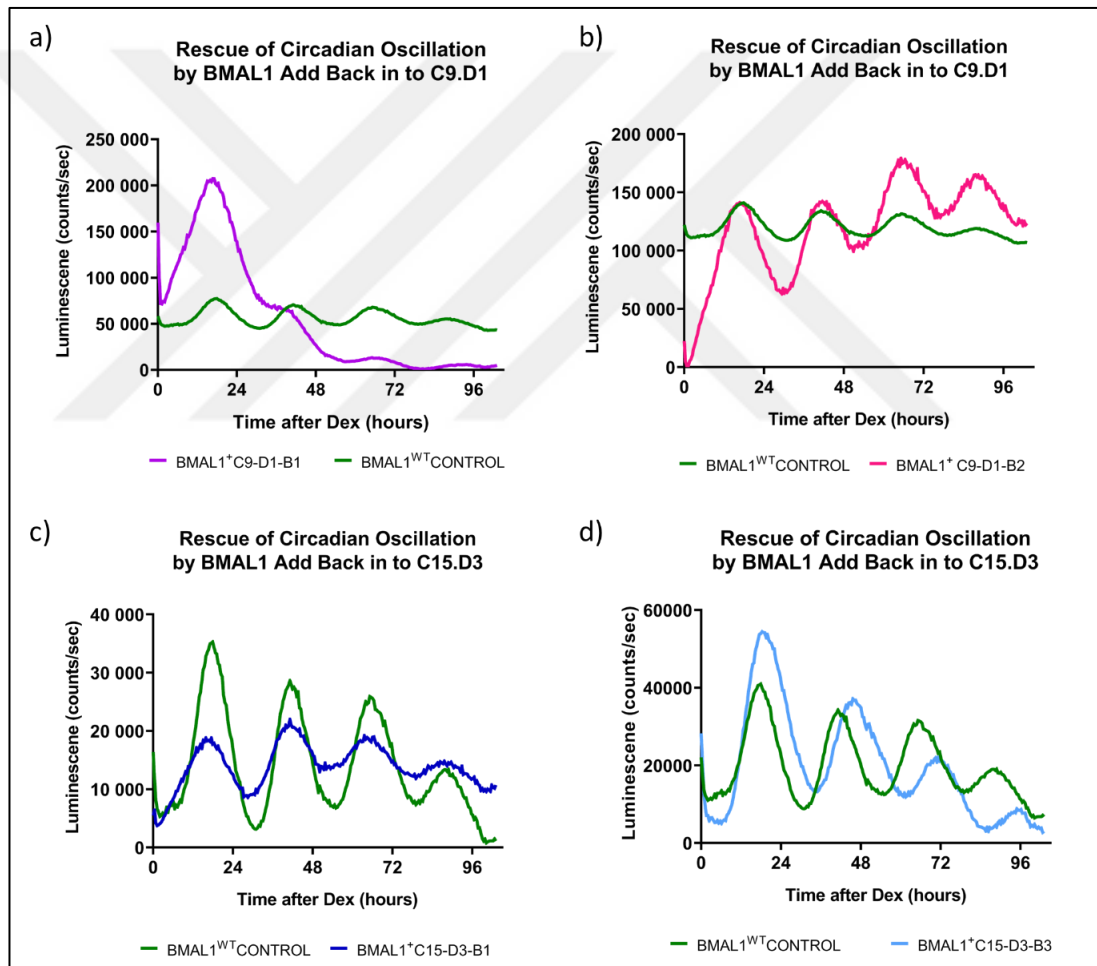


Figure 5.16: Rescue of circadian oscillation by *Bmal1* add back-in expressed under native Promoter for clone C9.D1.B1, C9.D1.B2, C13.D3.B1 and C15.D3.B3. B: *Bmal1*⁺ add back-in clone number, C: clone. (Data are mean $\pm$ s.e.m.; n=3 replicated in the experiments).

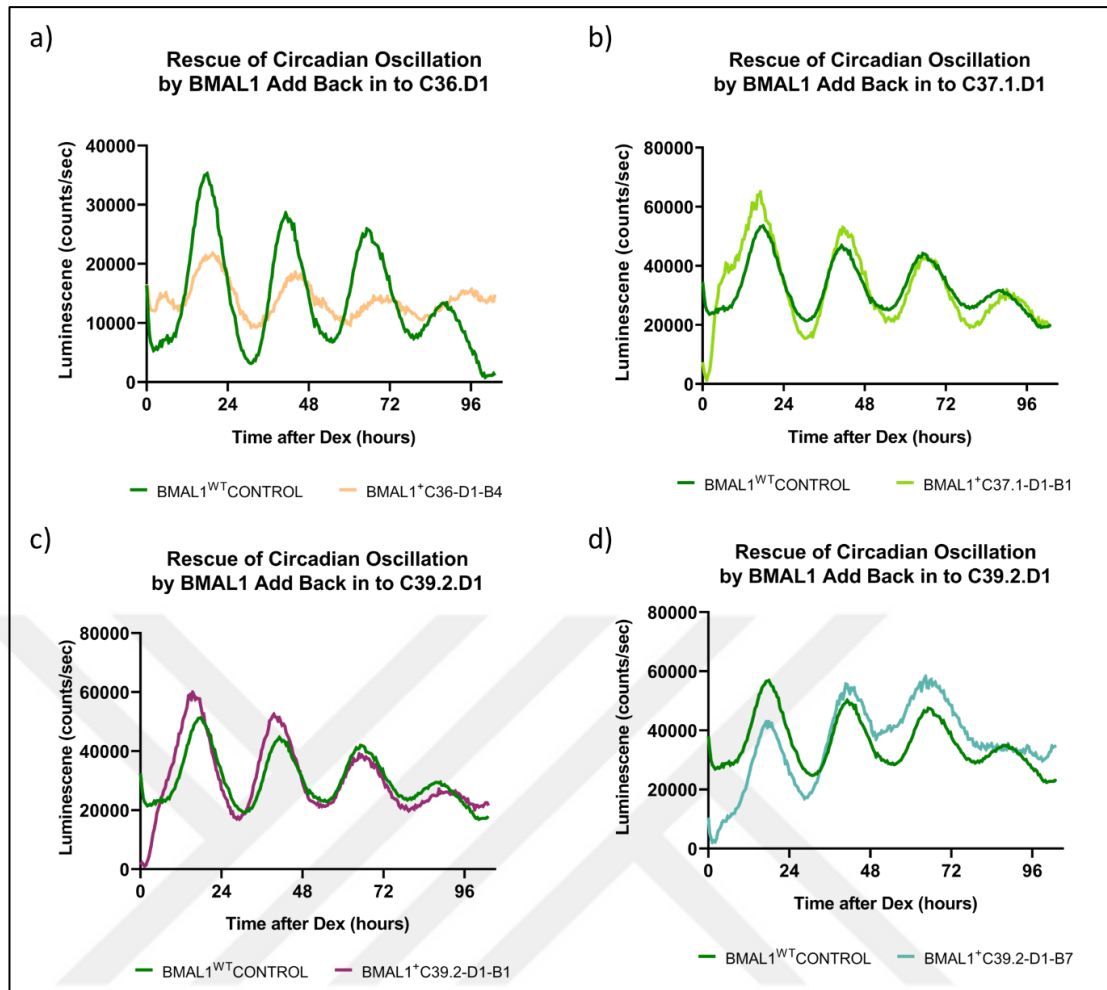


Figure 5.17: Rescue of circadian oscillation by *Bmal1* add back-in expressed under native promoter for clone C36.D1.B4, C37.1.D1.B1, C39.2.D1.B1 and C39.2.D1.B7. B: *Bmal1*⁺ add back-in clone number, C: clone. Data are mean $\pm$ s.e.m.; n=3 replicated in the experiments.

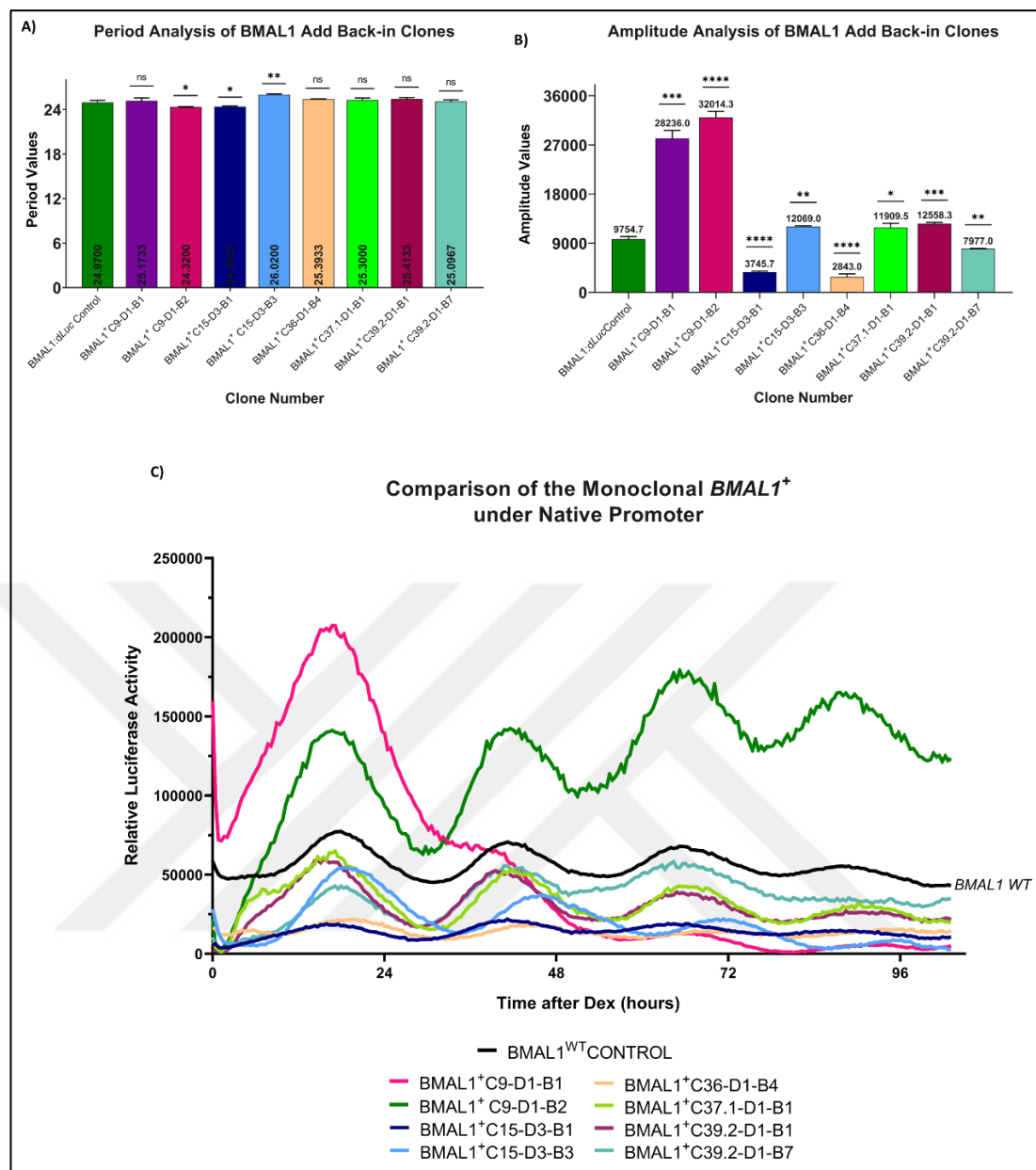


Figure 5.18: A) Period and B) amplitude values of *Bmal1* add back-in expressed under native promoter. C) Raw data of 8 clones which are oscillation rescued by *Bmal1* add back-in system under native promoter plotted with GraphPad Prism 8. Period analysis was performed with Biodare2 webtool. Input data were analysed with Amp&baseline detrending method. Period analysis method was adjusted to FFT NLLS option. Period and amplitude values were compared with control cell line by unpaired student's *t*-test (two-tailed, $*p < 0.01$). B: *Bmal1*⁺ add back-in clone number, C: clone. Data are mean $\pm$ s.e.m.; n=3 replicated in the experiments.

In this section, it was also compared the *BMAL1* native promoter activity with CMV promoter activity. Recall that in systems with *Bmal1* add back-in under two different promoters, the circadian oscillation was monitored by the activity of *dLuciferase* cloned under the *BMAL1* promoter. C37.1-D1-B1 clone which oscillation recovery

observed under native promoter was chosen for comparison the oscillation rescue with different promoter activity systems. Circadian oscillation data from 3 different cell lines were shown on “a” part of Figure 5.19. Oscillation was rescued in both systems. It seems that when the two promoter activities are compared, a significant difference in amplitude values is observed (“c” part of Figure 5.19) whereas period values were the same with the control cell line (“b” part of Figure 5.19). Data were analysed with unpaired student’s *t*-test with statistical confidence value $*p < 0.01$ two-tailed.

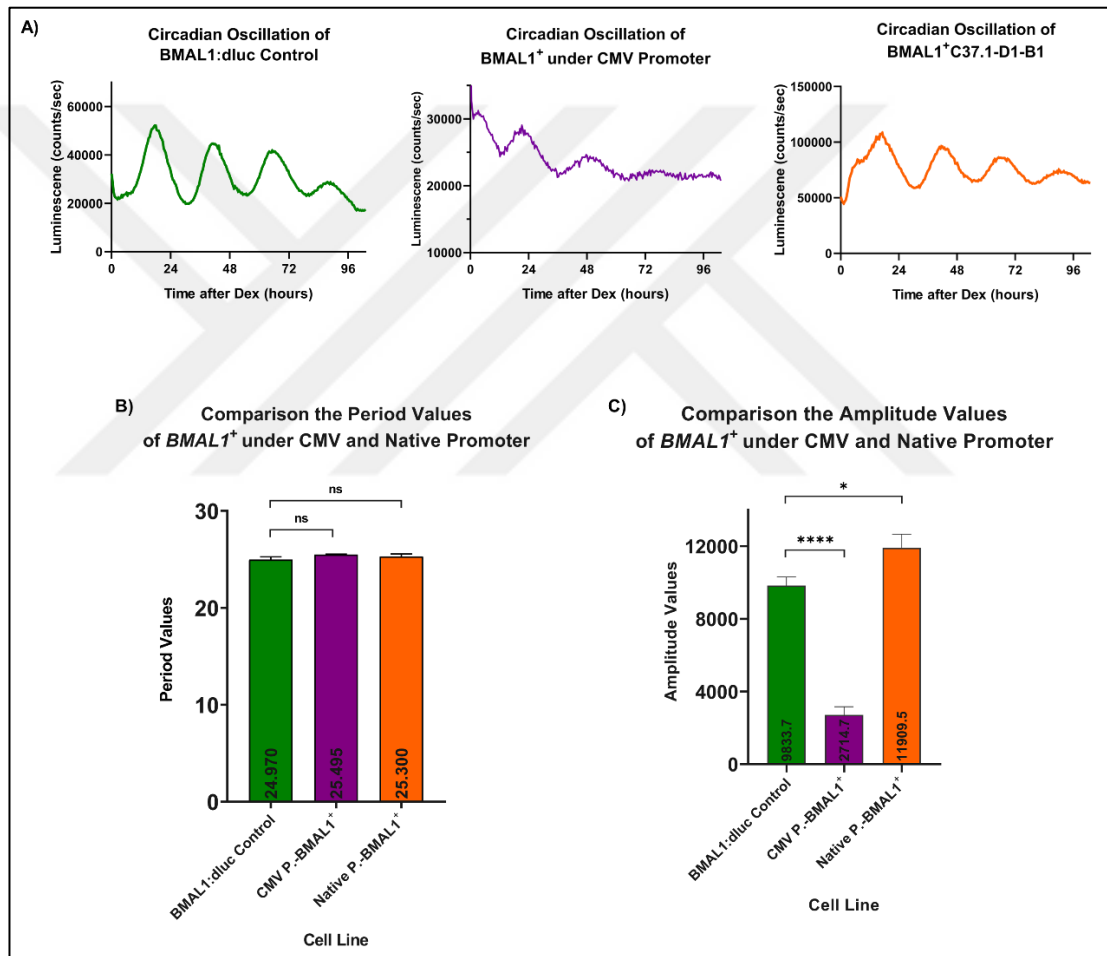


Figure 5.19: Comparison of *Bmal1* add back-in expressed under CMV and native promoter. a) Normalised data of *BMAL1* oscillation from U2OS-*BMAL1*^{WT}, U2OS-*Bmal1*⁺ under CMV promoter and U2OS-*Bmal1*⁺ under native promoter. b) Period analysis of circadian oscillation data. c) Amplitude analysis of circadian oscillation data. C: clone, P: promoter. Period and amplitude values were analysed with unpaired student’s *t*-test. (ns, non-significant), $p \leq 0.01$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***), $p \leq 0.00001$ (****). Data are mean $\pm$ s.e.m.; n=3 replicated in the experiments.

5.3.5. Investigation of Circadian Oscillation at the Bmal1 Protein Level

BMAL1 promoter activity in U2OS-*Bmal1*⁺ add backed-in cells was monitored by real time bioluminescence assay and rescue of circadian oscillation was demonstrated. Based on the circadian oscillation representing the day and night cycle, Bmal1 protein oscillates maximally at 12 am during the day and minimally at 12 pm during the night. For the oscillation rescued clones, after cells were reset with Dexamethasone, cells were harvested at CT36 (subjective day) and CT48 (subjective night) and proteins were isolated.

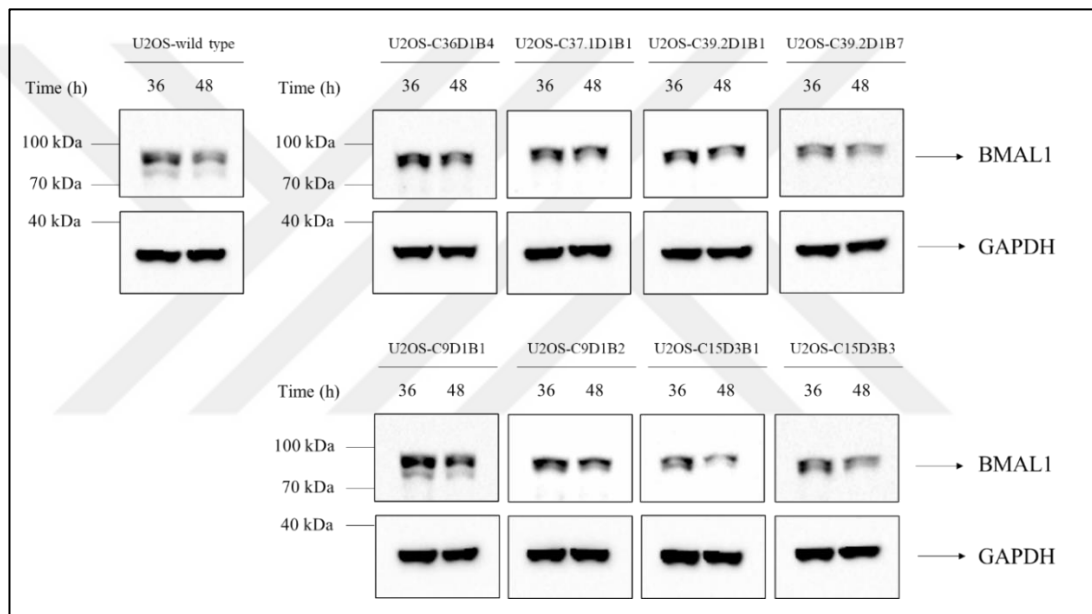


Figure 5.20: Immunoblotting of Bmal1 oscillation at CT36 and CT48 at the protein level on U2OS-*Bmal1*⁺ add backed-in cells. CT: circadian time, kDa: kilodalton, h: hour, C: *BMAL1*^{-/-} clone number, B: *Bmal1*⁺ add back-in clone number, D: *dLuc* reporter clone number.

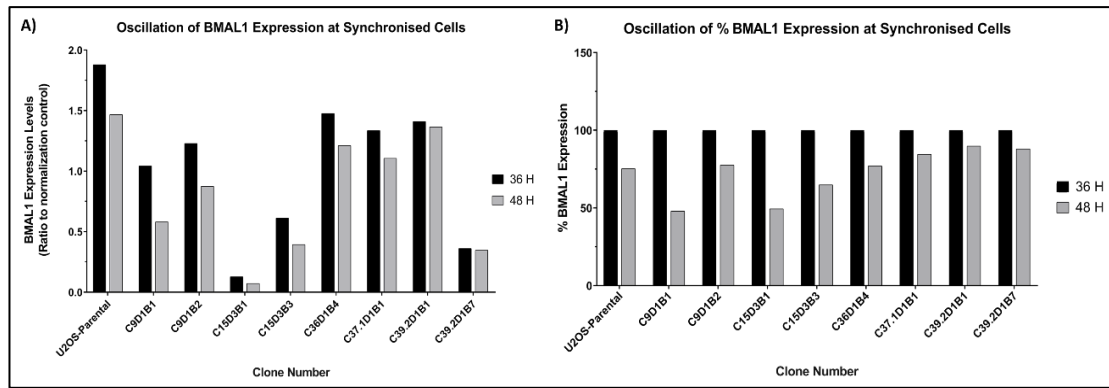


Figure 5.21: A) Analysis of immunoblotted Bmal1 oscillation at CT36 and CT48 at the protein level on U2OS-*Bmal1*⁺ add backed-in cells. B) CT36 of each clone was considered to have 100% Bmal1 expression and each clone was analysed on a pair-wise basis. CT: circadian time, kDa: kilodalton, h: hour, C: *BMAL1*^{-/-} clone number, B: *Bmal1*⁺ add back-in clone number, D: *dLuc* reporter clone number.

Protein samples were run on SDS-PAGE and immunoblotted with anti-BMAL1 antibody to show the difference in Bmal1 protein level in hours representing day and night (Figure 5.20). In each clone a difference between CT36 and CT48 was detected, as in U2OS-*BMAL1*^{WT} control cells (Figure 5.21).

5.3.6. Determination of Added-back in Bmal1 Functionality

Bmal1 functionality was tested in U2OS-*Bmal1*⁺ cells demonstrated for circadian oscillation rescue. The interaction between CLOCK and BMAL1, known to be interaction partners in the molecular TTFL mechanism, was investigated by co-immunoprecipitation method. Functionality test was not applied to all clones, for which clones C37.1.D1.B1 and C39.2.D1.B1 were selected for validation of protein functionality (Figure 5.22).

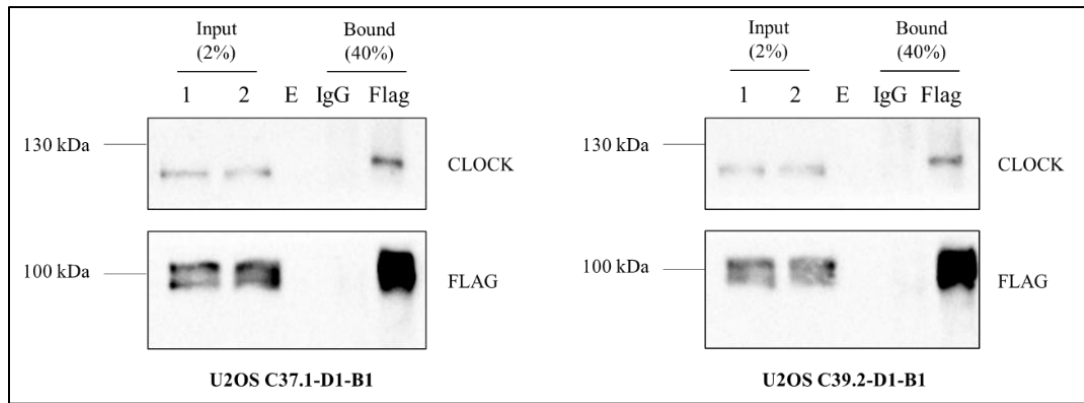


Figure 5.22: Determination of the functionality of re-inserted Bmal1 by co-immunoprecipitation method. kDa: kilodalton, IgG: immunoglobulin G.

In U2OS-*Bmal1*⁺ cells, mBmal1-FLAG was immunoprecipitated using anti-FLAG antibody and the presence of CLOCK, a known interaction partner of BMAL1, was evaluated with immunoblotting. When the presence of CLOCK was also detected in the samples in which mBmal1-FLAG was immunoprecipitated, Bmal1 and CLOCK interaction was confirmed and Bmal1 functionality was proven.

5.3.7. Polyclonally Rescue of Circadian Oscillation through *Bmal1* Add Back-in under the Native Promoter

The overall scope of this tool, designed for use in circadian research, was based on monoclonal cell isolation. Subsequently, we tested oscillation rescue in the polyclonal cell community by virally *Bmal1* add back in to U2OS- *BMAL1*^{-/-} cells followed by antibiotic selection. First, the sequence p(Bmal1)-mBmal1-3xFlag-6xHis was cloned into the pLenti III-no promoter-HA vector (Figure 5.23).

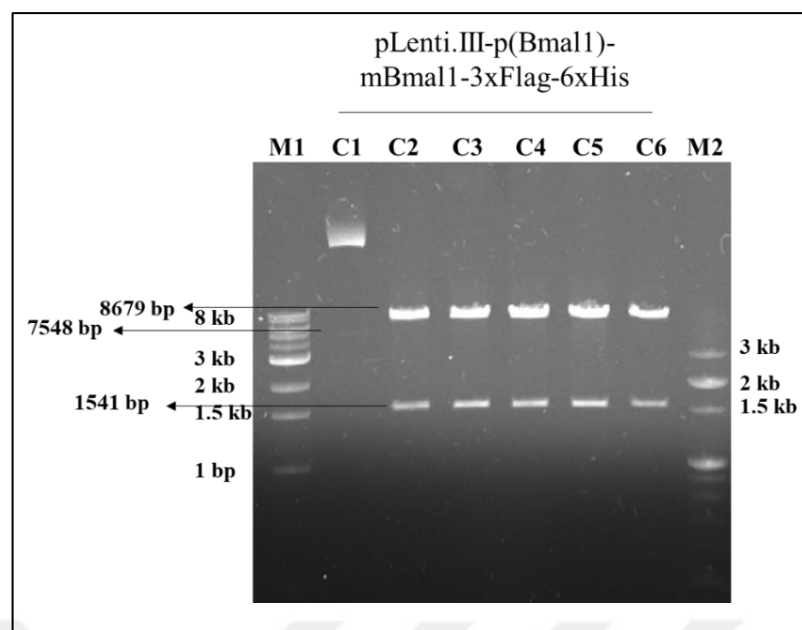


Figure 5.23: Restriction endonuclease analysis of Native *Bmal1* promoter construction pLentiIII-p(mBmal1)-m*Bmal1*-3xFlag-6xHis with *Stu* I. M: marker (M1 is 1 kb, M2 is 100 bp DNA markers), C: clone, bp: base pair, kb: kilobase pair.

The resulting positive clones C2, C3, C4, C5 and C6 were then screened for expression in HEK-293T cells by immunoblotting (Figure 5.24). Since C3 gave the most efficient result at the expression level, the C3 clone was selected for further studies.

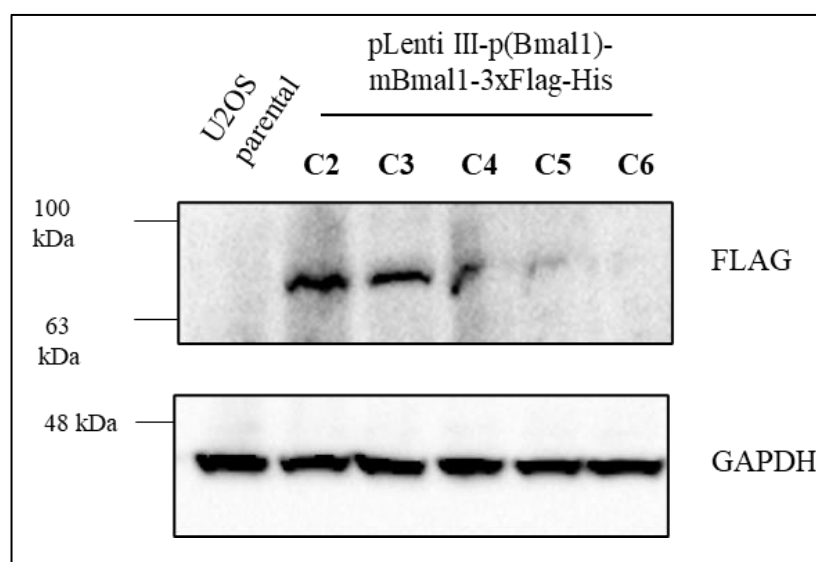


Figure 5.24: Control of pLentiIII-p(mBmal1)-m*Bmal1*-3xFlag-6xHis construction at protein level on HEK-293T cells. kDa: kilodalton, C: clone, p: promoter.

U2OS-*BMAL1*^{-/-} cells seeded in 12-well cell culture plates were transduced with virus particles containing pLenti III-p(*Bmal1*)-m*Bmal1*-3xFlag-6xHis and subjected to puromycin antibiotic selection 24 hours after transduction. Previously, puromycin resistance was conferred to the cells during *BMAL1* knockout generation and unfortunately, the pLenti III-no promoter-HA vector we had also contained the puromycin selectable marker. In this case, to select positive cells, the antibiotic was used at a concentration of 5 µg/mL according to the literature and their MTT assay results (Hu et al., 2020). Antibiotics were changed every 2 days for 4 weeks and the cell pool was passaged at very low confluency to eliminate cells without *Bmal1* re-insertion. The resulting polyclonally m*Bmal1* stably expressed cells were confirmed by immunoblotting in the presence of negative controls (Figure 5.25).

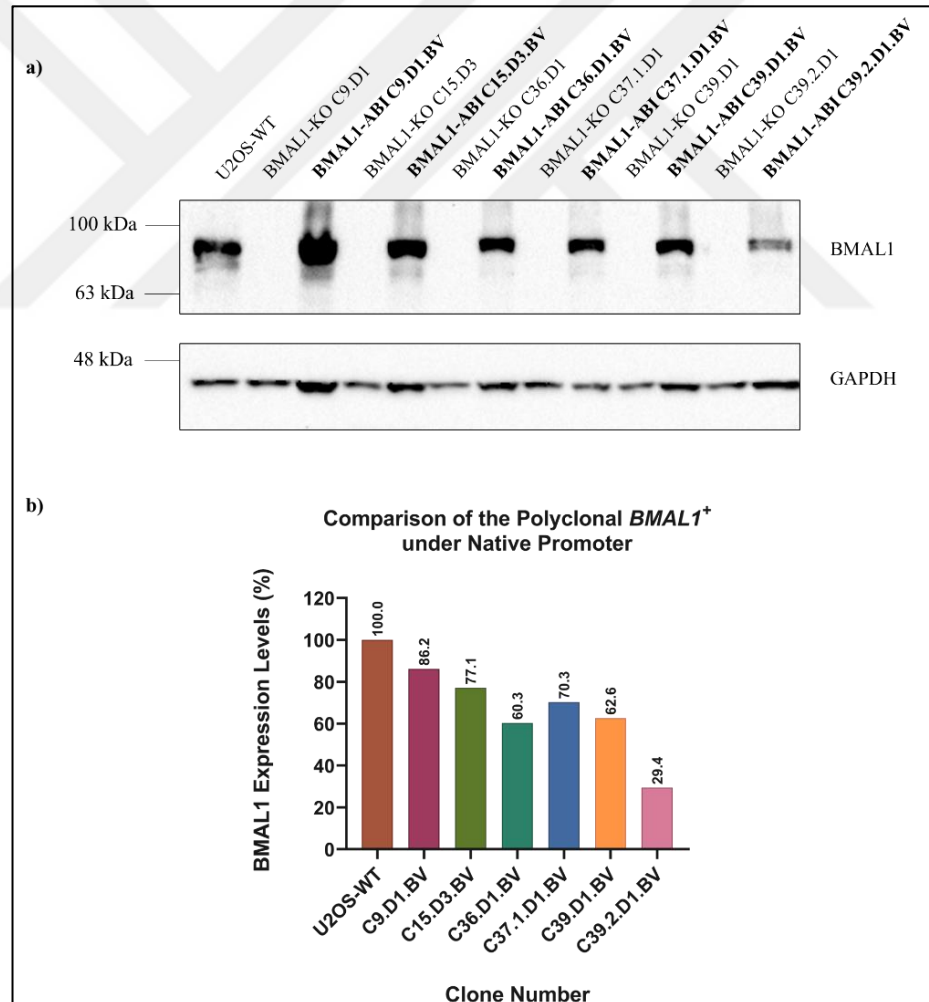


Figure 5.25: a) Immunoblotting of *Bmal1* whose U2OS *Bmal1* add back-in expressed under the native promoter without monoclonal isolation. b) Analysis of *Bmal1* expressions normalized with GAPDH. KO: knockout, WT: wild type, C: clone, BV (bold): *Bmal1*⁺ add back-in cells via virus transduction, D: d*Luc* reporter clones, kDa: kilodalton.

Finally, the resulting *Bmal1*⁺ containing polyclonal cell pool was subjected to real time bioluminescence assay to check circadian oscillation rescue (Figure 5.26). The circadian oscillation rescue was observed on polyclonally selected *Bmal1*⁺ cells then, the system will be a powerful tool to study circadian oscillation.

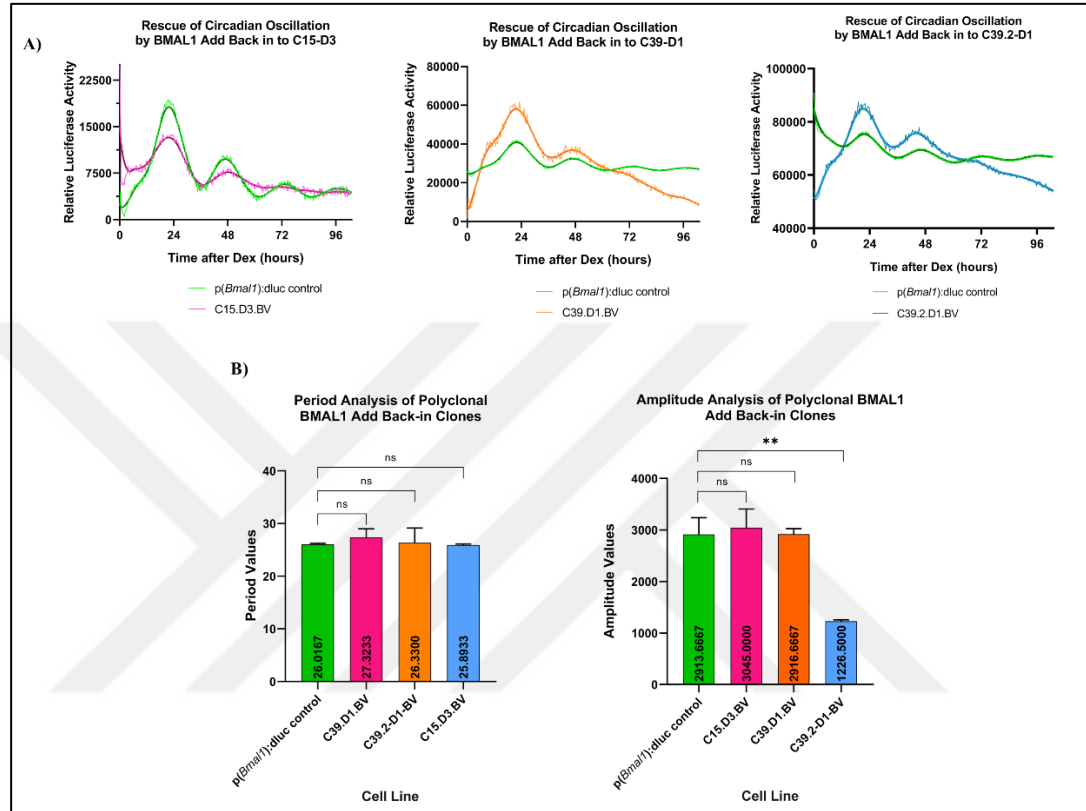


Figure 5.26: Rescue of circadian oscillation by *Bmal1* add back-in expressed under native promoter in polyclonal cells. a) real-time bioluminescence result baseline normalized with control cells. b) period analysis of *Bmal1*⁺ add back-in cells, C: clone, BV: *Bmal1* add back in via viral transduction selected polyclonally. Data were analysed by unpaired student's *t*-test (two-tailed, **p* < 0.01). Data are mean ± s.e.m.; n=3 replicated in the experiments.

However, due to the pre-existing resistance conferred to these cells by the selected antibiotic, the selection process could not be executed perfectly. Therefore, it was decided to utilize the data from the initial 72 hours of oscillation. Nevertheless, the methodology used proved that the circadian oscillation disrupted in *BMAL1*^{-/-} cells can be restored by *mBmal1* add back in expressed under the native promoter and without monoclonal isolation.

6. DISCUSSION

For years, the characterization of specific gene mutations occurring in DNA and the investigation of their outcomes have been the subject of research. This research contributes not only to fields directly impacting human health, such as genetic research and medicine or diagnosis, but also to scientific knowledge by tracking changes in biological processes involving the expression of these genes. It is a matter of serious curiosity what the consequences might be of mutations in key genes involved in the biological clock, which regulates various physiological and behavioural activities such as the sleep-wake cycle and metabolic processes, along with many other yet unknown controls.

In this thesis, considering the finding that *BMAL1* inactivation disrupts the circadian oscillation (Bunger et al., 2000; Padlom et al., 2022), we show once again that only *BMAL1* gene inactivation in U2OS cells indeed abolishes circadian oscillation (Figure 5.6). We preferred to use three different targeted sgRNAs and combined them in various combinations to obtain more powerful gene knockouts (Figure 5.3). Among these, T6 and T7 cut after the stop codon, while T8 cuts before the first ATG. Therefore, we paired T6 with T8 and then T7 with T8 to have targeted sgRNAs both at the beginning and at the end of the *BMAL1* sequence. The CRISPR/Cas9 construct used in this process was attempted to be introduced into the cells by both transfection and transduction. As expected, the transduction method, in which we used lentivirus, which has the feature of integration of DNA into the target cell genome, gave much more effective results than the transfection method, which usually provides transient gene expression (Figure 5.2).

BMAL1 knockout clones and parental U2OS cells, which will be used as oscillatory control in subsequent studies, were transduced with pLV7-Bsd-p(*Bmal1*)-*dluc* viruses to obtain stably expressing *luciferase* under the *BMAL1* promoter to enable monitoring of circadian oscillation. *BMAL1:dluc* cells were obtained in step 2 because it was desirable to protect them from the CRISPR/Cas9 target made in the first step. If *BMAL1:dluc* cells had been obtained first, the reporter could have been deleted by sgRNAs targeting the nearby of *BMAL1* promoter regions. It means that, if we introduced a reporter system at the beginning and then tried to get a *BMAL1* knockout, there would be 2 levels of control: first, was a successful knockout

achieved and second, is the reporter system still functional. To avoid this, we completed the knockout studies first and then the only thing that needed to be checked was the presence of the reporter.

Real-time bioluminescence experiments in *BMAL1*^{-/-} clones in which circadian oscillation was lost revealed a steadily increasing *BMAL1* promoter activity. In other words, *BMAL1*^{-/-} produced a substantial increase in *BMAL1* promoter activity (Figure 5.6 B and D) A study with similar findings suggests that the sustained and increased activity of the *BMAL1* promoter upon knockdown of *BMAL1* is attributed to the absence of a positive element in the circadian model (Baggs et al., 2009). Moreover, the knockdown of *BMAL1* or *CLOCK* leads to a dose-dependent disruption of circadian rhythms and functional defects, resulting in increased levels of the *BMAL1*-luciferase reporter (Baggs et al., 2009). When period analysis was performed on these cells, each of them showed abnormal values according to *BMAL1*^{WT}(Figure 5.6 E). From our point of view the absence of the CLOCK:BMAL1 transcriptional activator complex suggested that REV-ERBs, one of the CCGs that binds to the RRE region of *BMAL1* promoter and inhibits it, is highly controlled by CLOCK:BMAL1 complex, whereas the positive activator of BMAL1 expression, RORs, is also tightly controlled with other transcriptional activators independent of the CLOCK:BMAL1 complex.

Subsequently, we performed dual luciferase reporter assay to better understand transcriptional function of BMAL1. When we transiently transfected the *mPer1:dluc* reporter, sport6-*CLOCK*, “except *BMAL1*“ into each of *BMAL1*^{-/-} cell clones and subclones exhibiting disrupted circadian oscillations, we did not detect any significant change in reporter activity in unsynchronized cells (Figure 5.7 and Figure 5.8). When parental U2OS cells containing endogenous *BMAL1* were transiently transfected with increasing concentrations of *CLOCK*, then *mPer1:dluc* activity was increased in a dose-dependent manner. Furthermore, *BMAL1*^{-/-} cells were transiently transfected with sport6-Clock, and pcDNA4-*BMAL1*. This time we showed that overexpression of BMAL1 and CLOCK enhanced *mPer1-dluc* activity as previously shown (Cal-Kayitmazbatir et al., 2021) and *mPer1-dluc* enhancement in *BMAL1*^{-/-} cells as in parental U2OS cells (Figure 5.9 and Figure 5.10). In *BMAL1*^{-/-} cells, we demonstrated that dose-dependent increase of *CLOCK* alone could not enhance *mPer1-dluc* activity, whereas *mPer1-dluc* activity was enhanced in cells in which

CLOCK and *BMAL1* were together. Even, concentration of *mBmal1* increase resulted in transcriptional *mPer1-dluc* enhancement in a dose-dependent manner. We detected a high level of *mPer1-dluc* activity in some clones, although it is not dependent on Clock increase and not significant (e.g. Clone 36). However, in the parental cell, the increase in *mPer1-dluc* reporter activity induced by Clock increase is at least 3-fold greater, indeed, it was increased dose-dependently. We speculate that the excess of *mPer1-dluc* activity in these cells may be due to the different genetic structure of each clone, leading to variations in the way clock transactivation affects *mPer1-dluc* expression. Genetic variations in *BMAL1*^{-/-} U2OS cells may lead to different responses of *CLOCK* to different genetic bindings resulted in various *Per1* promoter activity which are different from each other.

Next, we attempted to rescue the oscillation by enabling the re-insertion of *Bmal1* stably expressed under two different promoters constitutive expression promoter (CMV) (Figure 5.11) and native *Bmal1* promoter (Figure 5.16 and Figure 5.17). Our findings revealed that we were able to rescue the circadian oscillation with *Bmal1* under a constitutive expression promoter, but the oscillation rescued with *Bmal1* under the native promoter was much more successful although, some studies conclude that rhythmic clock gene expression is not essential for the circadian clock (Liu et al., 2008). The rescue of oscillation is most likely related to the fact that the cellular presence of *BMAL1* is important for circadian rhythmicity, which is more suitable for the TTFL model but, *Bmal1* cloned under the CMV promoter was successful in oscillation rescue (although the proportion of positive clones obtained was very low), even though its protein level was approximately 3-fold higher than in parental cells. Also, the robustness of the core clock loop in the absence of cyclic *BMAL1* (as possible as low or high) is maintained by the regulation of transcriptional and post-translational mechanisms of *PERs* and *CRYs*, including protein cycling and synchronous nuclear translocation. Based on our results, we also detected rhythmic oscillations in cells with higher *Bmal1* protein abundance than parental cells (clone C9-D1-B2). Moreover, the period of these oscillations is similar to control cells. We found that the amplitude (indicating promoter activity) was also generally high in clones with high protein levels. It is normal that the luciferase activity depends on the *p(Bmal1)-dluc* number inserted into the genome, so amplitude values is possibly different each other. According to the immunoblotting

results, perhaps the minimum level of BMAL1 at the protein level may be important for oscillation, but the difference in protein level in the *Bmal1* add back-in clones we obtained with a 4-fold increase compared to the control (C9-D1.B2 as an example) did not prevent oscillation rescue.

Moreover, even the oscillation patterns rescued by re-insertion of *Bmal1* under the native promoter were not exactly identical even they were similar to each other and re-insertion of *Bmal1* under the CMV promoter. When period values were compared, there were no significant difference between CMV and native promoter and both of them exhibits such as approximately 25 hours (C37 was selected and figured as a native promoter oscillation rescue model). The period values of the clones obtained by *Bmal1* re-insertion, which are under the native *BMAL1* promoter, are already similar and approximately 25 hours (Figure 5.18 A). Nevertheless, the period values in some clones seem to have changed significantly compared to the control according to the student's *t*-test, but the variance between replicates and clones is almost similar and therefore very small changes are considered significant by statistical analysis. Also, Biodare2 algorithm tries to fit it using a Fourier transform and uses the signal inputs and decomposes them into their components, which allows it to detect false periods in the bioluminescence data, such as background or contaminations due to the low resolution of the Synergy H1. Nevertheless, we preferred to analyse and present the raw data without setting a cutoff value, in which case the biological significance should be questioned. In fact, the period of the oscillation rescued by *Bmal1* under the CMV promoter was approximately 27-hour, whereas it was 25-hour in subsequent readings. Again, the low resolution of Synergy H1 reduces the likelihood of getting exactly the same numerical results. For example, control cells have period values between 24-25 hours.

On the other hand, when amplitude values were considered, we found a considerable difference in almost all clones compared to control cells subjected to the same experiment (Figure 5.18 B). Either *Bmal1* add back-in clones dampen, or some of them heighten the amplitude. The highest amplitude values belong to C9-D1-B1 and C9-D1-B2. Even after 24 hours, the luminescence in C9-D1-B1 was damped due to the lack of luciferin in the medium. Oppositely, immunoblotting results was showed that C9-D1-B1 is not the most *Bmal1* expressed cell clone. According to this result, although promoter activity is very high, protein abundance may not be high in the

same direction. Perhaps in clone C9-D1-B1, although the promoter activity is very high, the exogenously expressed Bmal1 stability is much lower. The most damped *Bmal1* add back-in clones are C15-D3-B1 and C36-D1-B4. Clones C37.1-D1-B1 and C39.2-D1-B1 exhibit oscillations closest to the control cells (Figure 5.18). These results are suggested that the cellular abundance of BMAL1 does not affect the period of the circadian oscillation up to a certain level, but may affect the p(Bmal1) activity. A study also was demonstrated that *Bmal1* rhythmic patterns of mRNA and protein expression are not required for core clock function (Liu et al., 2008). In addition, the p(Bmal1)-*dluc* system was virally transferred into cells to insert into the genome. We do not know how much luciferase system is inserted into the genome during this transduction and cell selection and since the cells are single cell derived, this is the reason why we see differences in amplitude values between each clone. If they were polyclonal, a general evaluation could be made, but since each of these cells is unique, it is normal to see differences. In fact, amplitude values are not evaluable parameters that we can detect change or difference in the add back in system.

There are many biological factors that could cause differences between the rescued oscillations, the reason may be the protein abundance (flexible for certain levels), protein stability and promoter activity. Even if the same conditions are used experimentally (even ignoring the use of different guide RNAs between clones), in cellular studies, it is not known in which cell the DNA that is eventually used enters which cell, in what way, how and how much. Moreover, the system we use is not targeted, so we do not know where the new DNA enters. Even out of millions of positive cells, we only select few.

To detect the Bmal1 difference at the synchronized cells, we collected protein samples from parental cells and *Bmal1* add back-in clones under the native promoter and cells at the time points when Bmal1 was maximal (CT36) and minimal (CT48) at the protein level and performed Western blot analysis (Figure 5.20 and Figure 5.21). We had previously checked Bmal1 protein level in *Bmal1* add back-in clones under the native promoter, but they show overall Bmal1 expression, with unsynchronised cells (Figure 5.14). When we accepted the CT36 value as 100% within each clone, we indeed found a decrease in CT48 in most clones, which is evidence that Bmal1 is also rhythmic at the protein level, even the decrease was not exactly half-and-half in

all clones. In this case, it is reasonable to first evaluate the amount of Bmal1 expressed. We noticed that the data obtained by comparing Bmal1 levels in unsynchronized and synchronized cells are not consistent for some clones. In addition, in some clones with similar Bmal1 expression compared to the control, the difference between CT36 and CT48 is very small. It may be due to epigenetic phosphorylation of the BMAL1 protein as well as being influenced by experimental processes. Also, it is known that some of serine and threonine phosphorylation and dephosphorylation are crucial for the coordinated transcription of circadian genes and essential for protein accumulation in terms of the functioning of the TTFL (Schibler, 2021), so we attribute this to BMAL1 phosphorylation. The impact of PTMs, particularly phosphorylation, on the transcriptional activity of circadian elements, as well as its effectiveness in subcellular localization, suggests that Bmal1 functionality could be influenced by PTMs. Therefore, in studies where *BMAL1* is deleted and re-introduced into cells, the system's operation can be evaluated in conjunction with the restored Bmal1 functionality. In other words, the oscillation rescue in the *Bmal1* add-back system may be highly affected by the integrity of Bmal1 phosphorylation.

We designed a co-immunoprecipitation assay to test the functionality of Bmal1 under the native promoter. We used Flag tag from U2OS cells stably expressing p(Bmal1)-m*Bmal1*-3xFlag-6xHis for co-IP experiments. We coated the beads with Flag antibody and combined the protein lysate obtained from the cells 3xFLAG-mBmal1 were pulled down by anti-FLAG antibody coated magnetic beads. In both of the co-IP studies performed on two randomly selected clones, we detected CLOCK interaction via immunoblotting of beads pulled down, indicating that mBmal1, which we exogenously inserted to the cells, was functional (Figure 5.22). It is proved that the re-inserted Bmal1 can interact with CLOCK in terms of its functionality.

Bmal1 add backed-in cell lines obtained throughout all studies derived from single cells. Since the single cell isolation and growth of the cells at this stage takes time, we thought that the biological tool studied should be improved and for this reason, we tested the polyclonal cell screening system at the *Bmal1* add backed-in stage. It was important for us to transfer DNA into the cell in the most efficient way and therefore we preferred the viral transduction method. Firstly, pLentiIII-p(Bmal1)-m*Bmal1*-3xFlag-6xHis construct was transferred into U2OS-*BMAL1*^{-/-}-p(Bmal1)-

dluc cells with the help of lentiviruses. The problem we had was that there was a puromycin selective marker in the backbone we used, but we had already introduced this resistance to these cells (LentiCRISPR.v2.puro-*BMAL1*-KO). Nevertheless, we increased the amount of antibiotic appropriately and tried to select the infected cells. We subjected the cells to bioluminescence test without any monoclonal isolation and the results were promising. No significant difference was detected in terms of period in the 3 polyclonal *Bmal1* add back-in cells. Amplitude values were also close to the control cell (except clone C39.2.D11.BV). However, the rescued oscillation had difficulty in showing its pattern after approximately 60 hours. In this case, we think that there are contaminated U2OS- *BMAL1*^{-/-}-p(*Bmal1*)-*dluc* cells (*BMAL1*^{-/-}) present in the cell population and that two different luminescence dampen the oscillation pattern. However, it is obvious that this system will yield much better results if the plasmid backbone has a different selective marker than the antibiotic resistance previously imparted to the cells.

In conclusion, we succeeded in rescuing the oscillation, which was completely lost in the absence of *BMAL1*, with *BMAL1* cloned under both constitutive promoter and native promoter. We determined that the circadian oscillation is independent of the amount of *BMAL1* up to a certain level. Also, high luciferase activity which is represented by amplitude values is caused by the number of p(*Bmal1*)-*dluc* gene inserted into the genome. So, amplitude values are not evaluable parameters. We found that *CLOCK* increase alone is not sufficient to boost p(*Per1*) activity. Although we did not necessarily expect the protein amount to increase as a result of high promoter activity, we found this increase in some clones, but on the other hand, the fact that we detected low protein levels in clones with high promoter activity suggested that protein stability may be affected in some clones in the gene add back-in system. Finally, *BMAL1* rhythmic patterns of mRNA and protein expression are not required for core clock function.

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BIOGRAPHY

Büşra TİRYAKİ graduated from Gebze Technical University in 2021 with a thesis titled "Investigation of the Relationship Between Antidepressant Class Member Paroxetine and Biological Clock on Cancer Cells." Following her graduation, she was accepted to the MSc Program in Molecular Biology and Genetics at the Graduate School of Natural and Applied Sciences in Gebze Technical University in the same year. She worked under the supervision of Prof. Dr. Nuri Ozturk during her MSc program.



PRESENTATIONS FROM THE THESIS

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APPENDICES

APPENDIX A: PROTEIN AND DNA MARKERS.

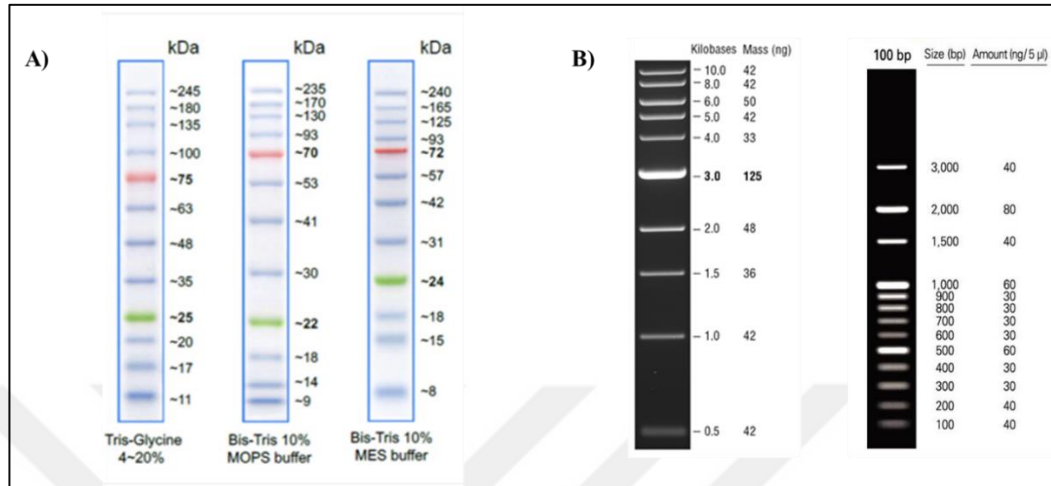


Figure A.1: A) GangNam-STAIN™ Prestained Protein Ladder. B) NEB 1 kb DNA marker and Enzymomics 100 bp DNA marker.

APPENDIX B: PERIODS OF CIRCADIAN OSCILLATIONS.

Table B.1: Period values of circadian oscillation data of *Bmal1* add back-in expressed under CMV and native promoter comparing with the control cell line. Data yielded Figure 5.19.

Data Label	Status	Period	Period Err
BMAL1:dluc Control		24.65	0.33
BMAL1:dluc Control		25.05	0.55
BMAL1:dluc Control		25.21	0.43
BMAL1+ under CMV Promoter	IGNORED	27.53	0.82
BMAL1+ under CMV Promoter		25.53	1.02
BMAL1+ under CMV Promoter		25.46	1.0
BMAL1+C37.1-D1-B1		25.38	0.33
BMAL1+C37.1-D1-B1		25.01	0.31
BMAL1+C37.1-D1-B1		25.51	0.24

Table B.2: Period values of circadian oscillation data of control cell line obtained with protamine sulfate (PS) and, U2OS-*Bmal1*⁻ clones. Data yielded Figure 5.6.

Data Label	Period	Period Err
U2OS-p(Bmal1)-dluc C3 PS	25.3	0.24
U2OS-p(Bmal1)-dluc C3 PS	25.17	0.21
C9-D1	37.51	2.13
C9-D1	38.86	1.91

Table B.2: Continued.

C15-D3	92.82	13.31
C15-D3	90.14	20.48
C16-D3	42.9	2.42
C16-D3	59.47	3.16
C36-D1	87.98	12.11
C36-D1	89.2	10.96
C37-D3	40.33	3.16
C37-D3	39.84	2.84
C39-D1	70.19	9.81
C39-D1	65.8	13.77
C15.2-D1	42.36	1.56
C15.2-D1	46.89	2.77
C16.1-D1	63.26	4.9
C16.1-D1	70.91	5.87
C36.1-D1	75.67	4.27
C36.1-D1	74.69	3.64
C37.1-D1	63.69	4.23
C37.1-D1	66.45	4.36
C39.2-D1	67.72	5.05
C39.2-D1	64.64	10.21

Table B.3: Period values of circadian oscillation data of control cell line and, U2OS-*Bmal1*⁺ clones under native promoter. Data yielded Figure 5.18.

Data Label	Period	Period Err
BMAL1:dluc Control	24.65	0.33
BMAL1:dluc Control	25.05	0.55
BMAL1:dluc Control	25.21	0.43
BMAL1+C9-D1-B1	25.29	0.41
BMAL1+C9-D1-B1	24.74	0.36
BMAL1+C9-D1-B1	25.49	0.29
BMAL1+ C9-D1-B2	24.4	0.25
BMAL1+ C9-D1-B2	24.27	0.24
BMAL1+ C9-D1-B2	24.29	0.24
BMAL1+C15-D3-B1	24.28	0.23
BMAL1+C15-D3-B1	24.44	0.24
BMAL1+C15-D3-B1	24.46	0.21
BMAL1+ C15-D3-B3	26.05	0.25
BMAL1+ C15-D3-B3	25.92	0.29
BMAL1+ C15-D3-B3	26.09	0.2
BMAL1+C36-D1-B4	25.38	0.41
BMAL1+C36-D1-B4	25.35	0.31
BMAL1+C36-D1-B4	25.45	0.26
BMAL1+C37.1-D1-B1	25.38	0.33
BMAL1+C37.1-D1-B1	25.01	0.31
BMAL1+C37.1-D1-B1	25.51	0.24
BMAL1+C39.2-D1-B1	25.28	0.24

Table B.3: Continued.

BMAL1+C39.2-D1-B1	25.32	0.23
BMAL1+C39.2-D1-B1	25.64	0.18
BMAL1+ C39.2-D1-B7	25.08	0.36
BMAL1+ C39.2-D1-B7	25.33	0.46
BMAL1+ C39.2-D1-B7	24.88	0.58

Table B.4: Period values of circadian oscillation data of control cell line and, U2OS-*Bmal1*⁺ clone C16D3CB3 under CMV promoter. Data yielded Figure 5.11.

Data Label	Period	Period Err
BMAL1:dluc Control	25.68	0.41
BMAL1:dluc Control	25.27	2.0
BMAL1:dluc Control	25.85	0.11
C16-D3-CB3	27.15	0.3
C16-D3-CB3	27.91	0.18
C16-D3-CB3	27.54	0.17
C16-D3-CB3-6 months later	25.58	1.13
C16-D3-CB3-6 months later	25.56	1.44
C16-D3-CB3-6 months later	25.46	1.0
C16-D3-BMAL1 KO	83.22	10.42
C16-D3-BMAL1 KO	86.18	12.11
C16-D3-BMAL1 KO	81.98	8.15

Table B.5: Period values of 60-hour circadian oscillation data of control cell line and, polyclonally selected U2OS-*Bmal1*⁺ cells under native promoter. Data yielded Figure 5.26.

Data Label	Status	Period	Period Err
Control Cells		25.9	0.57
Control Cells		26.3	0.46
Control Cells		25.85	0.41
C15-D3-BV		26.01	1.47
C15-D3-BV		26.04	1.56
C15-D3-BV		25.63	1.44
C39-D1-BV		26.28	1.18
C39-D1-BV	IGNORED	~~29.25~~	1.61
C39-D1-BV		26.44	1.89
C39.2.D1.BV	IGNORED	~~29.55~~	1.05
C39.2.D1.BV		24.37	0.67
C39.2.D1.BV		25.07	0.53