

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

**INVESTIGATION OF PROTEINS ASSOCIATED WITH
CIRCADIAN RHYTHMS USING CRISPR-CAS9 TECHNOLOGY
AND PROXIMITY LABELING**

FATMA YILMAZ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CRISPR-CAS9 TEKNOLOJİSİ VE
YAKINLIK ETİKETLENDİRMESİ
KULLANILARAK SİRKADİYEN RİTİM İLE
İLGİLİ PROTEİNLERİN İNCELENMESİ

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MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

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SUMMARY

The circadian clock generates daily rhythms in physiology and metabolism. At the molecular level, the circadian clock is generated by a transcription-translation feedback loop (TTFL) where CLOCK and BMAL1 transcription factors activate circadian clock-regulated genes (CCGs). The products of two of CCGs (*CRYPTOCHROME* and *PERIOD*) then inhibit CLOCK-BMAL1 activity and this activation/inhibition generates oscillations in 10-20% of transcriptome.

We aimed at finding novel proteins that regulate *BMAL1* gene. For this aim, we planned to label proteins in the regulatory region of *BMAL1* and identify them by mass-spectrometry (MS).

We used a catalytically-dead RNA-guided nuclease Cas9 (dCas9) fused to an engineered peroxidase APEX2 (CASPEX) to biotinylate proteins in the proximity of *BMAL1* promoter for subsequent enrichment and identification by MS analysis. CASPEX and two different sgRNAs targeting *BMAL1* promoter were stably expressed in HEK293T cells. Cells were then treated with doxycycline to induce the expression of CASPEX and phenol biotin was added into the medium to let proximity-dependent biotinylation to occur. After biotinylation, labeled proteins were purified using streptavidin-coated beads and identified by MS. The candidates were cleaned by excluding the proteins identified in a non-targeting sgRNA expressing cell line.

In summary, we identified novel proteins in the promoter region of the *BMAL1* gene. According to MS analysis results, transcription factors (such as MED19, LCOR) which are known to interact with the promoter of a gene and RNA polymerase were identified. These results show that the CASPEX system is effective for the identification of novel circadian clock regulatory proteins.

Key words: CRISPR-Cas9 technology, proximity labeling, circadian clock, BMAL1, transcription factor.

ÖZET

Sirkadiyen saat fizyoloji ve metabolizmada günlük ritimler üretir. Moleküler düzeyde, sirkadiyen saat, CLOCK ve BMAL1 transkripsiyon faktörlerinin sirkadiyen saatçe-düzenlenen genleri (CCG'ler) aktive ettiği bir transkripsiyon-translasyon geri besleme döngüsü (TTFL) tarafından üretilir. CCG'nin iki ürünü (CRYPTOCHROME ve PERIOD) daha sonra CLOCK-BMAL1 aktivitesini inhibe eder ve bu aktivasyon/inhibisyon, transkriptomun %10-20'sinde salınım oluşturur.

BMAL1 genini düzenleyen yeni proteinleri bulmayı amaçladık. Bu amaçla *BMAL1*'in düzenleyici bölgesindeki proteinleri etiketlemeyi ve bunları kütle spektrometrisi (MS) ile tanımlamayı planladık.

Teknik olarak modifiye edilmiş bir peroksidaz olan APEX2 enzimine birleştirilmiş olan katalitik olarak ölü bir RNA kılavuzlu nükleaz Cas9 (dCas9)'dan oluşan CASPEX sisteminin *BMAL1* promotorunun yakınındaki proteinlerin biyotinlenmesi sağlandıktan sonra bunların zengileştirilmesini takiben MS analizi ile tanımlanmasını kullandık. CASPEX ve *BMAL1* promotorunu hedefleyen iki farklı sgRNA, HEK293T hücrelerinde stabil olarak eksprese edildi. Hücreler daha sonra CASPEX ekspresyonunu indüklemek için doksisisiklin ile muamele edildi ve yakınlığa bağlı biyotinlenmeye izin vermek için fenol biyotin hücre kültür ortamına ilave edildi. Biyotinlemeden sonra, etiketlenmiş proteinler streptavidin boncukları kullanılarak saflaştırıldı ve MS ile tanımlandı. Adaylar, hedefleme yapmayan bir sgRNA eksprese eden hücre hattında tanımlanan proteinlerin çıkarılması ile temizlendi.

Özetle, *BMAL1* geninin promotor bölgesinde yeni proteinler belirledik. MS analiz sonuçlarına göre, bir genin promotoru ve RNA polimeraz ile etkileşime girdiği bilinen transkripsiyon faktörleri (MED19, LCOR gibi) tanımlanmıştır. Bu sonuçlar CASPEX sisteminin yeni sirkadiyen saat regülatör proteinlerinin tanımlanmasında başarıyla kullanılabileceğini göstermektedir.

Anahtar kelimeler: CRISPR-Cas9 teknoloji, yakınlık işaretleme, sirkadiyen saat, BMAL1, transkripsiyon faktörü.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Explanations

Acronyms

µg	: Microgram
µl	: Microliter
mW/cm ²	: Milliwatts per square centimeter
°C	: Celsius
Ab	: Antibody
AMP	: Adenosine monophosphate
APS	: Ammonium Persulfate
ATP	: Adenosine triphosphate
ARNTL1	: Aryl hydrocarbon receptor nuclear translocator like protein 1
bHLH	: Basic Helix-Loop-Helix
BMAL1	: Brain muscle arnt like protein-1 gene/protein
bp	: Base pair
CCG	: Clock Controlled Genes
CRY1	: CRYPTOCHROME 1 gene/protein
CRY2	: CRYPTOCHROME 2
CRISPR/Cas9	: Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein-9
ddH ₂ O	: Double-distilled water
dsDNA	: Double stranded DNA
DMEM	: Dulbecco's Modified Eagle's Medium
EDTA	: Ethylene-diamine-tetra-acetic acid
HDR	: Homology Directed Repair
HEK 293T	: An immortal human embryonic kidney cell line
HRP	: Horseradish peroxidase
LB	: Luria Bertani
MS	: Mass-Spectrometry
MT	: miniTurbo enzyme
ng	: Nanogram
NHEJ	: Non-homologous end joining

NPAS2	: Neuronal PAS Domain Protein 2
PAS	: PER-ARNT-SIM
PCR	: Polymerase Chain Reaction
PER	: PERIOD gene/protein
RPM	: Revolution per minute
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulphate
SDS-PAGE	: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	: Single-stranded DNA
T _m	: Melting temperature
TTFL	: Transcription Translation Feedback Loop
U2OS	: A human osteosarcoma cell line
WB	: Western Blot

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1. INTRODUCTION

The subject of this thesis is to identify proteins in the regulatory regions of the circadian clock gene *BMAL1*. The latest development in genome targeting technique based on the catalytically inactive genome editing enzyme Cas9 (dCas9) and proximity labeling resulted in a technique that allows the discovery of proteins in a specific genomic location. This method is called CASPEX [Myers et al., 2018]. In the CASPEX system, dCas9 is fused to an engineered peroxidase APEX2 and targeted a specific genomic locus via sgRNAs (single guide RNAs). APEX2 oxidizes the phenyl moiety of the biotinyl tyramide compound to phenoxyl radicals, which results in the biotinylation of the proteins in the close proximity of the enzyme when supplied with hydrogen peroxide for 1 minute. Biotinylated proteins are then precipitated using streptavidin-coated beads and identified by mass spectrometry. In summary, we used a guided CASPEX system to the promoter region of human *BMAL1* gene using specific sgRNAs in order to label the transcription factors and then to identify them by mass spectrometry.

1.1. The Purpose, Contribution and Content of Thesis

The main purpose was to test CASPEX system for identifying proteins in the close proximity of *BMAL1* gene regulator region and optimize the system for future experiments. For this purpose, we designed our viral plasmid to express sgRNAs targeting *BMAL1* gene regulator sequences, and adapt it to use with the CASPEX system. While we were trying to find out proteins in the regulator regions of *BMAL1* gene, we also tried to insert another proximity labelling enzyme in front of *BMAL1* and *CRY1* genes using the CRISPR/Cas9 mediated gene editing technique. The aim of this was to prepare a starting material which will be used to identify the interactome of *BMAL1* and *CRY1* proteins.

With the completion of this thesis, we aimed to contribute an initial list of proteins in the close proximity of *BMAL1* promoter and a model for the proximity labeling of *BMAL1* protein which can be later used for the identification of *BMAL1* interactome via mass spectrometry.

In the content of the thesis, 1) we used recombinant DNA technology to prepare constructs for sgRNAs targeting *BMAL1* promoter so CASPEX enzyme can be sent to a specific genomic locus or targeting a sequence around ATG start codon for genome editing, 2) generated lentiviral particles using our home-made plasmids, 3) generated stable cell line or genome edited cell lines, 4) analysed the expression of the recombinant proteins using immunoblotting, and 5) identified the proteins that were precipitated using streptavidin-coated magnetic beads through mass spectrometry.

2. LITERATURE REVIEW

2.1. Biological Clock

Many organisms regulate their biological activities in order to obtain maximum advantages from the daily changes in the environment which are formed as a result of the daily rotation of the world around itself. Biological clock is generally expressed as the mechanism by which organisms regulate the timing of their biological activities. The circadian clock is a mechanism that generates approximately 24-hour oscillations in physiology and metabolism and it's a type of biological clock. Other than the circadian clock, annual migrations, monthly rhythms, biochemical oscillations, and neuronal firing are just a few examples of rhythm that are controlled by the biological clock with the period from years to seconds.

The circadian (circa meaning about, and dies or diem meaning a day in Latin) literally means 'about a day'. The circadian rhythms or daily rhythms are found in almost all organisms, from bacteria to humans [Sancar, 2004]. Circadian clocks regulate the physiology, metabolism, and behaviour of organisms in synchrony with the daily solar cycle. The circadian clock is generated by a transcription translation feedback loop (TTFL) mechanism. In the positive arm of the TTFL, CLOCK and BMAL1 transcription factors transactivate the expression of circadian clock-regulated genes, or clock controlled genes (CCGs) [Griffin, Staknis, Weitz, 1999]. In the negative arm of the TTFL, CRYPTOCHROME and PERIOD genes of the CCGs are translated and their products inhibit CLOCK/BMAL1 transcriptional activity by protein-protein interactions. [Sato et al., 2004], [Shaerman et al., 2000], [Busino et al., 2007]. CLOCK and BMAL1 proteins mostly transactivate the expression of the CCGs by binding to the E-box sequences in the promoters. Even though there are 4 core circadian clock factors, there are more than one gene for some factors. Two CRY genes (CRY1 and CRY2) and three PERIOD genes (PER1-3) genes have overlapping functions. PER3 is not required for the molecular clock, and the molecular clock is disrupted when the PER1/2 genes are disrupted [Kang et al., 2009]. On the other hand, NPAS2 can partially fulfill the function of CLOCK protein [Huang et al., 2012]. Post-translational modification of clock proteins assigns

their subcellular localization and is critical for adjusting the 24-h periodicity of the circadian clock [Lee et al., 2001], [Miyazaki et al., 2004]. Besides that, PER and CRY proteins are degraded by the proteasome system following ubiquitination which ensures the oscillation of them [Busino et al., 2007], [Siepka et al., 2007], [Godinho et al., 2007], [Reischl et al., 2007] [Shirogane et al., 2005]. Other post-translational modifications are very common for clock proteins. For example, PER proteins are phosphorylated by kinase casein kinase I (CKI) ϵ and a related kinase, CKI δ [Lowrey et al., 2000], [Toh et al., 2001], [Xu et al., 2005]. The general overview of the core molecular circadian is shown in Figure 2.1 (Figure was modified from [Partch et al., 2014]).

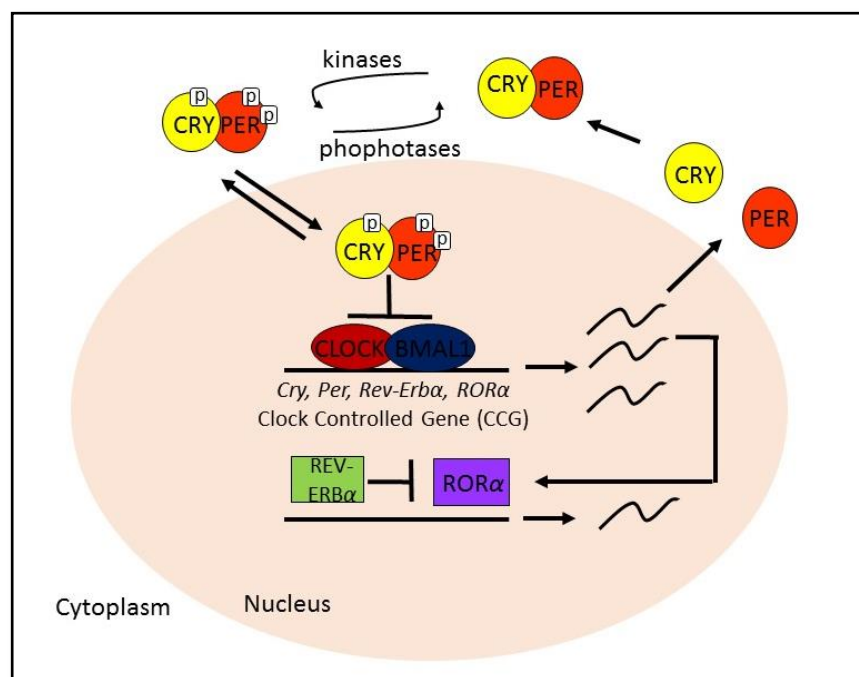


Figure 2.1: Temporal control of physiology via integral clock proteins. Two activators (CLOCK and BMAL1) and two repressors (PER and CRY) generates the oscillation in the core proteins which is tuned by secondary loops and post-translational modifications.

Circadian clock prepares the organism for orderly changes around itself so that the organism can take advantage of these changes. The synchronization of the internal (peripheral) clocks also orchestrates the best fitted processing of the metabolism, energy expenditure, and prevention/repair of DNA damages [Lowrey and Takahashi, 2004]. At the molecular level, the circadian clock controls temporal homeostasis with the environment by regulating gene expression to reach the top

point of protein expression once every 24-h when that specific protein's expression is required [Kang et al., 2009].

A 'master' clock located in the Suprachiasmatic nucleus (SCN) of the hypothalamus in the brain organizes the peripheral clocks which are located in nearly every organ in the body [Ko and Takahashi, 2006]. The master clock is formed by almost 20000 neurons [Mohawk and Takahashi, 2011]. Sunlight reset the master circadian clock through the eyes [Buhr Yoo and Takahashi, 2010]. Actually, the molecular clocks in master and the peripheral clock operates in the same mechanism at the molecular level [Yoo et al., 2004].

2.2. Mammalian Circadian Clock

In mammals, the circadian clock regulates physiological functions such as sleep-wake behaviour, feeding, heartbeat, and blood pressure [Gachon et al., 2004]. The light signal is received by retinal ganglion cells and is transmitted to the master clock through the retinohypothalamic tract. The photopigment melanopsin is believed to be the main photoreceptor for resetting the circadian clock [Pfeffer et al., 2017].

In humans and other mammals the molecular circadian clock is also generated by a self-sustaining TTFL mechanism [Lowrey and Takahashi, 2004], [Reppert and Weaver, 2002]. The CLOCK:BMAL1 heterodimer activates the transcription of the *PERIOD (PER)* and *CRYPTOCHROME (CRY)* genes [Bunger et al., 2000]. The products of *CRY* and *PER* genes accumulate gradually in the cytoplasm and they move into the nucleus where they inhibit the CLOCK:BMAL1 heterodimer activity. Because CLOCK:BMAL1 inhibition results in a decrease in CRY and PER protein level after some time, the removal of CLOCK:BMAL1 inhibition results in the activation of CLOCK:BMAL1 for the second cycle of the circadian rhythm. Secondary loops, post-translational modifications and controlled subcellular localization ensure the high amplitude in the oscillation with a period of around 24 hours [Lee et al., 2001]. It is therefore believed that post-translation modification is very important for the function of circadian clock TTFL [Eide et al., 2005].

2.2.1. Mammalian Cryptochromes

Cryptochrome (CRY) is a member of the photolyase/cryptochrome superfamily of flavoproteins. Photolyase enzymes repair the DNA damages induced by ultraviolet (UV) light and use photon energy from the blue light spectrum of sunlight as an energy source. Cyclobutane pyrimidine dimers (CPD) or pyrimidine-pyrimidone (6-4) photoproducts are the main damages caused by UV-light and they are repaired by either CPD or 6-4 photolyases, respectively. CRY proteins are structurally similar to photolyase enzyme, but they cannot repair DNA damages. Instead, CRYs function either as photoreceptors or as the regulator of the circadian clock. In addition to the N-terminal photolyase homology (PHR) domain, CRYs have C-terminal extension (CCE) regions. The mammalian CRYs do not have a photoreceptor function but they function in the negative arm of the TTFL model [Scheerer et al., 2015]. However, CRYs in insects and plant have a photoreceptor function, which resets the core clock in response to early sunlight [Hirayama et al., 2003].

2.2.2. BMAL1

BMAL1, also known as aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL 1) or Brain and Muscle ARNT-Like 1, is encoded by the *ARNTL1* gene in humans. BMAL1 is a transcription factor and it has a bHLH and two PAS domains. BMAL1 protein consists of 626 amino acids (a.a) and alongside with CLOCK protein functions in the positive arm of TTFL [Reppert and Weaver, 2002]. CLOCK:BMAL1 heterodimers transactivates the expression of *PER* (*PER1/PER2*) and *CRY* (*CRY1/CRY2*) genes [Mohawk et al., 2012]. Crystallographic studies showed that the BMAL1 structure contains 4 domains: bHLH, PAS/A, PAS/B, and a trans-activating domain. CLOCK:BMAL1 heterodimerization occurs through interactions between the bHLH, PAS/A and PAS/B regions of CLOCK and BMAL1 proteins [Huang et al., 2012].

There are some other regulators which contribute to the core clock. SIRT1 (Sirtuin 1) regulates the degradation of PER. CRY/PER complex cannot be formed when PER protein is removed, so BMAL1/CLOCK heterodimerization continues. [Asher et al., 2008]. CRY1 is ubiquitinated by FBXL3 E3 ligase substrate receptor, which leads to the degradation of CRY1 protein [Buhr and Takahashi, 2013]. On the

other hand, ROR α and REV-ERB α are major regulators of the cyclic expression of BMAL1. REV-ERB α expression is activated by the BMAL1/CLOCK. ROR α competes with REV-ERB α for binding of their shared DNA binding elements, in the promotor of the *BMAL1* gene. In summary, *BMAL1* expression is repressed by REVERB α and activated by ROR α . [Akashi and Takumi, 2005].

2.3. Proximity Labeling

In proximity labeling techniques, an enzyme fused to the protein of interest covalently attach some small groups such as biotin in the close proximity. Subsequently labeled proteins are pulled-down by affinity and identified by mass spectrometry. In this thesis, we have used proximity-dependent labelling with biotin. Biotin does not affect the protein function and therefore is used to label protein in vivo.

2.3.1. Proximity Labeling with Biotin Ligase

E. coli biotin ligase BirA enzyme biotinylates specific lysine residues of Acetyl-CoA carboxylase, it may also biotinylate other lysines nearby of short acceptor peptide. Chemically, the BirA enzyme activates biotin with ATP by forming biotinyl-5'-AMP. This non-stable intermediate is transferred to the substrate. A mutant form of this enzyme called BirA* biotinylates lysine residues without any requirement of a short acceptor peptide [Roux, 2013]. Firstly, Roux et al. have described that this enzyme as a fusion with the protein of interest can be used for proximity-dependent labelling with biotin in HEK 293T cell, and which can be identified by mass spectrometry following enrichment with streptavidin-coated beads. This method is called as BioID. As BirA* enzyme works very slowly, it requires a 24-hour biotinylation which made this enzyme unsuitable for the sub-circadian labelling work such as labelling for 3-4 hours at different circadian time points. Later, another biotin ligase mutant from *A. Aeolicus* (233 a.a) was identified as BioID2 which uses less biotin for labeling. However, still BioID2 enzyme required 16-hour labelling [Kim et al., 2016]. In 2017, another group used the BirA* as a starting material for directional mutagenesis and obtained TurboID (35 kDa) and

miniTurbo (28 kDa) enzymes that can reach to saturation in HEK293T cells in 10 minutes and 1 hour, respectively [Branon et al., 2018]. Therefore, the emergence of miniTurbo and TurboID made sub-circadian proximity-dependent biotin labelling possible.

2.3.2. Proximity Labeling with Ascorbic Acid Peroxidase (APEX)

Another method used as an alternative to the BioID method is peroxidase-based proximity labeling. Modified ascorbate-peroxidase (APEX/APEX2) or horseradish peroxidase (HRP) are used in this method [Han et al., 2018]. APEX is a triple mutant of ascorbate peroxidase of wild type soybean derived by structure-guided mutagenesis and screening [Rhee et al., 2013], [Martell et al., 2012]. APEX2 has an additional mutation and has been developed with yeast imaging selections [Lam et al., 2015]. APEX2 (27 kDa) carries a covalently unconnected heme cofactor and has a monomeric structure without disulphide bonds [Lam et al., 2015]. APEX catalyzes the oxidation of biotin-phenol to the short-lived (<1 ms) biotin-phenoxyl radical in the presence of H₂O₂. After adding biotinyl tyramide to the medium and incubating for 30 minutes, biotinylation of near proximity is induced by addition of hydrogen peroxide (H₂O₂). [Myers et al., 2018]. Cells are lysed and biotinylated proteins are isolated/enriched using streptavidin-coated beads for identification via MS.

2.4. Genome Editing or Targeting Specific Genomic Loci using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

CRISPR-Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cut foreign genetic material (Deveau et al., 2010), [Horvath and Barrangou, 2010], [Makarova et al., 2011], [Bhaya et al., 2011], [Garneau et al., 2010]. There are 3 types (I-II-III) defined in bacteria and archaea. Each system contains a cluster of CRISPR-associated (Cas) genes, noncoding RNAs, and a direct repeat. Exogenous DNA targets and direct repeats, defined as protospacers, function together to form CRISPR RNA (crRNA) sequences. Within the DNA target, each protospacer is always associated with a protospacer adjacent motif (PAM), which

can vary depending on the specific CRISPR system. The protospacer adjacent motif (or PAM for short) is a short DNA sequence (2-6 base pairs) that follows the DNA region targeted for cleavage by the CRISPR system [Marraffini and Sontheimer, 2008], [Brouns et al., 2008], [Barrangou et al., 2007].

The type II CRISPR-associated protein Cas9 protein recognizes and cleaves target DNA with the help from two guide RNAs (gRNAs; tracrRNA and crRNA) [Garneau et al., 2010], [Jinek et al., 2012], [Gasiunas et al., 2012]. These two noncoding RNAs can be fused into one single guide RNA (sgRNA). The Cas9/sgRNA complex binds target DNA sequences that contain a sequence match to the first 17-20 nucleotides of the sgRNA if the target sequence is followed by a PAM sequence (Figure. 2.2 was modified from [Ran et al., 2013]). In the CRISPR-Cas system from *Streptococcus pyogenes*, the PAM sequence is 5'-NGG.

crRNA and tracrRNA can form a single-guide RNA (sgRNA) (Jinek, et al., 2012). Thus, Cas9 can be again targeted almost to every target of interest directly by changing a 20 bp guide sequence within the sgRNA [Cho et al., 2013].

Cas9 nuclease has two conserved domains, HNH and RuvC. These two domains can be mutated to provide additional functions, so Cas9 can perform strand-specific splits [Saprunauskas et al., 2011]. An aspartate to alanine (D10A) mutation of the RuvC in the catalytic domain allows the Cas9 nickase mutant (Cas9n) to make it to the nick to form single chain breaks rather than cleavage DNA. Prepared sgRNA pairs increase the target recognition specificity by directing Cas9n to two strands of the target locus at the same time to mediate DSB [Ran et al., 2013]. Mutations in HNH and RuvC generates a completely nuclease dead Cas9 called dCas9. However, it can be still targeted to specific DNA sequences. PAM sequences in the human genome can be found approximately every 8-12 bp [Bhaya et al., 2011]. Therefore, dCas9 or Cas9 can be sent to nearly any location in the genomic region. When fused to different enzymes dCas9 can be engineered for many different tasks. For example, when it is fused to APEX2, dCas9 can be used to target this enzyme with dCas9 to virtually any promoter region of any given genes to label the proteins in the close proximity.

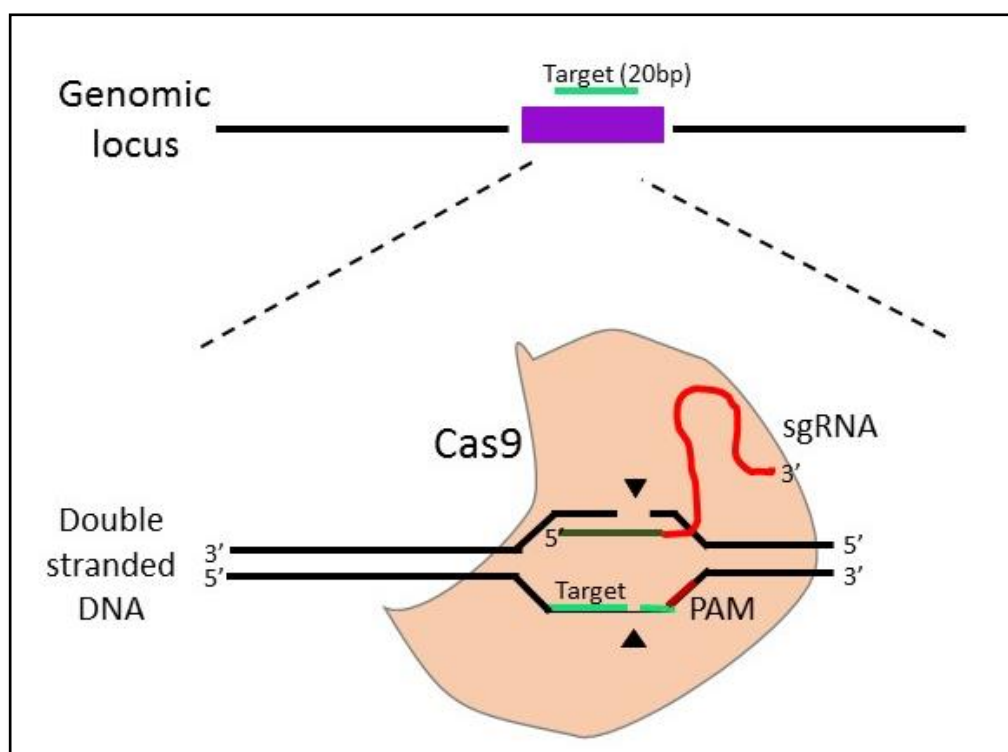


Figure 2.2: Schematic of the RNA-guided Cas9 nuclease. The Cas9 nuclease from *S. pyogenes* (in light pink) is targeted to genomic DNA by a sgRNA containing a 20 nucleotide guide sequence (green) and a scaffold (red). The guide sequence pairs with the DNA target (green bar on top strand), directly upstream of a necessary PAM sequence (dark red). Cas9 generates a double strand break (DSB) approximately 3 bp upstream of the PAM (black triangle).

Targeted nucleases are important tools for making genome alterations. The RNA-guided Cas9 nuclease from the microbial “clustered regularly interspaced short palindromic repeats” (CRISPR) adaptive immune system can be used for effective genome engineering in eukaryotic cells using specific guide RNAs [Ran et al., 2013]. Cas9 is a nuclease protein that guided by small RNA sequences through base pairing with target DNA sequences [Garneau et al., 2010], [Jinek et al., 2012], [Gasiunas et al., 2012] (Figure. 2.3 was modified from [Ran et al., 2013]). Cas9 supports genome editing by generating a DSB (double-stranded break) at a target region (or target genomic region) [Urnov et al., 2010], [Hsu and Zhang, 2012]. Cells have two important pathways to repair double-stranded DNA lesions. These pathways are the error-prone NHEJ (non-homologous end joining) and the highly-accurate HDR (homology-directed repair) systems. When Cas9 enzyme cuts the target sequence, the damage is repaired either by NHEJ and HDR pathways. NHEJ can be used to cause insertion/deletion (indel) mutation in the coding sequences of the target genes.

which generates either frameshift mutations and premature stop codons. These frameshift mutations or premature stop codons results in a non-functional proteins or knockout phenotype for the relevant gene [Perez et al., 2008]. HDR can be used to make very accurate modifications at a target locus with the co-delivery of a template DNA which will be used to repair DSBs. Under normal conditions, HDR works efficiently and therefore there are strategies to inhibit the NHEJ pathway to increase HDR efficiency. HDR is usually active only in dividing cells and its performance is extensively dependent on the genomic locus and repair template as well as on the cell type and conditions [Saleh-Gohari and Helleday, 2004].

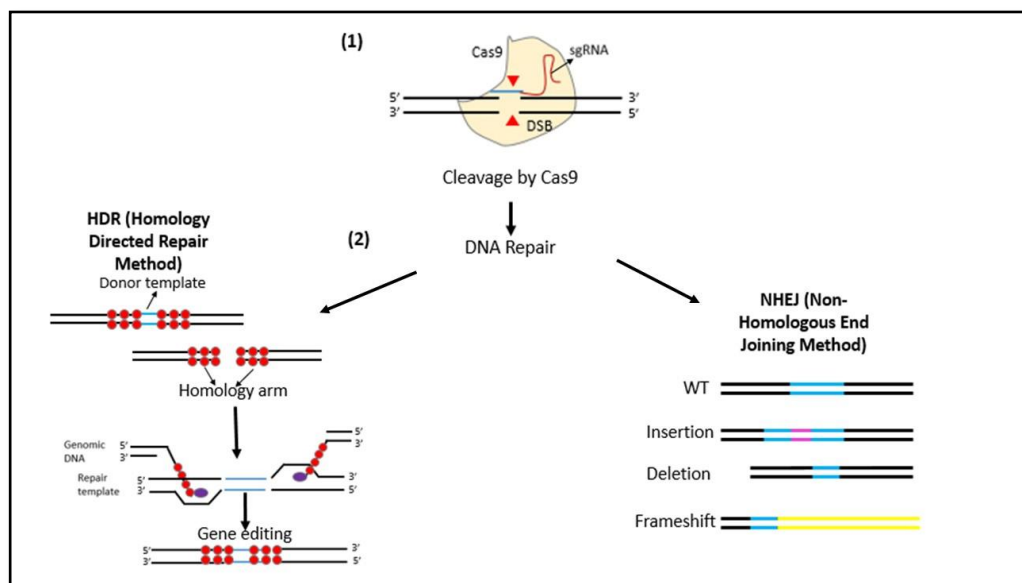


Figure 2.3: Schematic representation of the DSB repair mechanism induced by wild-type Cas9 nuclease.

In summary, (as shown in Figure 2.3.) DSBs can be repaired by two pathways: NHEJ and HDR. The NHEJ method is more prone to errors. In the NHEJ pathway, the ends of the DSB are repaired by the proteins involved in DNA repair but causing random indel mutations. Indel mutations occurring in a coding sequence of a gene might cause frame-shifts or early stop codons. Thus, the gene is knocked out in this way. In HDR pathway, DNA damage is repaired with high accuracy because it uses either double-stranded DNA fragments or single-stranded DNA oligonucleotides (ssODNs) as repair templates. When extra DNA sequences such as coding sequence for some enzymes or tags are covered by homologous DNA sequences at two ends of the extra sequences, homologous recombination results in the insertion of that extra

DNA in a very specific way. In this way, coding sequences in frame with the targeted genes are knocked-in in order to produce recombinant fusion proteins [Ran et al., 2013].

3. MATERIALS

3.1. General Kits and Reagents

Table 3.1: List of kits and reagents.

Nucleospin Plasmid Kit	740588.50 (Macherey Nagel-Germany)
PCR clean-up Gel extraction Kit	740609.50 (Macherey Nagel-Germany)
PureLink Genomic DNA Mini Kit	K1820-02 (Invitrogen-USA)
NucleoBond Xtra Midi	740410.10 (Macherey Nagel-Germany)
Protease Inhibitor Cocktail Tablets	S8830-2TAB (Sigma-Aldrich-USA)
Protein Molecular Weight Marker	26616 (Thermo Scientific-USA)
Chemiluminescent HRP Substrate	K-12043-D10 (Advansta-USA)
Taq DNA Polymerase	M0320S (New England Biolabs (NEB)-USA)
Standart 10X Taq Buffer (Mg-Free Buffer)	B9015S (New England Biolabs (NEB)-USA)
MgCl ₂ (25mM)	B9021S(New England Biolabs (NEB)-USA)
1 kb DNA Ladder	N3232S (New England Biolabs (NEB)-USA)
100 bp DNA Ladder	B7025 (New England Biolabs (NEB)-USA)
DNA Loading Dye	B7021S (New England Biolabs (NEB)-USA)
BCA Protein Assay Kit	23227 (Thermo Scientific-USA)
Streptavidin-coated Magnetic Beads	S1420S (New England Biolabs (NEB)-USA)
Biotinyl Tyramide	SML2135 (Sigma-Aldrich-USA)

3.2. Buffers and Solutions

Table 3.2: List of buffers and solutions.

1X-SDS PAGE Running Buffer	25 mM Tris-Base (pH:8.3) 190 mM Glycine 1% SDS
10X-Tris Buffered Saline (TBS)	200 mM Tris-HCl (pH:7.6) 1.5 M NaCl
1X-Transfer Buffer	10% 10X SDS-PAGE Running Buffer 20% Absolute Methanol
1X-TBS-T	1X TBS 0.1% Tween-20
5%-Blocking Buffer	5% non-fat dry milk in 1X-TBS-T
5X-Tris Borate EDTA (TBE)	54 g Tris-Base 27.5 g Boric-Acid 20 mL 0.5 M EDTA (pH:8.0)
4X-Laemmli Sample Buffer (100mL)	27.78 mL Tris-HCl (pH:6.8) 8 g SDS (4.4%) 44.4 mL Glycerol 10% β -mercaptoethanol 0.02% Bromophenol Blue
1X-Cell Lysis Buffer (RIPA)	150 mM NaCl 50 mM Tris (pH:8.0) 0.5% SDS 1% NP-40 8% IGEPAL
10X-Phosphate Buffer Saline (PBS)	BE-17-517Q (Lonza-Belgium)
Dulbecco's Modified Eagle Medium (DMEM)	41965-039 (Gibco Invitrogen-USA)
0.05%-Trypsin-EDTA	25300-054 (Gibco Invitrogen-USA)
Fetal Bovine Serum (FBS)	10270-076 (Gibco Invitrogen-USA)
100X Penicillin/Streptomycin	15140-122 (Gibco Invitrogen-USA)
Puromycin	P8833 (Sigma, Germany)
Zeocin	R25001 (Thermo Scientific-USA)
LB Broth Medium	LBP03 (Caisson Labs-USA)
LB Agar	LBP04 (Caisson Labs-USA)

Table 3.3: List of SDS-PAGE gel recipe.

<u>10%-separating gel</u>	<u>10 ml</u>	<u>15 ml</u>
Autoclaved ddH ₂ O	4.8	7.2
40%-Acrylamide/Bis-acrylamide	2.5	3.75
1.5 M Tris-HCl (pH:8.8)	2.5	3.75
10% SDS	0.1	0.15
10% APS	0.1	0.15
TEMED	0.004	0.006
<u>8%-separating gel</u>	<u>10 ml</u>	<u>15 ml</u>
Autoclaved ddH ₂ O	5.3	7.95
40%-Acrylamide/Bis-acrylamide	2	3
1.5 M Tris-HCl (pH:8.8)	2.5	3.75
10% SDS	0.1	0.15
10% APS	0.1	0.15
TEMED	0.006	0.009
<u>8%-separating gel</u>	<u>10 ml</u>	<u>15 ml</u>
Autoclaved ddH ₂ O	5.3	7.95
40%-Acrylamide/Bis-acrylamide	2	3
1.5 M Tris-HCl (pH:8.8)	2.5	3.75
10% SDS	0.1	0.15
10% APS	0.1	0.15
TEMED	0.006	0.009
<u>6%-separating gel</u>	<u>10 ml</u>	<u>15 ml</u>
Autoclaved ddH ₂ O	5.8	8.7
40%-Acrylamide/Bis-acrylamide	1.5	2.25
1.5 M Tris-HCl (pH:8.8)	2.5	3.75
10% SDS	0.1	0.15
10% APS	0.1	0.15
TEMED	0.008	0.012
<u>5%-Stacking Gel</u>	<u>2 ml</u>	<u>4 ml</u>
Autoclaved ddH ₂ O	1.46	2.92
40%-Acrylamide/Bis-acrylamide	0.25	0.5
1M Tris-HCl (pH:6.8)	0.25	0.5
10% SDS	0.02	0.04
10% APS	0.02	0.04
TEMED	0.002	0.004

3.3. Antibodies

Table 3.4: List of antibodies.

Name	Dilution	Cat. No.
Anti-HA	1:1000	#sc7392-Santa Cruz
Anti-CRY1	1:1000	#A302-614A-Bethylabs
Anti-CRY1	1:500	#PB9540-Boster
Anti-Bmal1	1:500	#A302-616A-Bethylabs
Anti- β actin	1:10000	#156511E2-Invitrogen
Anti-Flag-DYKDDDDDK Tag (9A3)	1:500	#8146-Cell Signaling Technology
Streptavidin-HRP	1:10000	#ab7403-Abcam
Anti-Mouse	1:10000	#31430-Invitrogen
Anti-Rabbit	1:10000	#31460-Invitrogen

3.4. Equipment

Table 3.5: List of equipments.

Agarose Gel Electrophoresis	1704406 (Bio-Rad-USA)
Centrifuges	521-1647 refrigerated (Micro Star 17R-VWR-USA) 521-1646 (Micro Star 17-VWR-USA) 521-2844 (Mini Star-VWR-USA) NF400-Nuve (02-1827)
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System (Bio-Rad-USA)
Incubator (CO ₂)	Thermo-Scientific-USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad-USA
Inverted Microscope	(Nikon/Eclipse/E100)
Laminar Flow Cabinet	Hera-Safe Class II Cabinet (Thermo Scientific-USA)
Microplate Reader	Varioskan Flash (Thermo Scientific-USA)
Power Supply	Bio-Rad-USA
PCR Thermal Cycler	41798 (Bio-Rad-USA)
TransBlot Transfer System	1704155 (Bio-Rad-USA)
NanoDrop	206-26300-48 (Shimadzu Biotech)
Shaker	Standard Analog shaker (VWR-USA)
Vortex	Scientific Industries-USA
Water Bath	Wisd-Germany
Magnetic Rack	DynaMag™-2 Invitrogen

3.5. Oligonucleotide Sequences

Table 3.6: List of primers.

cry-HDR1_F	AAAAGATCTGGGACCGGTCACCGGCCGGCAA
cry-HDR1_R	AAAGTCGACGGGGGCGCGGCGGGTCCTCCA
cry-HDR2_F	AAAGTCGACATGGGGGTGAACGCCGTGCAC
cry-HDR2_R	AAAGCGGCCGCTTCTCCATACTATGAGTTACC
Xho-MT_F	AAACTCGAGGCCCCCGGCATGTACCCGTATGATGTTC
Xh-MT_R	TTTCTCGAGCTTGAAACGGCGCGCCGAT
Bmal-HDR_F	AAAGCGGCCGCTGAGACTTTGTCTCAAAACAAAT
Bmal-HDR_R	AAAGCGGCCGCGACCTCCACTCATTCCTTCCA
KpnLnkrBio_R	AAAGGTACCTGAGCCTCCACCACCTGAGC
EcoRI_F	AAAGAATTTCGCAGACCAGAGAATGGACATTTCTTCAA
wBMAL1-T1_F	GACAAAGATGACCCTCATGGA
wBMAL1-T1_R	AGCCAGTATGGAGAGCATGG
wBMAL1-T2_F	CCTGGCAGTGAGACCATTTT
wBMAL-T2_R	TCATTTCATTTGGCATCACG

4. METHODS

4.1. Molecular Cloning

4.1.1. Restriction Enzymes Digestion

Plasmid or amplified DNA fragments were digested with suitable restriction enzymes (RE) in a microcentrifuge tube. RE reactions were performed in a total volume of 100 µl which contained 1x Restriction Enzyme buffer, 1-3 µg of plasmid DNA, or 0.5-3 µg of amplified DNA fragments, and an appropriate amount of RE. Digestions were carried out at 37°C for 1-4 hours unless stated otherwise. The reactions using two RE were performed according to the manufacturer's instructions using a suitable buffer.

4.1.2. Agarose Gel Electrophoresis

DNA samples which were amplified by PCR or digested with RE were run on a 0.5-2% agarose gel containing 1X SYBR Safe DNA Gel Stain in a horizontal electrophoresis system filled with 1X TBE. After separating the DNA fragments on the agarose gel, the fragments of interest were excised from the gel using a sterile blade under 366-nm illumination. The DNA was isolated/extracted from the gel using Macherey-Nagel Nucleospin Gel and PCR Clean-up kit by following the manufacturer's instruction except that DNA was eluted in 50 µl of double-distilled molecular grade water.

4.1.3. DNA Ligation and Cloning

Inserts (either from PCR products or another plasmids DNA) fragments and the vectors digested with the suitable restriction enzymes and isolated from agarose gels were used for the ligation reaction. Insert and vectors fragments (150 ng at total) were mixed at 3:1 molar ratio in a 10-µl ligation reaction containing 1x T4 ligation buffer and 1 unit of T4 ligase enzyme and incubated at 4-8°C overnight.

4.1.4. Transformations of Ligation Products in Component Bacteria

Escherichia coli DH5 α /NEB stable or Stbl3 competent strains were used for transformation. Competent cells were taken from -80°C freezer and melted on ice. Half of the ligation products (5 μ l) was added into 80 μ l of competent cells and were incubated for 25 minutes on ice. Heat shock was applied at 42°C for 1 minute in a heat block filled with water and then samples were returned onto the ice for a 2-minute incubation. Approximately, ~900 μ l of Luria Broth (LB) was added on cells and cultured in a rotation shaker at 37°C for 1 hour. This culture allowed the cells which took properly ligated plasmids to express the bacterial resistance marker such as ampicillin or kanamycin. At the end of this incubation, 100 μ l of the culture were plated out on the LB agar plates that contained an appropriate antibiotic. After a five-minute incubation, plates were inverted and incubated at 37°C overnight to allow the formation of single cell-derived colonies which took the proper ligation products. Single colonies were taken into 3.5 ml of LB media containing the appropriate antibiotic and cultured overnight.

4.1.5. Plasmid Isolation

For the initial screening of the cloning candidates, cells were precipitated from 3.5 ml overnight cultures. Macherey-Nagel Nucleospin Plasmid Isolation kit was used for plasmid isolation following the manufacturer's instructions. Candidates were screened by RE analysis. When a large amount of plasmids were required, a 10 ng of plasmids from miniprep isolation was used for transformation into bacteria again. Finally, single cell-derived colonies were cultured in 100 ml of LB media that contained the appropriate antibiotic. Pelleted cells from large scale culture were used to isolate transfection quality plasmids DNAs using Macherey-Nagel MidiPrep Plasmid Isolation kit. Plasmid DNA was eluted into 1x TE buffer, and the concentration were measured using a NanoDrop spectrophotometer.

4.2. Mammalian Cell Culture

4.2.1. Maintenance of Cell Lines

Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in high glucose Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin & 100 µg/ml streptomycin, 1% nonessential amino acids (NEA). Routine passages were performed by trypsinisation when cells reached to 90-100% confluency.

4.2.2. Transfection of Mammalian Cells

Polyethyleneimine (PEI) which is a cationic polymer was used as a transfection agent. PEI binds to negatively charged DNA.

HEK293T human embryonic kidney cell line and U2OS human osteosarcoma epithelial cell lines were used for transfections. For transfection, cells were seeded into 60-mm culture dishes at 60% confluency and incubated overnight. To form transfection material, 1-3 µg of plasmid was added into 150 µl of transfection buffer containing 150 mM NaCl, 10 mM Tris.Cl pH: 7.5, and 1 mM EDTA. PEI (1 mg/ml) was then added into the transfection buffer at three fold amount of plasmid DNA, mixed well by pipetting a few times and incubated at room temperature for 15 minutes. Then cell culture was removed from dishes and the transfection reaction solution was mixed with 5 ml of complete medium and added onto cells. After 24-hour incubation, the medium of the cells was replenished, and transfected cells were incubated for 2 days and harvested for western blotting using a plastic scraper.

Transfection was also used to prepare lentiviral particles for sgRNA expression. For this purpose, HEK293T cells were transfected with 1.5 µg of packaging plasmids (pCMV-VSV-G and pCMV-dR8.2 dvpr from Addgene with Cat No: 8454 and 8455, respectively) and 1.5 µg of target plasmid. In this study, the target plasmids were Lenti-CRI-zeo-sgRNA that were generated in our laboratory and constructed to express sgRNA plasmids targeting *BMAL1* promoter region. Lentiviral particles were collected from culture media after 2-3 days following transfection. For this purpose, the media was centrifuged at 3000 r.p.m for 3 minutes

to pellet cell debris, and then supernatant was filtered through a 0.22- μ m filter. This viral stock was either used immediately or stored at -80°C until infection procedure.

4.2.3. Generation Stable Cell Line

To generate a stable cell line, cells were first seeded onto 6-well plates at 50-60% confluency and transfected or infected. Transfection was performed as described above by scaling down the transfection reaction. Infection was performed using lentiviral stocks. Approximately, 0.25 ml of lentiviral containing media was completed to 1 ml with fresh complete DMEM. Polybrene at 6-8 μ g/ml concentration was then added into this media, mixed well and transferred onto cells. After 24-36 hour of either transfection or infection, cells that integrated the plasmid DNAs were selected for stable expression. For this, cells were trypsinized and seeded into 96-well plates by serial dilutions, and incubated in media containing the appropriate amount of selection antibiotics for 10-15 days. Stable cell lines derived from single cells were taken from the latest growing wells, and transferred into 24-, 12- and 6-well plates gradually. Cells were screening for the proper expression by culturing candidates in duplicates into 6-well or 12-well plates. For the screening purpose, the expression of the recombinant proteins was analysed by immunoblotting assay.

4.3. Immunoblotting (Western Blotting)

4.3.1. Sample preparation for immunoblotting

For immunoblotting, the medium was removed from cell culture dishes and cells were harvested by scraping in 1 ml of ice-cold 1X PBS using a plastic scraper. Cells were then pelleted in microcentrifuge tubes by spinning at 3000 to 5000 r.p.m. for 3 to 5 minutes, and the supernatant was removed. The cell pellet was kept at -80 °C until use or lysed directly in sample loading buffer.

4.3.2. Cells Lysis and Measuring of Total Protein Concentration

Cell pellets were lysed with 1X RIPA (radioimmunoprecipitation assay) lysis buffer supplemented with 1X protease inhibitor (PI) complex on ice for 20 minutes. For efficient lysis, lysate was pipetted at every 5 minutes. Cell lysates were then centrifuged at 13300 r.p.m. at 4°C for 15 minutes. After completion of the centrifugation, the supernatant (as cleared lysate) was transferred into a new 1.5-ml microcentrifuge tube.

The total protein concentration of the cleared lysates was determined by using the Pierce BCA Protein Assay Kit (Catalog number: 23225). Bovine Serum Albumin (BSA) samples with known concentrations were used as standards in this assay. Either lysates or BSA standard in 5 µl volume were added into 200 µl of BCA mixture in 96-well plates, and incubated at 37°C for 30 minutes. At the end of reaction development, plates were read at 562 nm. Protein concentrations were equalized using RIPA buffer. Approximately 20 µg of each sample is diluted with a 4X Laemmli loading buffer that includes to reach 1X Laemmli buffer concentration at the final.

In the same experiment, for example, while screening recombinant protein expression from 12-well plates, cells were directly lysed in 1X Laemmli buffer with 10% 2-mercaptoethanol. For this, cells were washed with 1X PBS once, and then 100 to 200 µl of Laemmli buffer added onto cells directly and incubated on a rotating shaker for 10 minutes. At the end of the lysis, samples were taken into 1.5-ml microcentrifuge tubes. The samples in 1X Laemmli buffer were boiled at 95°C for 10 minutes and kept on ice for 2 minutes to prevent refolding of proteins before loading samples for SDS-PAGE.

4.3.3. SDS-PAGE Gel Preparation and Electrophoresis

Firstly, the appropriate glass plates were cleaned and placed in the gel casting apparatus. Then, 10 ml of separating polyacrylamide gel solution (Table 3.3) was prepared in a 15-ml falcon tube. The components were added in the order shown in the table. After making sure that it was homogeneously mixed, the gel was poured between the glass plates. Approximately 500 µl of isopropanol was added to prevent

bubble formation and protect the gel solution of the radical scavenger oxygen. While waiting for the polymerization of separating gel, the stacking gel solution was prepared by the following the recipe in (Table 3.3). Isopropanol was removed before pouring the stacking gel solution onto the polymerized separating gel. An appropriate comb was inserted into stacking gel solution and allowed to polymerize for 15 to 20 minutes, the comb was then removed, and wells were washed with dH₂O. After removing unpolymerized acrylamide or small particles from the wells, the plates were set up into the PAGE system. SDS-PAGE was performed in a 1X Running buffer (Table 3.2) by running the gel for 15-20 minutes at 80 volts and then at 120 volts until bromophenol blue leaves the glass plates.

4.3.4. Transferring (Electroblotting) of Proteins from the Gel to the Membrane

A trans-blot system (Table 3.5) was used for transferring the proteins from the gel to the membrane. Four blotting papers and 1 nitrocellulose membrane were cut at the size of the separating gels and incubated in 1X Transfer Buffer (Table 3.2) for 10 minutes before setting up the blotting (transfer) sandwich. Then a blotting sandwich was prepared as follows: Two blotting papers were put on the transblot cassette. The nitrocellulose membrane was put on top of the blotting papers and the gel was placed onto the nitrocellulose membrane. Finally, two more blotting papers were put onto the gel and air bubbles were removed from the sandwich using a roller. In the transfer apparatus, (-) cassette electrode (cathode) was at the top and (+) cassette electrode (anode) was at the bottom. The sandwich was placed into the electroblotting apparatus and proteins were allowed to transfer to the nitrocellulose membrane by running trans-blot system at 12 volts for 1 hour.

4.3.5. Antibody Incubation (Immunoblotting)

After the completion of the electroblotting the sandwich was disassembled, and the membrane was passed through 1X TBS. Then, the membrane was blocked in a 5% blocking solution (skim milk in 1X TBS-T which is 1xTBS with 0.1% Tween-20) for at least 1 hour at room temperature. After blocking, the membrane was

incubated with the relevant primary antibody in blocking solution on a shaker at 4°C or at room temperature for 1 hour. In order to remove unbound antibodies, the membrane was washed four times with 1X TBST for 5, 15, 5, and 5 minutes washing-incubations for 30 minutes at total. The membrane was then incubated with the appropriate HRP-secondary antibody conjugates in a blocking solution at room temperature for 1 hour on a shaker. Again, unbound antibodies were washed away with 1X TBST for 30 minutes with 5, 15, 5, and 5-min intervals.

For the immunoblotting of biotinylated samples, a single step immunoblotting with Streptavidin-HRP was performed. For this, the membrane was blocked in a modified blocking solution which contained 5% BSA in 1x PBST (1X PBS containing 0.1% Tween-20). The blocked membrane was incubated with Streptavidin-HRP in the modified blocking solution at 4°C overnight. The immunoblotted membrane was then washed with 1X PBST for 30 minutes with 5, 15, 5, and 5-minute intervals.

4.3.6. Chemiluminescence Imaging of the Membrane

After the last wash with 1X TBST, the membrane was drained briefly, enhanced Chemiluminescence substrate [WesternBright Sirius HRP substrate (Advansta Inc., CA, USA)] were then added onto the membrane and incubated for 2 minutes. Chemiluminescence images were captured using the ChemiDoc XRS+ system (Bio-Rad).

4.4. Genomic DNA Isolation

PureLink Genomic DNA Mini Kit (Table 3.1) was used for genomic DNA (gDNA) isolation. The cells were scraped from 60-mm dishes and pelleted by centrifugation. The pellet was suspended in 200 µl of 1X PBS, then 20 µl Proteinase K and 20 µl RNase A were added in a 1.5-ml eppendorf tube. The sample was then briefly vortexed and incubated at room temperature for 2 minutes. The Lysis/Binding Buffer was added onto the sample, and the sample tube was incubated at 55°C for 10 minutes for protein digestion. Absolute ethanol was then added onto lysates and loaded on the PureLink Column (Thermofisher Scientific) following a brief

vortexing. The column was centrifuged at 10000 g for 1 minute at room temperature for binding of the DNA onto the membrane in the column. After binding, the column was washed with 500 µl of Wash Buffer 1 and 500 µl of Wash Buffer 2 (at 10000 g for 1 minute and at 17000 g for 1 minute, respectively). Then the column was centrifuged at 17000 g for another 1 minute to remove any residual washing buffer. For the DNA elution, 50 µl of elution buffer was added onto the column and incubated for 2 minutes at room temperature. The column was then centrifuged at 17000 g for 1 minute in order to get the eluted DNA into the microcentrifuge tubes, and the eluted DNA was transferred into a new microcentrifuge with a cap. DNA concentration was measured using a NanoDrop spectrophotometer (ThermoFisher Scientific) by taking absorbance at 260 and 280 nm, and the concentration was calculated for OD_{260/280} values.

4.5. Polymerase Chain Reaction (PCR)

PCR was performed to amplify specific genetic regions. PCR Master Mix (ThermoFisher Scientific) was used according to manufacturer's directions. The components were added and gently mixed into a 0.2-µl microfuge tube and placed into a thermal cycler.

Table 4.1: PCR reaction contents.

<u>Concentrations of Stock Solutions</u>	<u>μl for 20 μl total reaction)</u>
Taq Buffer (10X) Mg-Free NEB (B9015S)	2
MgCl ₂ (25mM) NEB (B9021S)	1.2
dNTP (10 mM) NEB (N0447S)	0.4
Primer (10 μM) Forward and Reverse Mix	0.8
Taq DNA polymerase (5,000 units/ml) NEB (M0320S)	0.1
DNA (25 ng/μl)	2
ddH ₂ O	13.5

Table 4.2: The thermocycling conditions used in PCR.

	<u>Temperature</u>	<u>Time</u>	<u>Step</u>
	95°C	5 minutes	Initial Denaturation
40 cycles	95°C	20 seconds	Denaturation
	58°C	30 seconds	Annealing
	68°C	1 minute per kb	Extension/elongation
	68°C	2 minutes	Final extension
	4°C	∞	Hold

PCR products were analysed on a 1% agarose gel. In some cases, PCR products were cleaned through PCR cleaning kit and then digested with a restriction enzyme to make sure that the amplified product matches the expected genetic locus.

4.6. BioID-Pulldown

Proximity-dependent biotin identification (BioID) is a method that relies on the biotinylation of neighbouring (in the immediate environment) by a biotin ligase enzyme (a mutant form of BirA) which is fused to the protein of interest, and the identification of the biotinylated proteins by mass spectrometry.

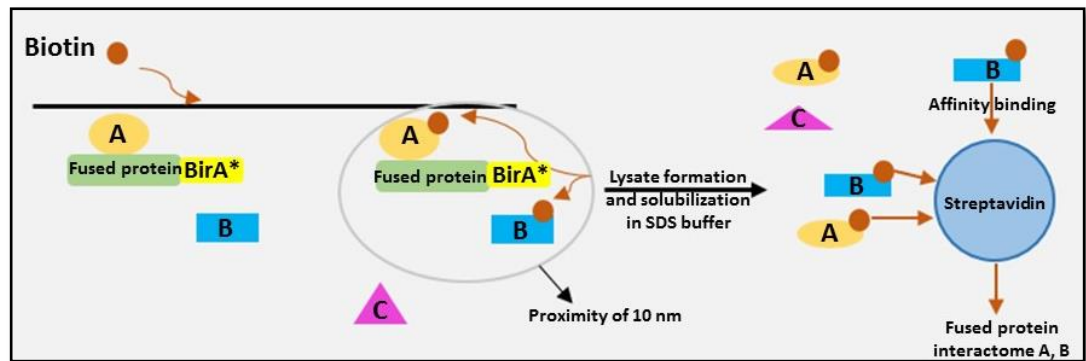


Figure 4.1: The basis of BioID technique.

In figure 4.1, the principle of the BioID technique was illustrated [Roux, 2013]. In this thesis, miniTurbo which is a fast-acting form of the mutant BirA (BirA*) was fused to fused protein as the protein of interest. BirA* has a mutation to eliminate the requirement of a recognition motif in the substrate protein in contrary to the wild-type BirA enzyme [Branon et al., 2018]. The miniTurbo enzyme was generated with several rounds of directional mutagenesis and reaches saturation in 1 hour in contrast to BirA* reaching saturation in 16 to 24 hours in mammalian cells. Here, biotin is added to the medium for miniTurbo to label proteins in the close proximity for 1 hour. After the biotinylation reaches the saturation, the cells are lysed in an SDS-containing lysis buffer, and biotinylated samples are purified using streptavidin-coated beads and then identified by mass spectrometry. In this illustration, A and B represents the proteins in the close proximity while protein C is not within the biotinylation range. Therefore, A and B but not C will be identified by mass spectrometry.

4.7. APEX mediating labelling

In this thesis, CASPEX system was used in which catalytically inactive Cas9 (dCas9) was fused the APEX2 enzyme which also biotinylate the proteins in the close proximity that is targeted to any genomic locations by single-guide RNAs [Myers et al., 2018]. Briefly, dCas9-APEX2 fusion protein is targeted to a specific locus via specific sgRNAs and then biotinyl tyramide phenol is added to cell culture media for 30-min incubation. At the end of 30-min incubation, H_2O_2 was added to the cells for 1 minutes, and cells were harvested after washing with 1X PBS to remove excess H_2O_2 (details were given below). In this way, the proximity-dependent biotin labelling is managed and identification of biotinylated proteins are done as in the BioID technique. In the CASPEX, the dCas9-APEX2 expression is controlled by the doxycycline (Dox)-inducible system which allows the relatively large dCas9-APEX2 protein to locate to a specific location so that the native organization of that genomic locus is disturbed minimally. Near *BMAL1* promoter genetic locus was targeted using CASPEX in this thesis.

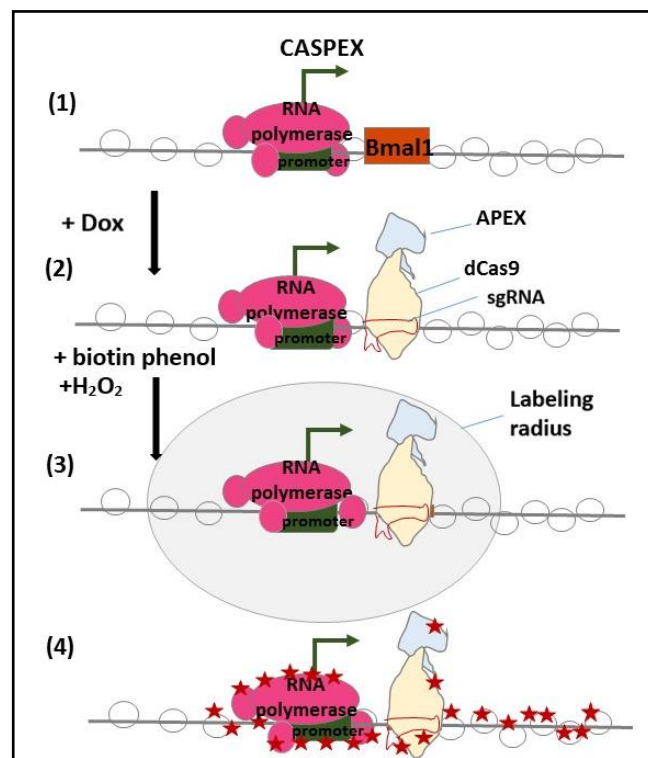


Figure 4.2: Illustration of the currently CASPEX system.

In figure 4.2 was modified from [Myers et al., 2018]). A sgRNA is designed to target dCas9-APEX2 fusion protein to a specific genomic locus (orange bar). (2) CASPEX expression is induced by doxycycline (Dox) to guide dCas9-Apex to the genomic region of interest. (3) APEX2 biotinylates the proteins within “labeling radius” in the presence of biotinyl tyramide and H₂O₂. (4) Labeled proteins with biotin (red star) are next sedimentated with streptavidin-coated beads for identification by mass-spectrometry.

4.8. Sample Preparation for Mass Spectrometry

4.8.1. Labeling with Doxycycline (Dox)-inducible APEX2

Firstly, cells were seeded into 100-mm cell culture dishes at 40-60% density. After overnight incubation, doxycycline was added to the DMEM to at the final concentration of 2 µg/ml to induce dCas9-APEX2 expression for 16 hours. For the biotinylation, biotinyl tyramide (500 mM) in DMSO was added directly to the DMEM to a final concentration 250 µM and the cell was incubated at 37°C for 30 minutes. After incubation, H₂O₂ was added into the medium to a final concentration of 100 µM with very gentle swirling and incubated for 1 minute at room to induce biotinylation. The cell culture medium was removed quickly, and cells were washed twice with 1X PBS before harvesting with a scraper. The pellet was snap-frozen and kept at -80°C until processing for mass spectrometry. In the case of analysis of biotinylation with immunoblotting, a small fraction of the cell pellet was lysed in 1X Laemmli buffer or RIPA lysis buffer and run on a 10% SDS-PAGE.

4.8.2. Pull-down (Enrichment) of Biotinylated Samples

Cells were harvested from five of 100-mm culture dishes and approximately 4% of the cell pellet was reserved for the testing of the biotinylation pattern using the immunoblotting assay. Cell pellets were lysed in 750 µl RIPA lysis buffer (1mM DTT- 10% PI were added before use) by sonication for 6 times with 10-second strokes and 20-second intervals on ice using a Bandelin Sonopuls HD 2070 Max. The lysate was then cleared by centrifugation at 13300 r.p.m for 15 minutes, 4°C.

After taking a 10- μ L sample for BCA assay and immunoblotting, the rest of the supernatant was mixed with 30 μ L of the streptavidin-coated magnetic bead and incubated overnight at 4°C (Table 3.5). On the next day, the beads were washed in the following way: Once with 1 mL of RIPA lysis buffer, once with 1 mL of 1M KCl, once with 1 mL of 0.1 M Na₂CO₃, once with 1 mL of 2 M urea in 10 mM Tris-HCl (pH: 8.0), twice with 1 mL RIPA lysis buffer, and once with 1 mL of 10 mM Tris-HCl (pH: 7.4). Then approximately 1% of the proteins bound to the bead were removed into a new tube for the western blotting after resuspending in 50 μ L of 2X Laemmli lysis buffer. The samples were sent to Koç University Proteomics Facility (Istanbul) for the identification of samples with mass spectrometry.

4.9. Identification of Biotinylated Samples by Mass Spectrometry

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

4.9.1. Significance Analysis of INTeractome Express (SAINTexpress) Software

Objective likelihood scoring of protein-protein interaction (PPI) is an important for the selecting significant interactome in MS data. SAINT is a statistical way for the probability scoring of PPI data. In addition, SAINTexpress includes extraneous interaction data to calculate an additional topology-based score to increase the possibility of objectively identifying the co-purified protein complex (Teo, et al., 2014).

4.9.2. InteractiVenn Tool

In biological sciences, it is frequently required to compare data sets such as genes and proteins. Although Venn diagrams can be built for any number of sets, the layout becomes increasingly challenging after four data sets. InteractiVenn is a tool that lets the user to build and analyse Venn diagrams for up to six sets of interactions. InteractiVenn provides the ability to interactively visualize the effect of successive unions of up to six sets, enhancing the user capacity of analysing data. Additionally, set unions are allowed in any order, including along a union list or a binary tree, enabling different perspectives on the data, for instance in the analysis of sets of genes across species related by a phylogeny. InteractiVenn lets the detailed investigation of cluster troops in venn diagrams. Thus, the interactions of sets can be analysed by additional surveillance gained by new interactions among additional sets [Heberle et al., 2015].

4.9.3. Analysis of the Interactome Using Cytoscape Software

Cytoscape, a software program enables meaningful analysis of biomolecular interaction networks in high output data. When used with various databases (such as StringApp, ReactomeFI), cytoscape enables analysis of protein-protein interactions (PPI), protein-DNA interactions, and genetic interactions that exist for a number of model organisms and humans. It can be used for a variety of functions, including investigating ways of interaction related to changes in gene expressions, investigating complexes involved in repairing DNA damage, and creating physical/functional interaction networks for an organism [Shannon et al., 2003].

5. RESULTS

The main purpose of this thesis was to generate tools that would enable us to discover new proteins that are associated with the circadian clock. Firstly, we selected *CRY1* and *BMAL1* genes for two different approaches. As the main purpose of this thesis, we used the APEX2 enzyme fused to dCas9 so we could target APEX2 to the promoter of *BMAL1* gene using two specific sgRNAs. This allowed us to label proteins/transcription factors with biotin molecules on the promoter region of *BMAL1* and purify them using streptavidin-coated magnetic beads to identify them through mass spectrometry. Secondly, we aimed to insert a biotin ligase enzyme (called miniTurbo) before the start codon of *CRY1* and *BMAL1* using CRISPR/Cas9-mediated genome editing so we could have a tool to identify proximity labelled proteins (interactomes) of *CRY1* or *BMAL1* interactome in future studies.

5.1. Knock-in into the Mammalian Cells Using CRISPR-Cas9-HDR Method

To insert a 28 kDa enzyme coding sequence, such as miniTurbo coding sequence, 5' and 3' homology arms must be around at least size of 500 bp for an efficient insertion. We cloned miniTurbo coding sequence and 5' and 3' homology arms for *CRY1* or genetic loci into the pGEX4T1 vector as the carrier. The reason for choosing the pGEX4T1 vector is that it would not express miniTurbo enzyme even if the coding sequence is inserted in the genome randomly. To generate the pGEX-4T1-based donor vector, the miniTurbo sequence was amplified from the original vector (3xHA-miniTurbo-NLS_pcDNA3 plasmid from Addgene with Cat No: 107172). Then the 5' and 3' HDR arms were amplified from U2OS cell genomic DNA and inserted sequentially to the 5' and 3' ends of miniTurbo coding sequence in pGEX-4T1 using restriction enzyme digestion and T4 ligase-based cloning technique. This was a short description of the knock-in process for *CRY1*, a more detailed of knock-in procedure of miniTurbo enzyme coding sequence into *CRY1* and *BMAL1* genes is given below.

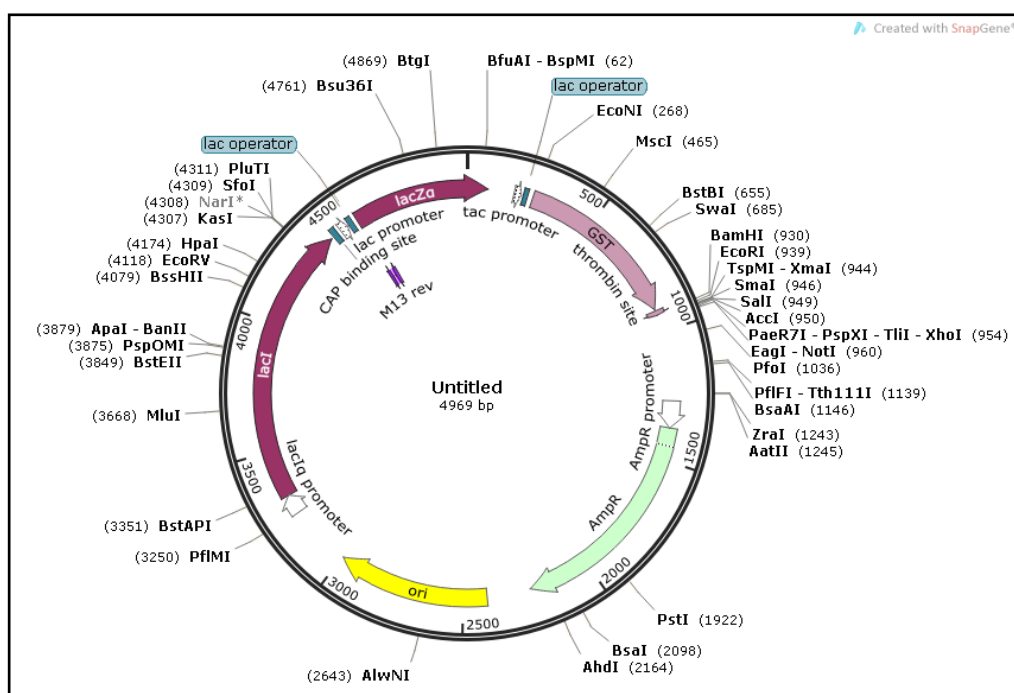


Figure 5.1: pGEX-4T1 vector map. (The plasmid map was generated using SnapGene Viewer Program).

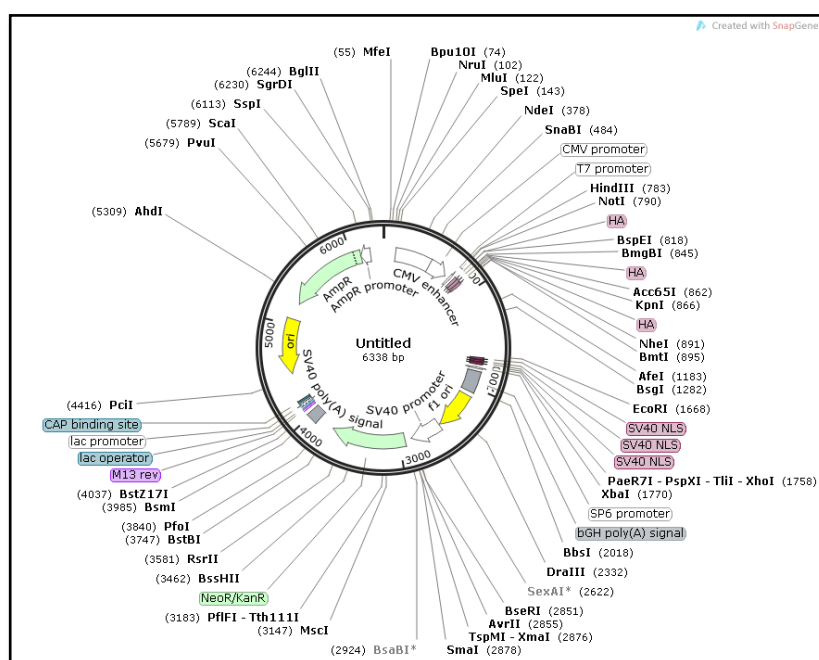


Figure 5.2: 3xHA-miniTurbo-NLS_pcDNA3 vector. (Addgene Cat No: 107172).
(The plasmid map was generated using the SnapGene viewer program).

5.1.1. Designing of Donor Plasmids for *CRY1* and *BMAL1* genes

pGEX-4T1-HDR-3xHA-MiniTurbo-13xLinker-CRY1:

The cloning strategy is given in figure 5.3. MiniTurbo biotin ligase was inserted in front of the 3XHA-CRY1 coding sequence. To create to this construct; (1) pGEX-4T1 vector was cut with BamHI and SalI enzymes. (2) HDR1 (5' arm of CRY) was amplified with cry-HDR1_F and cry-HDR1_R and HDR2 (3' arm of CRY) was amplified with cry-HDR2_F and cry-HDR2_R. Then HDR1 and HDR2 were inserted into the pGEX-4T1 plasmid. (3) 3xHA-miniTurbo-13xLinker was amplified from pAc5.1-3xHA-miniTurbo-13xLinker-V5-dCRY with Xho-MT_F and Xho-MT_R oligos and the amplified region was inserted into the pGEX-4T1-HDR-HDR2 plasmid. Thus, we obtained the pGEX-4T1-HDR-MiniTurbo-13xLinker-CRY1 plasmid.

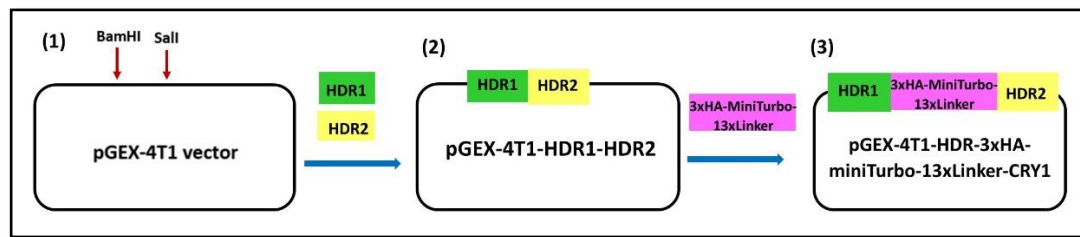


Figure 5.3: Schematic visualization of the pGEX-4T1-HDR-3xHA-MiniTurbo-13xLinker-CRY1 plasmid.

pcDNA3-HDR-3xHA-MiniTurbo-13xLinker-Bmal1:

The cloning strategy is schematized in figure 5.4 the donor plasmid was designed to insert miniTurbo biotin ligase near the ATG codon of the *BMAL1* gene. To create this construct; (1) HDR1 (5' arm of Bmal1) was amplified with BMAL1-HDR_F and KpnLnkrBio_R oligos and HDR2 (3' arm of BMAL1) was amplified with EcoRI_F and XhoI_R. Then HDR1 and HDR2 were inserted into the 3xHA-miniTurbo-NLS_pcDNA3 plasmid. Thus, we obtained the pcDNA3-HDR-3xHA-MiniTurbo-13xLinker-Bmal1 plasmid.

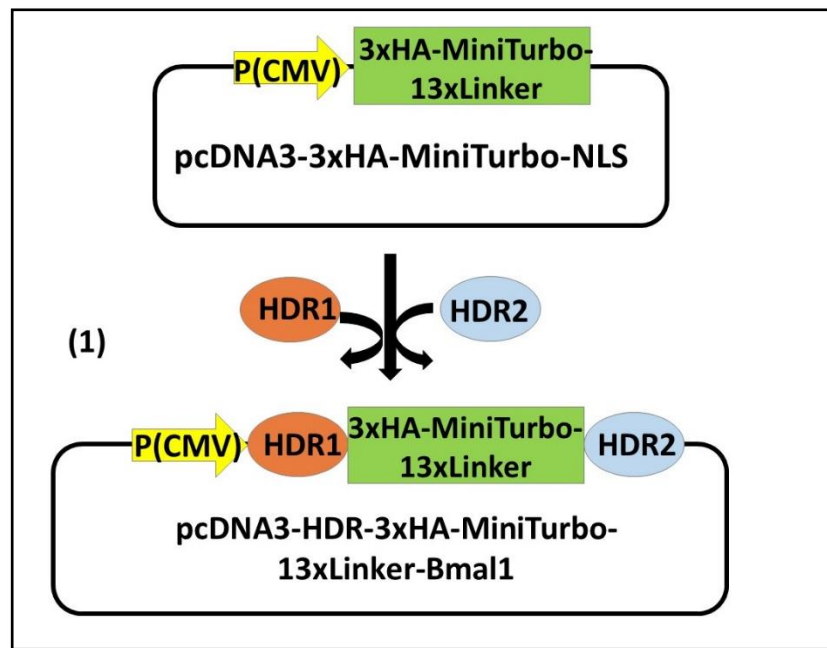


Figure 5.4: Schematic visualization of the pcDNA3-HDR-3xHA-MiniTurbo-13xLinker-Bmal1 plasmid.

The restriction analysis (RE analysis) method was used to confirm the candidate colonies which had correctly ligated constructs during the cloning steps. Figure 5.5 (A) is the RE analysis of the pGEX-4T1-HDR-3xHA-miniTurbo-13xLinker-CRY1 plasmid clones. In this RE analysis, the expected bands in positive clones were determined as 209 and 445 bp. The RE products were separated on a 2% agarose gel and expected bands C2 and C3 were observed (red rectangle). Figure 5.5 (B) is the RE analysis of the pcDNA3-3xHA-MiniTurbo-13Xlinker-Bmal1 plasmid clones. In this RE analysis, the expected band in the positive clone is about 2 kb. The RE products were separated on a 1% agarose gel and expected bands were observed in C1 and C2. The correct colonies were selected, and these colonies were co-transfected alongside LentiCRISPRv2.Bm-MT or LentiCRISPRv2.Cr-mT (which causes double-strand breaks at the knock-in sites for *BMAL1* and *CRY1* loci, respectively) into HEK 293T cell line.

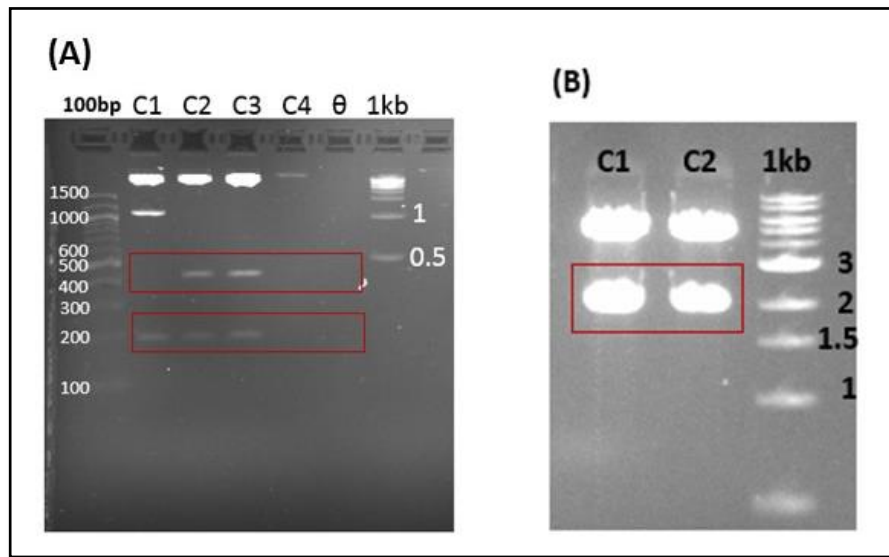


Figure 5.5: Restriction analysis of donor plasmid candidates with a restriction enzyme. (A) RE analysis of HDR-MT-CRY1 candidates with *EcoRI* digestion. Column 1; 100 bp DNA ladder. Column 2-5; probable HDR-MT-CRY1 plasmid clones. Column 6; empty well. Column 7; 1 kb DNA ladder. (B) RE analysis of HDR-MT-Bmal1 candidates with *NotI*-*XhoI* digestion. Column 1-2; probable HDR-MT-Bmal1 plasmid clones. Column 3; 1 kb DNA ladder.

5.1.2. Transfection for Knock-in

For knock-in processing with the CRISPR-Cas9-HDR method, Lenti-CRISPR.v2 plasmids (expressing Cas9 and sgRNAs) and linearized donor plasmids were co-transfected into HEK 293T cells. Lenti-CRISPR.v2 was used to generate double stranded breaks (DSBs) in the endogenous *CRY1/BMAL1* genomic loci while the plasmid HDR-MT-CRY1 and HDR-MT-BMAL1 donor plasmids were used to generate a repair template which can be used for HDR.

Figure 5.6 (A) shows the Lenti-CRISPR.v2-Cr-MT and pGEX-4T1-HDR-3xHA-miniTurbo-13xLinker-CRY1 plasmids were co-transfected into HEK 293T cells. The transfection was done as described in section 4.2.2. In order to check whether the transfection occurred and whether the plasmids were working, their expression at the protein level was examined with this immunoblotting technique. The numbers on the clones are defined as follows: The first numbers refer to the clone numbers of the Lenti-CRISPR.v2-Cr-TM plasmid, while the second numbers refer to the numbers of the selected pGEX-4T1-HDR-3xHA-miniTurbo-13xLinker-CRY1 plasmid. In anti-HA tag blotting we can only see MT-CRY because the HA

tag is not included in the endogenous/un-edited CRY1, while in CRY1 blotting, we can see both endogenous and recombinant CRY1. These samples were loaded in duplicates and checked separately with both anti-HA Ab and anti-human CRY1 Ab. When we look at the endogenous CRY1 band, it was observed that expression was high level in WT, while expression was decreased if the double strand cuts caused the indel instead of HDR. One reason that expression is not completely lost here is that the cells used for immunoblotting were from a polyclonal pool. Even though, MT-CRY1 band was merged with a cross-reacting band we could differentiate the recombinant protein from the cross-reacting band based on the intensity.

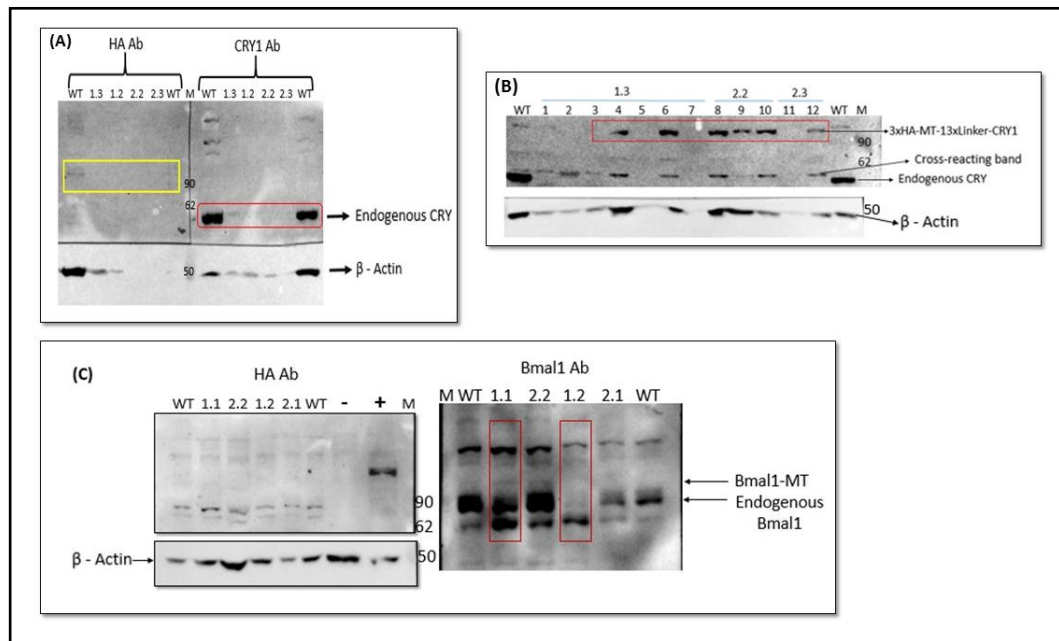


Figure 5.6: Screening of HDR-MT transient candidates in HEK 293T cells by WB. (A) Screening of MT-CRY1 knock-in candidates. The portion marked with the red rectangle indicates endogenous CRY1. (B) Screening of HDR-MT-CRY1 transient candidates in HEK 293T cells. CRY1 weighs about 62 kDa. MT-CRY1 weighs approximately 100 kDa. (C) Screening of MT-BMAL1 knock-in candidates in HEK 293T cells. (Courtesy of Senanur OLFÄZ in the Biological Clock Laboratory). BMAL1 weighs about 78 kDa. MT-BMAL1 weighs approximately 112 kDa. As we could not detect a clear knock-in for BMAL1 (weak bands were indicated as Bmal1-MT), we analyzed candidates with PCR for a possible very low knock-in as described below in the text. Actin, that used as a loading control, weighs about 46 kDa. M, Prestained Molecular Weight Marker (PM6).

The transfection process was repeated by increasing the ratio of the donor plasmids for MT-CRY1 plasmid, and the WB technique was applied to control the

protein expression of growing cells. Figure 5.6 (B) shows the immunoblotting results are included as stated in Figure 5.6 (A), there are 4 different combinations of two different plasmids (1.2, 1.3, 2.2, 2.3). The cells were transfected with the same combinations as indicated in section 4.2.2. and to obtain clearer results, the gene pool was shrunk a little and subclones of these clones were obtained and immunoblotting was performed. In these results, endogenous CRY1 was knockout in all clones and MT-CRY1 expression was seen in clones 4, 6, 8, 9, 10, 12.

Figure 5.6 (C) shows the results of the experiment that the Lenti-CRISPR.v2-Bm-MT and pcDNA3-3xHA-MiniTurbo-13Xlinker plasmids were co-transfected into HEK 293T cells. The transfection was done as described in section 4.2.2. To check whether the transfection occurred and whether the plasmids were working, their expression at the protein level was examined with this immunoblotting technique. The numbers on the clones are defined as follows. The first numbers refer to the clone numbers of the Lenti-CRISPR v2-Bm-MT plasmid, while the second numbers refer to the numbers of the selected HDR-MT-Bmal1 plasmid. In anti-HA tag blotting, we expected to see only MT-BMAL1 because the HA tag is not included in the endogenous BMAL1, while we expected to see both endogenous and recombinant BMAL1 in anti-BMAL1 blotting. These samples were loaded in duplicates and checked separately with both anti-HA tag antibody and anti-BMAL1 antibody. Looking at the results, we observed that Lenti-CRISPR v2-Bm-MT C1 was working and endogenous *BMAL1* is knockout as 1.1 and 1.2 (red rectangle). For the knock-in of MT into BMAL1, very weak bands could be seen in Figure 5.6 C, where samples 1.1 and 1.2 were as labelled as Bmal1-MT to indicate the possible recombinant protein signal. However, the results were not conclusive. Because we could not see miniTurbo knock-in into the *BMAL1* gene in HEK293T cells clearly, we wanted to check if the knock-in occurred but at very low efficiency. In other words, because HEK293T knock-in study was a relatively transient process with samples from polyclonal cells, we analyzed the possible knock-ins using RE analysis as described below.

5.1.3. PCR/RE-based Analysis of Knock-in Candidates in HEK 293T Cells

Instead of proceeding with single colony pickup and screening many clones with immunoblotting from HEK293T cells, we just analyzed the presence of the knock-in in clones using PCR analysis so we can proceed with knock-in process in U2OS cells which is the final aim for the knock-in procedure in this thesis for BioID. The genomic DNA of the selected clones (both HDR-MT-CRY1 and HDR-MT-Bmal1) were isolated and PCR was performed with the appropriate primers. First, PCR was performed with all HDR-MT-CRY1 clones, and then clones 1, 2, and 3 were selected, carrying the bands more clearly. Then, a clear result was obtained with these three clones after PCR optimization.

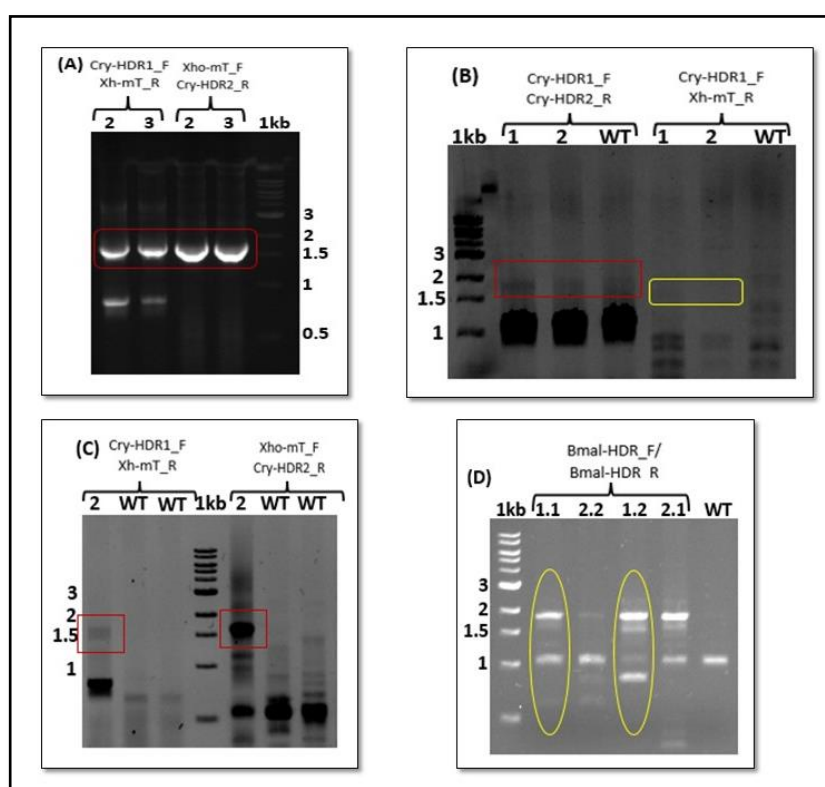


Figure 5.7: Confirmation of MT in *CRY1/BMAL1* locus in HEK 293T cells using *Taq* DNA polymerase. (A), (B), and (C) PCR of MT in *CRY1* locus using *Taq* DNA polymerase. (D) PCR of MT in *BMAL1* locus using *Taq* DNA polymerase. Samples were run on 0.8% gel. 1kb DNA marker was used as a ruler.

Figure 5.7 (A) is PCR analysis for MT in *CRY1* locus using *Taq* DNA polymerase for HDR-MT-CRY1 C2 and C3 in HEK 293T cells. Two different

primer pairs were used for clone 2 and 3. The first of these is Cry-HDR1_F/Xh-mT_R pair that amplifies the 5' arm of CRY- miniTurbo coding sequence. This region is approximately 1.5 kb. The second primer pair is Xho-mT_F/Cry-HDR2_R pair that amplifies the region starting from miniTurbo to 3' arm of CRY1. This region is approximately 1.5 kb. Expected bands were seen in both clones with PCR results. Figure 5.7 (B) is PCR for MT in CRY1 locus using *Taq* DNA polymerase for HDR-MT-CRY1 C1 and C2 at HEK 293T cells. Two different primer pairs were used for clone 1 and 2. The first primer couple was Cry-HDR1_F/Cry-HDR2_R pair that amplifies the 5' arm of CRY1 to 3' arm of CRY1. This region is approximately 2 kb. The gDNA was obtained from a polyclonal pool of cells. Therefore, some cells carry the knock-in, while others do not. Therefore, when PCR is performed with this primer pair, if MiniTurbo is located in the CRY1 locus, the band can be seen as approximately 2 kb. On the other hand, if there is no MiniTurbo in the CRY1 locus, the band can be seen at about 1 kb. For this reason, we could see both bands, but our main goal was to see the expected bands more dominantly than the other bands. As a result of PCR, bands were observed in both areas. The presence of bands in the section marked with the red rectangle proved the presence of MiniTurbo in the CRY1 locus, but the predominance of the band in the unexpected region indicates that more single cells should be selected from these clones. The second primer pair is Cry-HDR1_F/Xh-mT_R pair and replicates the region starting from 5' arm of CRY1 to MiniTurbo. This region is approximately 1.5 kb. The expected band was seen very slightly (yellow rectangle). It might be due to poor PCR conditions. Clone 2 was selected based on the result of previous PCRs. Figure 5.7 (C) is PCR performed once more for the primer pairs previously used to confirm for HDR-MT-CRY1 C2.

Figure 5.7 (D) is PCR for MT in *BMAL1* locus using *Taq* DNA polymerase in HEK 293T cells. PCR was established with appropriate primers using genomic DNA from all clones (1.1, 2.2, 1.2, and 2.1). One primer pair was used for clones that Bmal-HDR_F/Bmal-HDR_R that amplifies the 5' arm of BMAL1 to 3' arm of BMAL1. This region was approximately 2 kb. When PCR samples were run on 0.8% agarose gel, the expected band could be seen in clone 1.1 and 1.2 (in agreement with weak Bmal1-MT protein band in these clones). After confirming that knock-in for both *CRY1* and *BMAL1* locus was successful at least based on the PCR from gDNA of transiently transfected polyclonal cells. Then we sought to obtain knock-in clones in U2OS because the circadian clock can be reset in this cell line but not in

HEK293T cells. However, U2OS is difficult to make for genome editing while HEK293T is easy to use for genome editing studies.

5.1.4. Transfection and Biotinylation Activity of HDR-MT in U2OS Cell Line

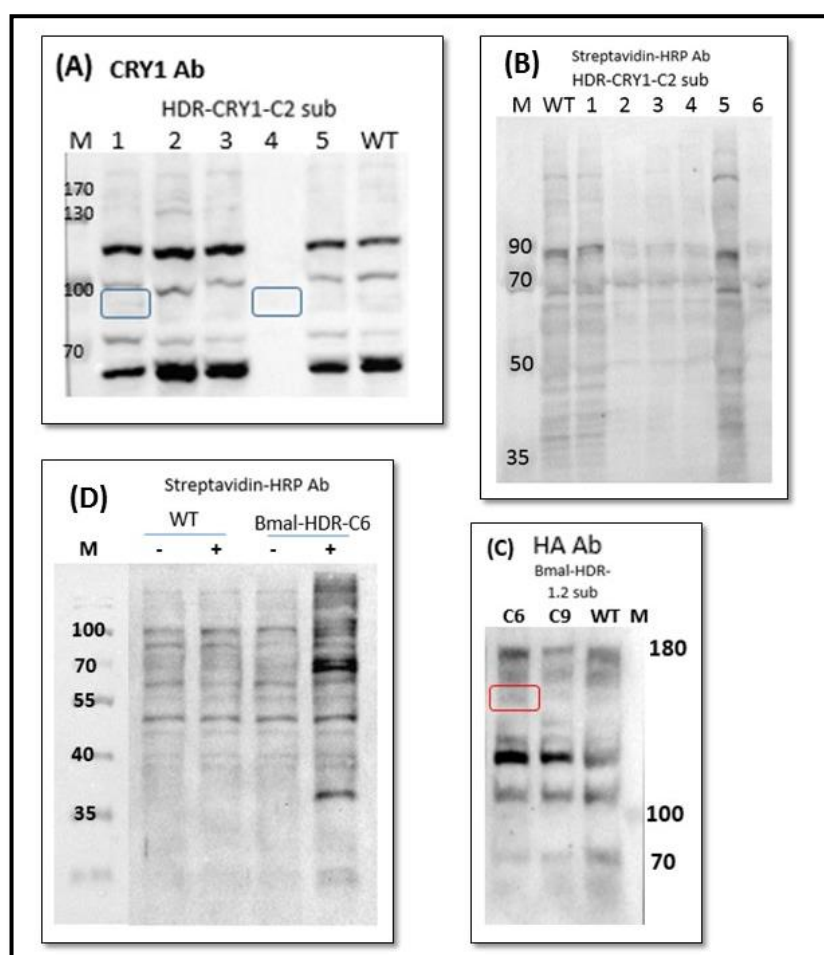


Figure 5.8: Screening of HDR-MT subclones in U2OS cells. (A) Testing MT knock-in into *CRY1* using anti-*CRY1* blot (Courtesy of Senanur Olfaz). (B) Biotinylation activity of MT-*CRY1* as analyzed with streptavidin-HRP immunoblotting. (C) Testing MT knock-in into *BMAL1* as analyzed with anti-HA blot. (D) Biotinylation activity of MT-*BMAL1* as analyzed with streptavidin-HRP immunoblotting. In this figure, the - symbol indicates that the cells were not incubated with biotin, while the + symbol indicates that the cells were supplemented with biotin. M indicates the prestained molecular weight marker (PM7).

Figure 5.8 (A) confirms the MT-*CRY1* expression or MT knock-in in *CRY1* gene. Although there were non-specific bands, we saw the expected band (approximately 100 kDa) in clone 1 (blue rectangle). Although clone 4 has a very

small amount of protein, the presence of a light band at the expected location (blue rectangle) also indicates this clone had the knock-in. Figure 5.8 (B) shows the biotinylation activities of the cells based on Streptavidin-HRP blotting. Biotinylation activity was observed in clones 1 and 5, but the appearance of a similar profile in wild-type cells prevented us from reaching a clear conclusion. Therefore, based on Figure 5.8, C1, C3, and C4 were selected to check whether there was a MiniTurbo in the *CRY1* locus by PCR.

Then protein expressions and biotinylation activities were examined with immunoblotting for MT knock-in in *BMAL1* gene. Figure 5.8 (C) shows the MT-BMAL1 expression in two clones (red rectangle in C6). Figure 5.8 (D) Shows the biotinylation activities of the MT-BMAL1 in C6 as determined by Streptavidin-HRP immunoblotting. As a result of the studies, C6 was selected to check whether the MT knock-in could be also detected in *BMAL1* locus by PCR.

5.1.5. Confirmation of MiniTurbo knock-in into *CRY1* and *BMAL1* Locus in U2OS Cells

Genomic DNA isolation of selected clones (C1, C3, and C4) of the HDR-MT-*CRY1* C2 subclones was performed. Knock-in and its surrounding relevant sequence were amplified using appropriate primers. From all clones, clones 1, 3, and 4 were selected, the band corresponding to the knock-in was prominent. In figure 5.9 (A) two different primer pairs were used for clones 1, 3, and 4. The first of these is Cry-HDR1_F/Xh-mT_R pair that amplifies the 5' arm of *CRY1* to MiniTurbo. This region corresponds to a DNA of 1.5 kb. The expected band was seen in both clones 1 and 3 in the PCR result (red ellipse). The second primer pair is Xho-mT_F/Cry-HDR2_R that amplifies the region starting from MiniTurbo and up to 3' arm of *CRY1*. This region covers 1.5 kb sequence. The expected band was seen in only clone 1 in the PCR analysis.

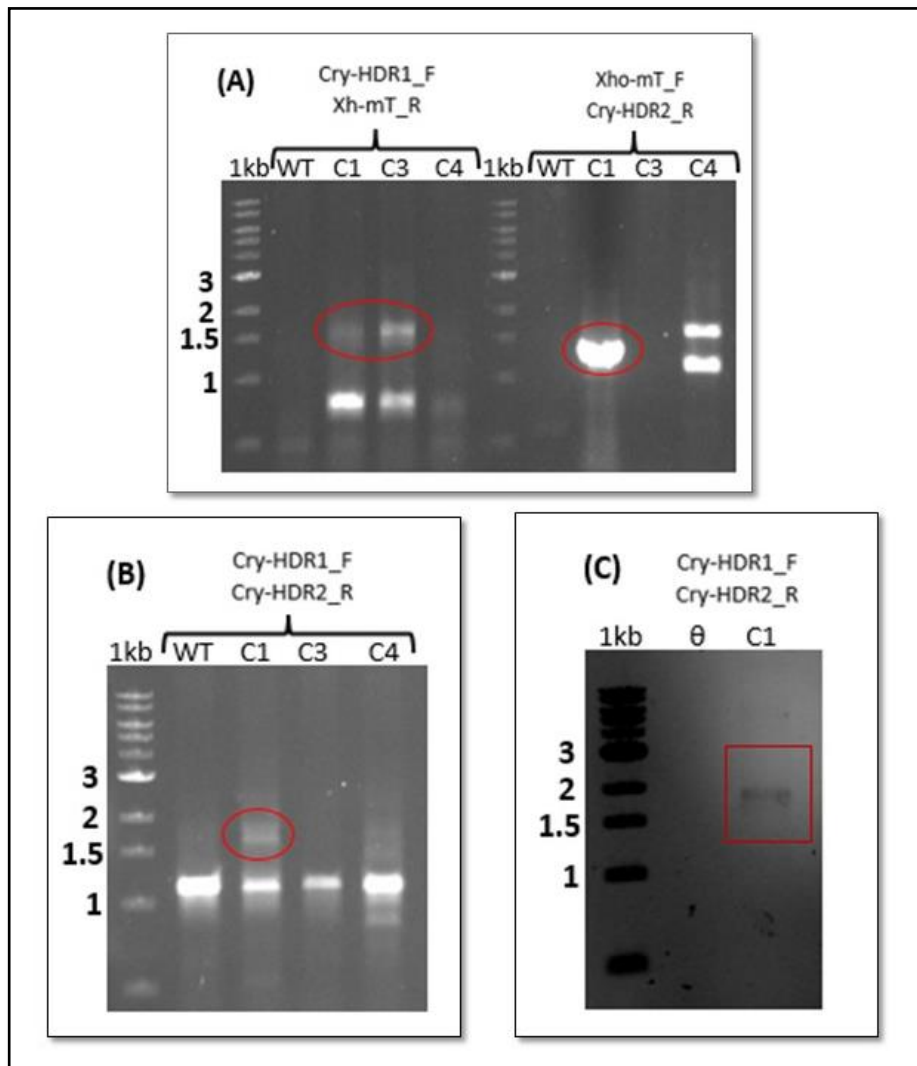


Figure 5.9: Confirmation of MT in *CRY1* locus in U2OS cells using *Taq* DNA polymerase. Samples were run on 0.8% gel. 1kb is the DNA marker.

In figure 5.9 (B) one primer pair was used for clones 1, 3, and 4. The primer couple Cry-HDR1_F/Cry-HDR2_R amplified the 5' arm of *CRY1* to 3' arm of *CRY1*. This region is approximately 2 kb. As mentioned in Figure 5.7 (B), while the band we expect is 2kb, it amplifies a 1-kb fragment if MT is not inserted into *CRY1* locus. The bands corresponding to knock-in were observed in clone 1. The presence of bands in the section marked with the red ellipse proved the presence of miniTurbo in the *CRY1* locus. In figure 5.9 (C), C1 is selected according to the PCR analysis and PCR was repeated in large volumes for sequence analysis. The amplified region covers the part starting from 5' arm of *CRY1* and up to 3' arm of *CRY1*.

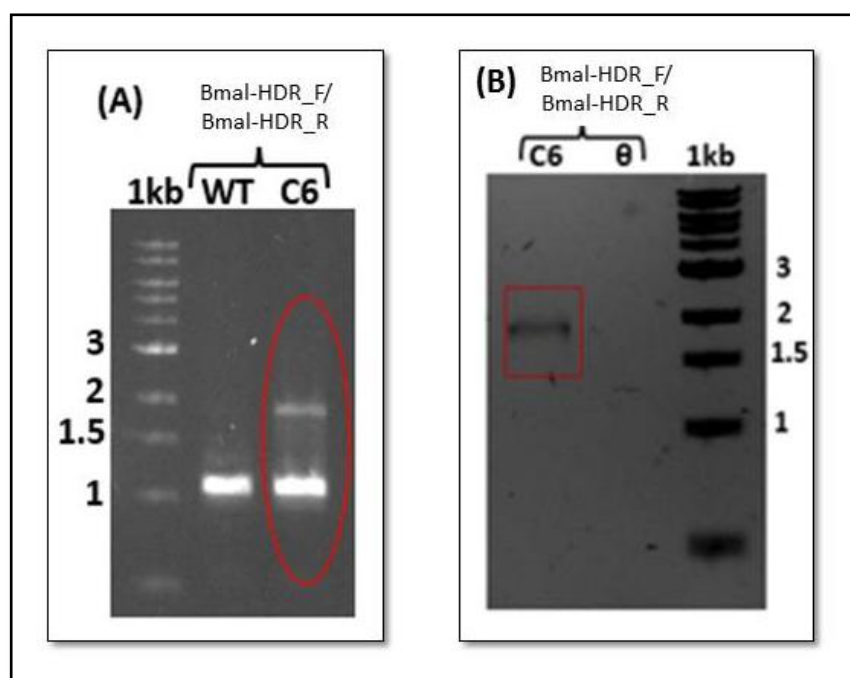


Figure 5.10: Confirmation of MT knock-in *BMAL1* locus in U20S cells. (A) PCR of MT in *BMAL1* locus using Taq DNA polymerase. (B) PCR of MT knock-in *BMAL1* locus using Taq DNA polymerase for sequence analysis. 0.8% agarose gel. 1 kb is the DNA ladder.

Genomic DNA was isolated from C6 from MT knock-in in *BMAL1* gene candidate, and relevant knock-in and its surrounding sequence were amplified with appropriate primers. In figure 5.10 (A) the amplification of knock-in in C6 is shown. The primer pair Bmal-HDR_F/Bmal-HDR_R amplifies a sequence corresponding to a fragment starting from the 5' arm of *BMAL1* to 3' arm of *BMAL1*. This region is approximately 2 kb. When PCR samples were run on 0.8% agarose gel, the expected band was seen in clone C6 (red ellipse). We amplified the corresponding band and isolated from gel for sequencing as shown in figure 5.10 (B).

5.2. CASPEX System for Discovery of Proteins in *BMAL1* Promoter

The inducibility CASPEX system is advantageous because it prevents the disturbing the native structure of targeted genomic locus. In our system, sgRNA expression was constitutively expressed. For the constitutive sgRNA expression, we generated the LentiCRI-sgRNA-Zeo plasmid in our laboratory. Briefly, the Cas9

cassette was removed from LentiCRISPRv2 and puromycin resistance cassette was replaced with a zeocin resistance cassette from Lenti-sgRNA (MS2) zeo backbone (Addgene plasmid #61427). Plasmids that were used in this project are outlined in Figure 5.11. Inducible CASPEX is puromycin resistant and stable candidates were selected for the best inducibility. Then, sgRNA plasmids were transfected/infected and selected with zeocin to obtain stables with different sgRNAs to scan genomic locus.

After CASPEX transfection, puromycin was added to cells a final concentration of 0.5 µg/ml and those carrying the target sequence were selected. The cells were checked for the doxycycline-inducibility of dCas9-APEX2 using anti-FLAG antibody immunoblotting. Subsequently, sgRNA constructs were transfected into selected CASPEX-HEK 293T stable cell line and zeocin was added to a final concentration of 200 µg/ml.

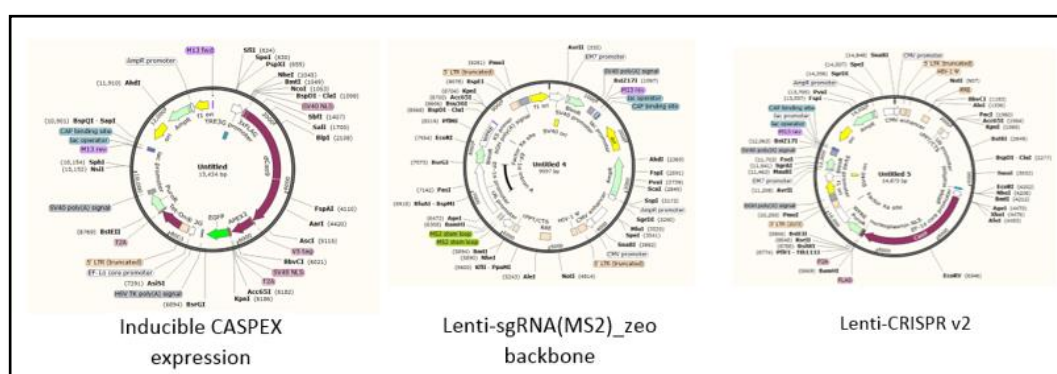


Figure 5.11: Maps of plasmids that were used in the CASPEX system. Inducible CASPEX and Lenti-sgRNA (MS2) zeo backbone and Lenti-CRISPR-v2 were obtained from Addgene. Plasmid maps were generated using the SnapGene viewer program.

5.2.1. Generation of Lenti-CRI-sgRNA-zeo-Bmal1_T1/Lenti-CRI-sgRNA-zeo-Bmal1_T2 Plasmids

Specific sgRNAs (Bmal1_T1 and Bmal1_T2) were used to guide catalytically inactive Cas9 (dCas9)- APEX2 onto promoter of *BMAL1* gene to biotinylate proteins in close proximity. T indicated target, and the sequence of T1 and T2 is given previously [Korkmaz et al. 2018]. LentiCRI-sgRNA-Zeo plasmid was linearized and

sgRNAs corresponding to two targets on *BMAL1* gene close to the ATG start codon were inserted to this plasmid.

The restriction analysis method was used to determine the correct colony during the cloning steps. Figure 5.12 (A) is RE analysis of the Lenti-CRIS-sgRNA-zeo-Bmal1_T1 plasmid clones. In this RE analysis, the expected band in positive clone was determined as 651 bp. and the expected band was observed at C1 (red rectangle). Figure 5.12 (B) is RE analysis of the Lenti-CRIS-sgRNA-zeo-Bmal1_T2 plasmid clones. In this RE analysis, the expected band in positive clones were determined as 175 and 651 bp. and expected bands were observed at C1 and C2 (red rectangle). Here, both the clones were cut with the restriction enzyme and uncut sample was also run next to them.

The Bmal1_T1 C1 plasmid and Bmal1_T2 C1 and C2 plasmid were transfected into a HEK 293T cell line to check whether they work. Figure 5.12 (C) shows the immunoblotting of Lenti-CRI-sgRNA-zeo-T1 and -T2 co-transfected transiently with a wild-type Cas9 expressing plasmid (pLenti-Cas9-Blast) into HEK 293T cells. Lenti-CRI-sgRNA-zeo-T1 and -T2 plasmids were designed to target the sequence of *BMAL1* close to the ATG coding part. In figure, the first number refers to the targeted region of BMAL1 (T1 or T2), while the second number refers to the clone number of that plasmid. C1s for both plasmids were selected for stable transfection into HEK293T-CASPEX cell line.

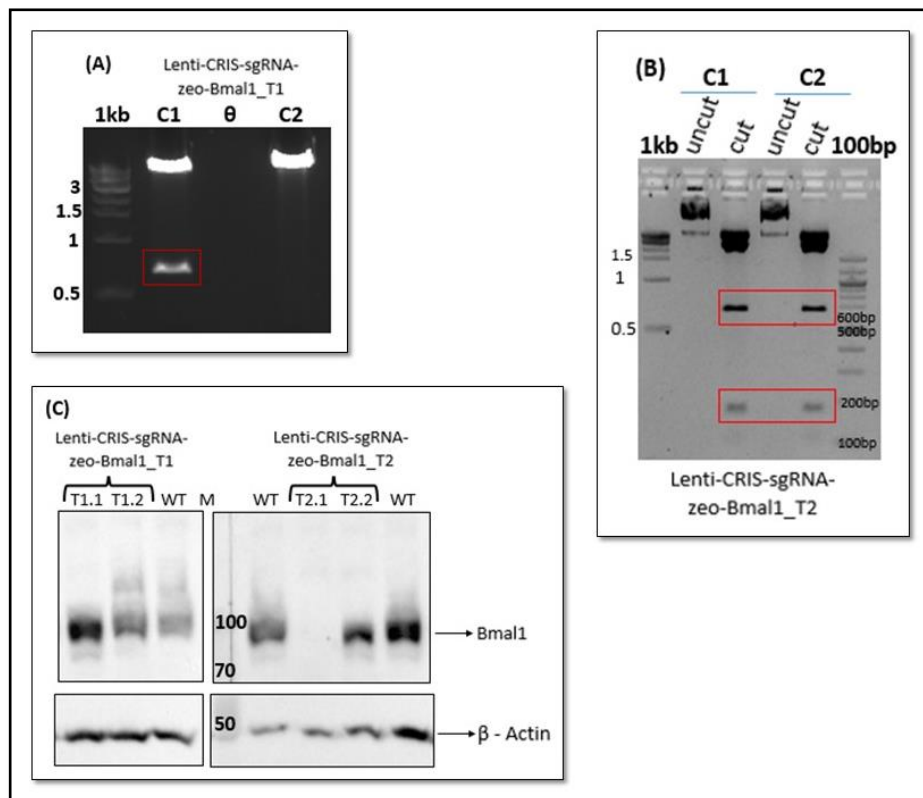


Figure 5.12: Restriction Analysis and Screening of Lenti-CRIS-sgRNA-zeo-Bmal1 clones in HEK 293T cells. (A) Restriction Analysis of Lenti-CRIS-sgRNA-zeo-Bmal1_T1 with EcoRI digestion. (B) Restriction Analysis of Lenti-CRIS-sgRNA-zeo-Bmal1_T2 with NdeI-EcoRI digestion. (C) Immunoblot of HEK293T cells after co-transfection Lenti-CRIS-sgRNA-zeo-sgRNAs for BMAL1 alongside wild-type Cas9. A decrease in BMAL1 protein intensity indicates that sgRNA were functional. M, prestained molecular weight marker (PM7); 1 k DNA ladder; 100 bp DNA Ladder. Actin was used to the loading control.

5.2.2. Inducible-CASPEX-HEK 293T System for Targeting Specifically *BMAL1* Locus

While preparing these sgRNA plasmids (Bmal1_T1 and Bmal1_T2), a control sgRNA which do not hit any loci was prepared. The CASPEX plasmid expresses the FLAG tagged-dCas9, and anti-FLAG antibody was used to check the induction of CASPEX following induction using Dox. Figure 5.13 (A) shows the immunoblot of induction of CASPEX candidates in HEK 293T cells. C1 was chosen for future studies.

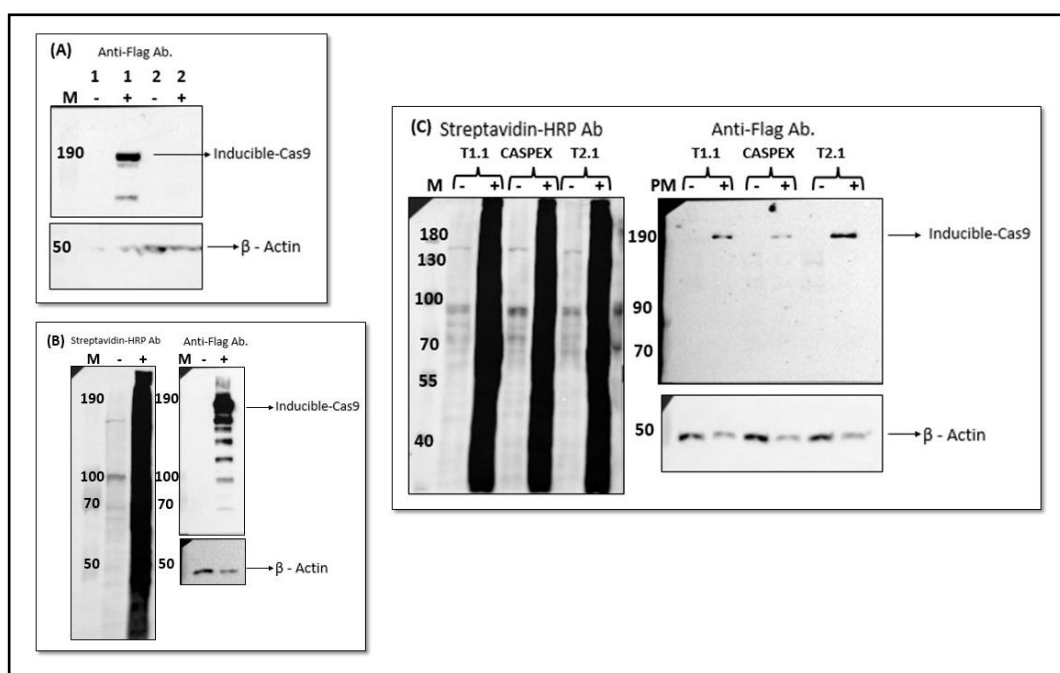


Figure 5.13: Screening of Inducible CASPEX in HEK 293T cells. (A) Immunoblotting of inducible CASPEX HEK293T candidates. dCas9-APEX2 fusion proteins run around 180 kDa. (B) Biotinylation activity of inducible CASPEX candidates. (C) Analysis of Lenti-CRI-sgRNA-zeo-Bmal T1/T2 -CASPEX-HEK293T cells. CASPEX between T1 and T2 samples correspond the CASPEX-HEK293T cell line with Lenti-CRI-sgRNA-zeo-NT (non-targeting). - and + indicate Dox treatment. PM homemade prestained molecular weight marker (PM7), M indicates commercial prestained molecular weight marker.

After controlling the induction of dCas9, we checked the biotinylation activity by APEX2. Figure 5.13 (B) shows the biotinylation activity of the Inducible CASPEX-HEK 293T cells. Figure 5.13 (C) shows screening of cells after the transfection of Lenti-CRI-sgRNA-zeo-Bmal T1 and T2 plasmids into inducible CASPEX -HEK 293T cell line. According to results, stable transfection of sgRNA plasmids did not affect CASPEX system and seemingly system worked well for biotinylation. Lenti-CRI-sgRNA-zeo-NT plasmid expressed a non-targeting sgRNA.

5.2.3. Preparation of Samples for Mass Spectrometry

Samples were prepared in large volumes to identify proteins in close proximity of dCas9-APEX2 when it targets to T1 or T2 sites on *BMAL1* locus. Samples for mass spectrometry were prepared as described in section 4.7. After the samples were induced with Dox overnight, biotinyl tyramide was added into culture media and

incubated for 30 minutes. A 1-min incubation with H₂O₂ completed the biotinylation in the vicinity of APEX2. Biotinylation was confirmed before enrichment using streptavidin-coated magnetic beads. Figure 5.14 (A) shows the immunoblotting of samples before sending for MS. Biotinylated proteins were enriched/pulled down on streptavidin-coated magnetic beads for on bead digestion with trypsin and identification through mass spectrometry.

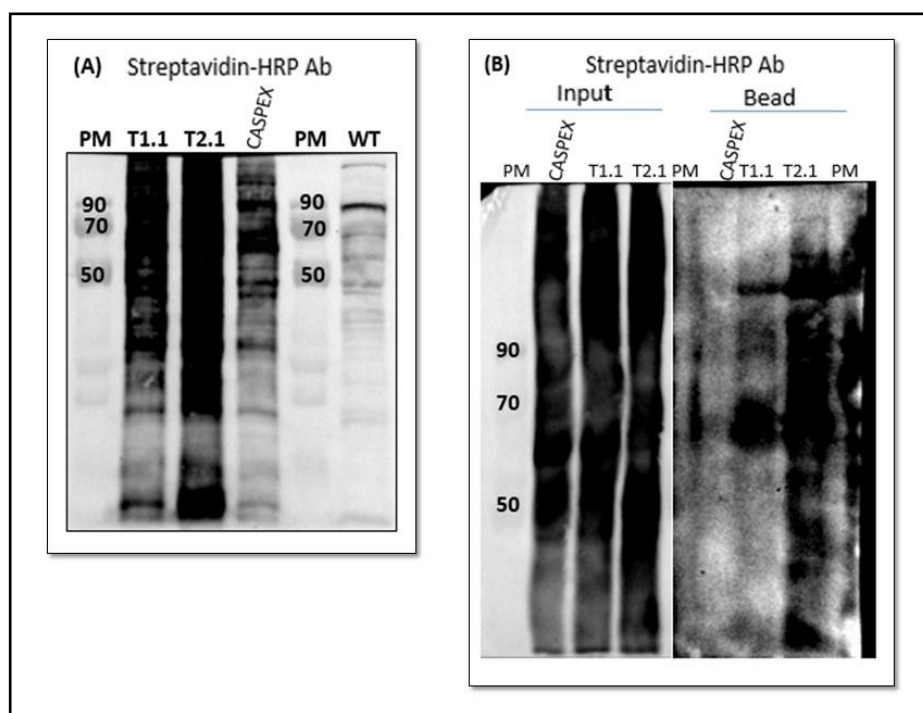


Figure 5.14: Screening of MS Samples. (A) Screening of Large Scale MS Sample before enrichment. (B) Immunoblotting of biotinylation in cell lysates and on-bead samples before submitting for mass spectrometry. PM, Homemade prestained protein marker (PM7). CASPEX indicates that non-targeting sgRNA expression CASPEX-HEK293T cell line.

5.2.4. Analysis of Mass Spectrometry Results

The results from mass spectrometry were analyzed using SAINTexpress software. T1 and T2 list were corrected against CASPEX (NT) list. Saint scores which were equal or above of 0.95 were considered as significant, and a list of proteins was created with a saint score of 0.95 and above (Appendix A). In this Appendix, the common proteins between T1 and T2 are given. T1 and T2 sequences are close so the list of common proteins between T1 and T2 indicates that these are

real interactions. Figure 5.15 shows the venn diagram created with T1, T2, and T1-T2 common lists from SAINTexpres analysis. This venn diagram was created using the InteractiVenn tool.

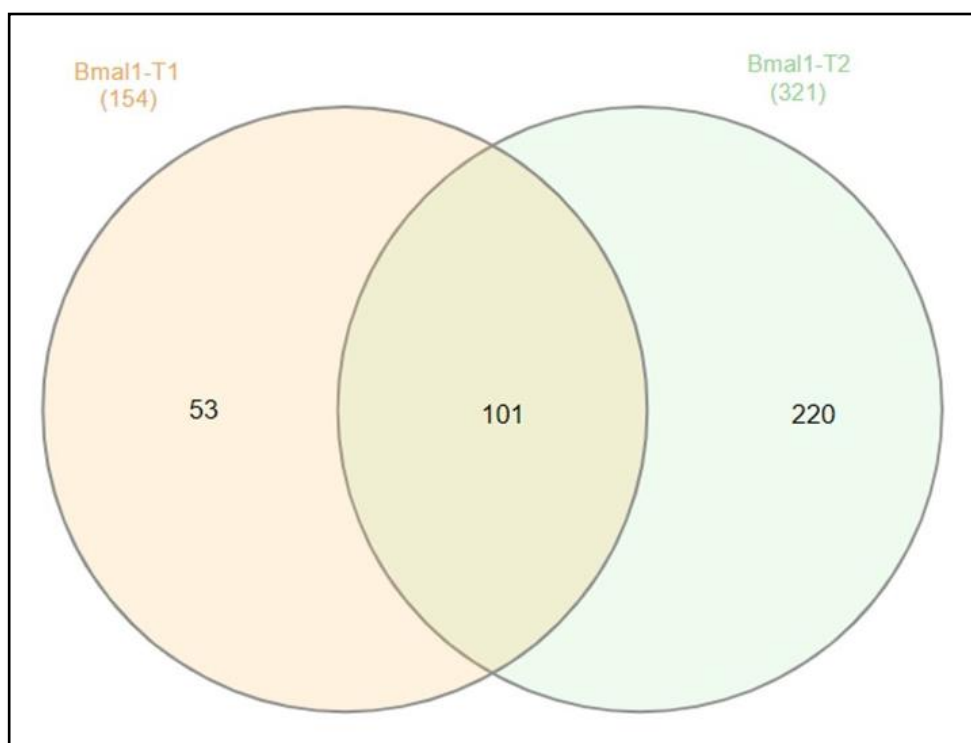


Figure 5.15: The venn diagram of the T1 and T2 protein lists obtained from SAINTexpress (SaintScore ≥ 0.95). The venn diagram was generated using the InteractiVenn tool.

The number of proteins common to T1 and T2 is 101. We created a network through Cytoscape with these proteins (Figure 5.16). This study aims to detect transcription-related proteins in target region, and T1 and T2 plasmids were designed to target around the promoter of *BMAL1* or just after the promoter. Thus, we expected to see proteins that mostly function on the transcription initiations. When we analyzed the network, we noticed that the presence of RNA polymerase and transcription factors confirm that our system was working. The list of six proteins selected from the network in this notion was given in Table 5.1. When we looked at the general functions of the proteins in this table, they all seemed involved in the transcription process.

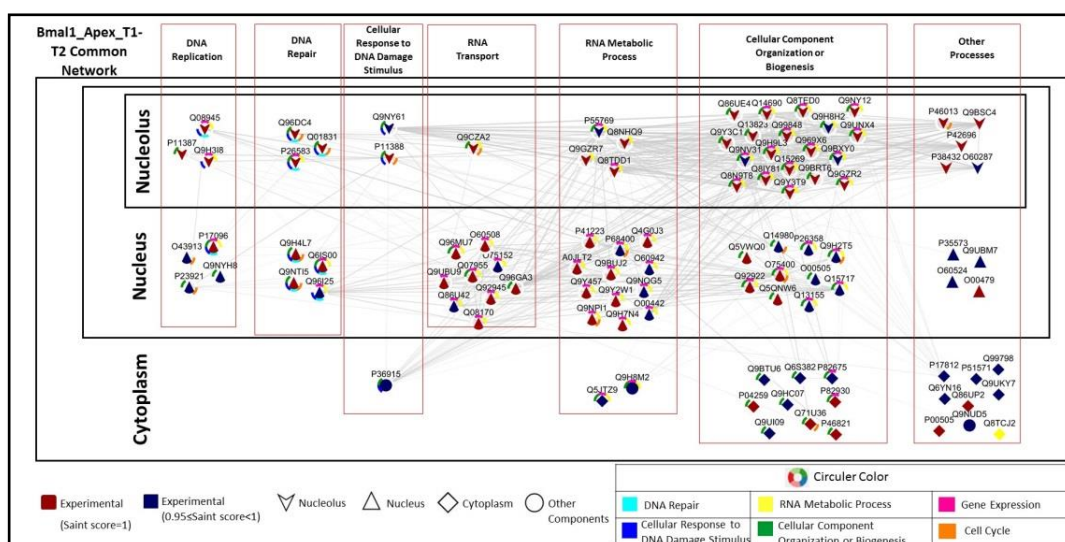


Figure 5.16: Protein network of T1 and T2 common proteins that was created using Cytoscape with StringApp algorithm.

Table 5.1: Selected proteins from T1-T2 common network.

UniprotID	Gene Name	Name	Saint Score
Q8N655	C10orf12 (LCOR)	Chromosome 10 open reading frame 12	1
A0JLT2	MED19	Mediator of RNA polymerase II transcription subunit 19	1
P68400	CSNK2A1	Casein kinase 2, alpha 1 polypeptide	<1
Q9Y2W1	THRAP3	Thyroid hormone receptor-associated protein complex 150kDa component	1
P11387	TOP1	Topoisomerase (DNA) I	1
P11388	TOP2A	DNA topoisomerase II, alpha isozyme	1

C10orf12 (LCOR) (Chromosome 10 open reading frame 12); Ligand-dependent corepressor protein is encoded by the *LCOR* gene in humans. It is able to act as a transcription activator and binds DNA with the sequence 5'-CCCTATCGATCGATCTCTACCT-3'. It functions as a repressor of ligand-dependent transcription activation by target nuclear receptors [Fernandes et al., 2003].

MED19 (Mediator of RNA polymerase II transcription subunit 19); Mediator complex subunit 19 (Med19) protein is encoded by the *MED19* gene in humans. It is a component of the Mediator complex, a coactivator involved in the regulated

transcription of nearly all RNA polymerase II-dependent genes. It functions as a bridgework to get over information from gene-specific regulatory proteins to basal RNA polymerase II transcription machinery [Allen and Taatjes, 2015]. The mediator is engaged to promoters by direct interactions with regulatory proteins and serves as a scaffold for the mounting of a functional preinitiation complex both RNA polymerase II and the general transcription factors [Bernecky et al., 2011].

CSNK2A1 (Casein kinase 2, alpha 1 polypeptide); Casein kinase II subunit alpha is an enzyme that in humans is encoded by the *CSNK2A1* gene [Lozeman et al., 1991]. The kinase is a tetramer consisting of 4 subunits. These subunits are an α -prime, and two β subunits. The two β subunits undergo autophosphorylation, while the α subunit is responsible for catalytic activity [Faust and Montenarh, 2000]. This enzyme plays a role in the regulation of many cellular processes such as cell cycle, apoptosis, transcription and circadian rhythm. It is involved in the regulation of transcription by directly involved in the phosphorylation of RNA polymerase I, II, III and IV [Meggio and Pinna, 2003]. In addition, it is involved in the phosphorylation of transcription factors such as NF- κ - β , STAT1, CREB1, and IRF1 [Miyata, 2009]. This enzyme also plays a role in the circadian clock function. It plays an important role in the interaction with CLOCK protein by phosphorylation of BMAL1. At the same time, this enzyme controls the nuclear entry of CLOCK [Bae et al., 2015].

CK2A is involved in the posttranslational modification of BMAL1. A mutation occurring in the 'Ser 90' region of the BMAL1 protein, or a mutation occurring in the region where the CK2A performs phosphorylation, leads to impaired accumulation of nuclear BMAL1 protein and disruption of CLOCK/BMAL1 dependent CCG expression [Tamaru et al., 2009]. In addition, the BMAL1 protein undergoes hyper-phosphorylation in CRY1 & 2 knockout mice which suggest that the CRY protein is involved in the rhythmic BMAL1 posttranslational modification [Lee et al., 2001], [Tamaru et al., 2003].

THRAP3 (Thyroid hormone receptor-associated protein complex 150 kDa component); Thyroid hormone receptor-associated protein 3 is a protein that in humans is encoded by the *THRAP3* gene. It is involved in the pre-mRNA splicing process and can trigger mRNA decay, which appears to be independent of nonsense-mediated decay and allows to recognize premature stop codons (PTC). It also plays a role in alternative splicing arrangements depending on the signal [Lee et al., 2010]. In addition, this protein might be involved in the positive regulation of the circadian

clock. By acting as a coactivator in CLOCK/BMAL1 dimerization, it supports transcriptional activator activity and binding to the target circadian clock genes [Lande-Diner et al., 2013].

TOP1 (Topoisomerase (DNA) I;_DNA topoisomerase 1 is an enzyme encoded by the *TOP1* gene in humans. This enzyme mediates the progress of transcription and replication by influencing the topological state of DNA during transcription that releases the supercoiling and torsional tension of DNA [Pommier, 2013]. It plays a role in the circadian transcription of the core circadian clock component BMAL1 by changing the chromatin structure around the ROR response elements (RORs) on the *BMAL1* promoter [Onishi and Kawano, 2012].

TOP2A (DNA topoisomerase II, alpha isozyme);_DNA Topoisomerase 2-alpha is encoded by the *TOP2A* gene in humans. This enzyme controls the topological states of DNA during transcription and replication and catalyzes the temporary breakage and recombination of a particular region in dsDNA [Tsai et al., 2000]. However, this enzyme may act to regulate the period length of the transcriptional oscillation of BMAL1 [Ogawa et al., 2014].

6. DISCUSSION

In line with the new developments in molecular biology, the field of discovering new interactions have been expanded. These developments have made it possible to illuminate the function of a protein, its location in the cell, its interactions, and the mechanisms, and even paved the way for studies that led to the discovery of new proteins. One of these developments, CRISPR-Cas9 technology can be used to regulate genes in organisms. It is a unique technology that allows adding or removing a specific gene region in the genome. This regulation process includes a wide range of application areas such as basic biological research and disease treatment. We combined CRISPR-Cas9 technology and proximity dependent labelling techniques in this thesis as explained below.

Various techniques are available for studying protein-protein interactions (PPI), such as yeast two hybrid and mammalian hybrid screening systems. However, these techniques do not detect interactions in their natural environment and therefore, their use is limited. Co-immunoprecipitation is a good tool to detect new interactions when combined with mass spectrometry, but it might fail to detect weak or transient interactions during the procedure. Another technique used for PPI is the proximity labeling technique. Proximity labeling is based on the principle that an enzyme fused to the protein of interest causes some covalent modifications in adjacent proteins which can be pulled down later for the identification with mass spectrometry. Various enzymes are used in this technique. For example, BirA* (a mutant form of BirA biotin ligase from *E. coli*) and its mutant and fast acting form, miniTurbo enzyme used biotin to label proteins in the close proximity. On the other hand, the APEX2 enzyme requires biotin phenol instead of biotin and hydrogen peroxide (H_2O_2) completes biotinylation and it requires only 30-min incubation in total. The proximity labeling technique can identify even weak and transient protein interactions with sub-circadian (less than 24 hours) biotinylation within the living cell. Therefore, the proximity labeling technique was used in this study to identify protein interactions.

Circadian clock regulates the physiology and biochemical activities of organisms. The disruption of the circadian clock is associated with many diseases such as cancer, diabetes, neurological disorders. Therefore, the identification of

proteins responsible for the regulation of the circadian clock proteins (either at the transcriptional level or proteins' interactions with other proteins) can contribute to the discovery of new proteins or interactions which can be targeted for therapeutic purposes.

In this thesis, we aimed to examine proteins related to circadian clock proteins using CRISPR-Cas9 technology and proximity labeling. For this purpose, we selected the *CRY1* and *BMAL1* genes located in the opposite arms of the molecular TTFL. We first designed a suitable plasmid to insert MiniTurbo biotin ligase in front of *CRY1* and *BMAL1* using CRISPR-Cas9 mediated genome editing tool. In this thesis, we just aimed to obtain miniTurbo knock-in *CRY1* and *BMAL1* genes which will be used for labelling and identification by mass spectrometry in future studies. On the other hand, in this thesis, we aimed to obtain a system to identify the proteins in the promoter of *BMAL1* gene using the dCas9-APEX (CASPEX) system. Transcriptional regulation is a coordinated process involving many proteins. In order to get a better understanding of transcriptional regulation and chromatin structure, the chromatin immunoprecipitation (ChIP) method is of great importance. This method is of great importance in understanding the transcriptional regulation of genes, including circadian clock genes. However, its use in mammalian cells is limited due to the need for ChIP specific antibodies and various disadvantages (such as loss of chromatin content, loci disruption). But recently, a biotechnological approach of CRISPR/Cas9 technology that uses dCas9 to target APEX2 enzyme with proximity labelling capacity to any genomic location has provided a very important tool to study transcriptional regulation. A method called CASPEX was applied to identify proteins associated with a specific genomic locus in vivo [Myers et al., 2018]. In CASPEX system, specific sgRNAs were used to direct the biotin ligase APEX2 enzyme fused dCas9 to the promoter of the *BMAL1* gene, and biotinylated proteins were precipitated using streptavidin-coated magnetic beads to identify them by mass spectrometry.

Within the scope of the thesis, two systems were designed by using the methods mentioned above. The first of these was to transfer the miniTurbo coding sequence (MT) in front of *BMAL1* and *CRY1* using the CRISPR/Cas9- mediated genome editing. To this end, suitable plasmids were prepared as outlined in section 5.1. The plasmids were then transfected into HEK 293T cells in order to see the efficiency of the knock-in process. Then U2OS cell line was used to obtain

miniTurbo knock-ins because this cell line has a functional or resettable circadian clock while HEK293T cells do not have an overt circadian rhythm. The knock-ins of miniTurbo in *CRY1* and *BMAL1* genes in U2OS cells will be starting material for the identification of CRY1 and BMAL1 interactome at the different phases of the circadian clock. However, before proceeding obtaining more clones especially with MT knock-ins in both alleles is important to get the best results. So far, we have obtained knock-ins only in one allele.

The second system was based on the Inducible-CASPEX system [Myers et al., 2018]. The main purpose here was to identify proteins on a certain part of *BMAL1* regulatory sequence. Here, we primarily created the Inducible-CASPEX-HEK 293T system. In this system, dCas9 fused with APEX2 can be induced in the presence of Dox, and APEX2 can biotinylate proteins in its close vicinity when biotinyl tyramide is added to the medium. We designed plasmids to express sgRNAs for targeting dCas9-APEX2 to the promoter of *BMAL1*. These plasmids are Lenti-CRI-sgRNA - based constructs that target different regions of *BMAL1* (designated as BMAL_T1 and BMAL1_T2). They target the DNA sequences close to 5' of the ATG coding sequence. Because they have approximately 100 bp between them and they can reach away from 100 bp, we assumed that we can catch proteins in the promoter region of the *BMAL1* genes. The common proteins identified by T1 and T2 would increase the significance of the interactome. Sometimes, sgRNAs may not be functional. Therefore, we tested our sgRNAs first to the knockout ability by contransfecting them with a wild-type Cas9 in HEK293T cells. Then selected sgRNA plasmids were stably transfected into the inducible-CASPEX-HEK 293T cells. We used a sgRNA which does not target any genomic sequence in human cells as a control (labeled as CASPEX). We observed biotinylation in all inducible CASPEX-HEK293T cells (figure 5.13 C and figure 5.14). Biotinylated proteins were enriched on streptavidin coated magnetic beads and these proteins were identified by mass spectrometry. We have used the SAINTexpress program to test the significance of the interactome. SAINTexpress is a software frequently used in the analysis of PPI studies. In this software, saint score 1 is used because it indicates very strong protein interactions. However, we used saint score 0.95 value to select proteins because we considered the common proteins from two targets (BMAL1_T and T2) would increase the probability of the being real interactions. To interpret the interactome list, a network analysis was created with proteins common in T1 and T2 using Cytoscape software.

In the network, proteins were grouped according to their known interactions and functions. The interactome included proteins involved in processes such as gene expression, RNA metabolic process, cell cycle, RNA transport and transcription. This was within the range of our expectations. For example, we detected proteins known to be involved in transcription process (such as LCOR, WHSC1, and Med19) as well as proteins involved in transcription process that are also known to interact with BMAL1 (such as CSNK2A1 and TOP1) or suspected to interact with BMAL1 (such as THRAP3 and TOP2A). We believe that including more sgRNAs to cover whole promoter sequence would reveal a larger interactome data with a higher significance value.

The fact that general transcription factors are included in the interactome list confirms that the CASPEX system is working. However, it must be confirmed that these proteins take part in the regulation of BMAL1 using various techniques. A few techniques can be applied to this. The first technique is the ChIP-qPCR. Chromatin immunoprecipitation (ChIP) technique is an immunoprecipitation technique used to investigate interactions between proteins and DNA within the cell. Therefore, it can be used for the validation of LCOR, THRAP3, TOP1, and TOP2A. In an alternative approach, we can transfect the plasmids for selected proteins. They can be transfected alongside a luciferase reporter for *BMAL1* promoter. Thus, changes in the luciferase signal will tell us that these proteins are true regulators of the *BMAL1* gene. If there is no definitive answer from these techniques, the interaction between the promoter sequence and the selected proteins can be tested using the electrophoretic mobility shift assay (EMSA) technique.

The data obtained as a result of the study showed that the CASPEX system worked for the purpose and gave the expected results. The tissue-specific arrangement of the circadian clock is known. And, the effects of circadian clock proteins on various systems are known in different tissues. For this reason, it is of great importance to study the tissue specific effects on the circadian clock. Based on the CASPEX system, this study can be expanded as follows, the CASPEX system can be tested on cell lines of different origin, and tissue-specific transcription factors can be identified, interactions with these transcription factors can be investigated. Finally, it is possible to study the circadian regulation of the novel transcription factors identified by the CASPEX system.

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APPENDICES

Appendix A: Lenti-CRIS-sgRNA-zeo-Bmal1_T1/Lenti-CRIS-sgRNA-zeo-Bmal1_T2 common protein list by SAINTexpress Analysis

Table A1.1: A list of Bmal1_T1 and Bmal1 T2 common proteins was created using SAINTexpress software (saint score of 0.95 and above).

UniprotID	SaintScore	UniprotID	SaintScore	UniprotID	SaintScore
P11387	1	P17096	1	O75152	0,99
Q7Z6E9	1	Q9Y3C1	1	Q8TCJ2	0,99
P11388	1	Q08170	1	Q9NQG5	0,99
Q13823	1	Q86UE4	1	Q96EY1	0,99
Q71U36	1	Q96MU7	1	P23921	0,99
Q9H7N4	1	P00505	1	Q86U86	0,99
P04259	1	Q9NPI1	1	Q14980	0,99
P46013	1	Q9H8M2	1	P51571	0,99
Q9GZR7	1	O00479	1	P82675	0,99
Q5VWQ0	1	Q15269	1	Q99798	0,98
P42696	1	Q92922	1	Q9BXY0	0,98
Q8IY81	1	Q8NHQ9	1	O60524	0,98
Q08945	1	P26583	1	P36915	0,98
Q01831	1	Q8TDD1	1	Q6YN16	0,98
Q5QNW6	1	Q9Y2W1	1	Q9HC07	0,98
Q9BRT6	1	Q9Y3T9	1	Q9H8H2	0,98
Q14690	1	O60508	1	Q9NY61	0,98
P62826	1	Q9H4L7	1	P35573	0,98
P06748	1	P38432	1	P26358	0,98
Q07955	1	Q969X6	1	Q86U42	0,98
O96028	1	P41223	1	P55769	0,98
Q9GZR2	1	Q8N9T8	1	Q9BTU6	0,98
Q99848	1	P46821	1	Q9UI09	0,98
Q9NY12	1	Q9BSC4	1	O43913	0,98
A0JLT2	1	Q8TED0	1	Q9UKY7	0,98
Q8N655	1	Q9H9L3	1	Q13155	0,98

Table A1.1: Continued					
Q9BUJ2	1	Q9UBU9	1	Q5J TZ9	0,98
Q86UP2	1	P82930	1	Q9NUD5	0,98
Q9NTI5	1	Q15717	0,99	Q96I25	0,98
Q92945	1	Q9NV31	0,99	O00505	0,98
O75400	1	Q15149	0,99	O60942	0,98
Q96GA3	1	O60287	0,99	Q9UBM7	0,98
Q9UNX4	1	O00442	0,99	P68400	0,97
Q4G0J3	1	P17812	0,99		

Appendix B: Protein and DNA Markers

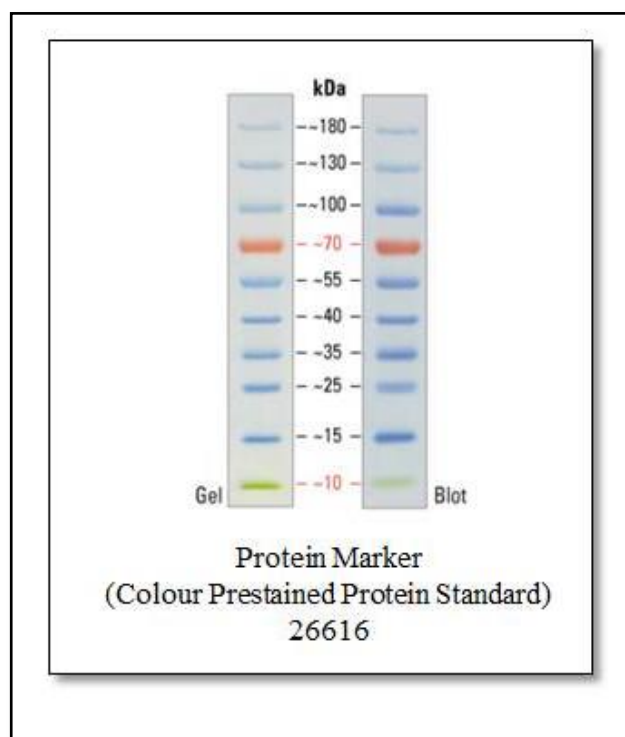


Figure B1.1: Protein Marker.

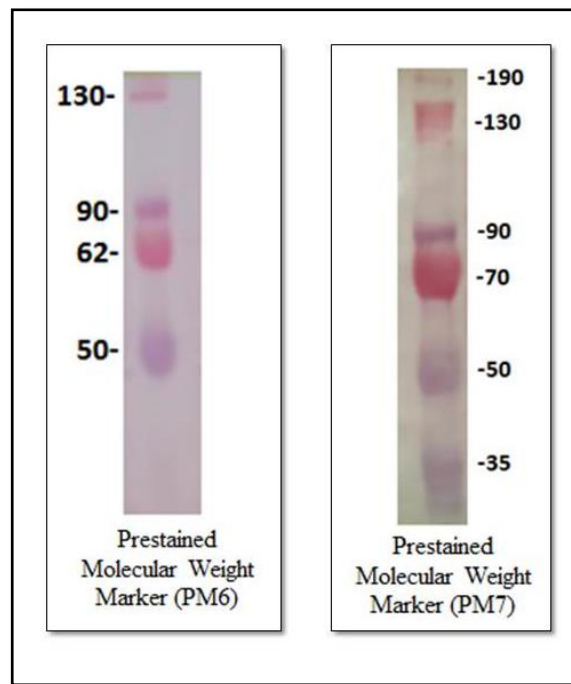


Figure B1.2: Homemade Prestained Molecular Weight Markers

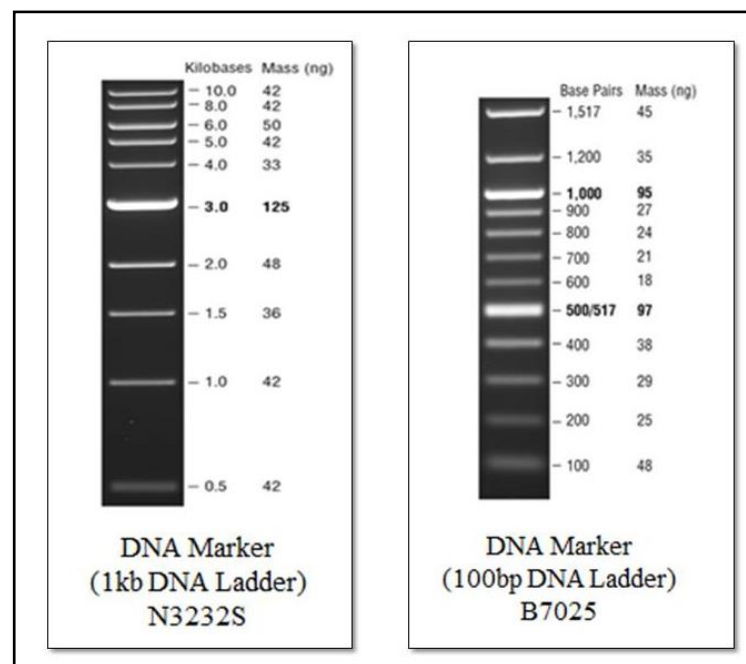


Figure B1.3: DNA markers.