

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

SYSTEMATIC ANALYSIS OF THE RELATIONSHIP BETWEEN
BIOLOGICAL CLOCK AND CANCER DEVELOPMENT

TUBA KORKMAZ
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
ASSOC. PROF. DR. NURİ ÖZTÜRK

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SİSTEMATİK ANALİZİ

TUBA KORKMAZ
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
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SUMMARY

The circadian clock confers daily rhythmicity on many biochemical and physiological functions and its disruption is associated with increased risks of developing obesity, diabetes, heart disease and cancer. Although, there are studies on the role of *BMAL1* in carcinogenesis using germline, conditional or tissue-specific knockouts, how *BMAL1* gene affects cancer-related biological events at the molecular level is not well understood. In contrast to other circadian clock gene knockouts, *BMAL1* is the only gene whose deletion alone leads to complete loss of rhythmicity. Therefore, it was aimed to taken an *in vitro* approach to understand the contribution of *BMAL1* in this molecular mechanism using human breast epithelial cell lines by knocking out *BMAL1* gene with CRISPR technology. Epithelial cells were preferred over fibroblasts as the most of cancers originate from epithelial cells. After obtaining *BMAL1* knockouts by targeting the gene at two different sites from non tumorigenic MCF10A and invasive tumorigenic MDA-MB-231 cells, apoptosis and invasion properties of the cell lines were analysed as representative events in tumor development. *BMAL1* disruption sensitized both cell lines to a bulky-DNA adduct forming agent (cisplatin) and a double-strand break-inducing agent (doxorubicin), while it enhanced the invasive properties of MDA-MB-231 cells. These results show that the disruption of clock genes may have opposing carcinogenic effects.

Key Words: Circadian Rhythm; Cancer; CRISPR/Cas9; *Bmal1*.

ÖZET

Sirkadiyen saat, birçok biyokimyasal ve fizyolojik fonksiyonda günlük ritmi sağlamaktadır ve sirkadiyen saatin bozulması, obezite, diyabet, kalp hastalıkları ve kanser gelişim riskinin artması ile ilişkilendirilmektedir. Her ne kadar, *BMAL1*'in karsinogenez üzerindeki rolü üzerine germline, koşullara bağlı ya da doku spesifik knockoutların kullanıldığı çalışmalar bulunsun da; *BMAL1* geninin kansere bağlı biyolojik olayları moleküler seviyede nasıl etkilediği iyi anlaşılamamıştır. Diğer sirkadiyen saat gen knockoutlarının aksine; *BMAL*, delesyonu tek başına tam bir ritim kaybına sebep olan tek gendir. Bu nedenle, CRISPR teknolojisi ile, insan meme epitel hücre hatlarında *BMAL1* geni knockout edilerek, *BMAL1* geninin bu moleküler mekanizmaya katkısının anlaşılması için *in vitro* bir yaklaşım alınması amaçlanmıştır. Pek çok kanser epitel hücrelerden kökenlendiğinden, fibroblastlar yerine epitel hücreler tercih edilmiştir. Tümörjenik olmayan MCF10A ve invaziv tümörjenik MDA-MB-231 hücrelerinde, *BMAL1* geninin iki farklı bölgede hedeflenmesi ile *BMAL1* knockoutların elde edilmesinden sonra, hücre hatlarının, tümör gelişiminde belirleyici olaylar olan apoptoz ve invazyon özellikleri analiz edilmiştir. *BMAL1* hasarı, her iki hücre hattını da DNA adduct yapısı oluşturan ajan cisplatin ve çift iplik kırığı oluşumunu indükleyen doxorubicine'e duyarlı hale getirirken; MDA-MB-231 hücrelerinin invaziv özelliğini arttırmıştır. Bu sonuçlar, saat genlerinin bozulmasının, ters karsinojenik etkiler verebileceğini göstermektedir.

Anahtar Kelimeler: Sirkadiyen Ritim; Kanser; CRISPR/Cas9; *Bmal1*.

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
µg	: Microgram
µl	: Microliter
APS	: Ammonium persulfate
bHLH	: Basic helix-loop-helix
BMAL1	: Brain and muscle Arnt-like 1
CLOCK	: Circadian locomoter output cycles protein kaput
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
CRY	: Cryptochrome
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
ECM	: Extracellular matrix
EDTA	: Ethylenediaminetetraaceticacid
EGF	: Epithelial Growth Factor
EMT	: Epithelial Mesenchymal Transition
FBS	: Fetal Bovine Serum
HEK293T	: Human embriyonic kidney 293T cells
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid)
HRP	: Horseradish peroxidase
LB	: Luria Bertani
MEBM	: Mammary Epithelial Basal Medium
MEGM	: Mammary Epithelial Growth Medium
MMP	: Matrix metalloproteinases
NaCl	: Sodium chloride
PAS	: Period-Arnt-Single-minded
PBS	: Phosphate Buffered Saline
PCR	: Polimerase chain reaction
PER	: Period gene
REV-ERBA	: Retinoic acid-related orphan nuclear receptors
RHT	: Retinohyptothalamic tract
RIPA	: Radio Immuno Precipitation Assay

ROR α	: Retinoid-related orphan receptor α
RT-qPCR	: Real-time quantitative polymerase chain reaction
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sgRNA	: Single guide RNA
TBE	: Tris/Borate/EDTA
TBS	: Tris Buffered Saline
TBS-T	: Tris buffered Saline with Tween 20
TEMED	: Tetramethylethylenediamine
TRIS	: Hydroxymethylaminomethane
TTFL	: Transcription translation feedback loop

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1. INTRODUCTION

The circadian clock regulates ~10% of the transcriptome in any given cell [Partch *et al*, 2014]. When second order genes and posttranslational modifications are considered, it is accepted that 20-30% of pathways are under control of the circadian clock. Considering that the circadian clock directly or indirectly affects all metabolic processes and signalling pathways [Sahar & Sassone-Corsi, 2009], it would be expected that the disruption of the clock would have an effect on cancer development and progression.

The lack of a general theory on the circadian clock and cancer connection and the presence of unanswered questions require to work on the circadian clock and cancer relationship. So, biological experiments have been conducted to provide approaches to the unanswered question of a circadian disruption–cancer connection.

Breast cancer is one of the most common diseases among women. Night shift work exposure has been recently thought to be one of the most significant breast cancer risk factors in industrialized countries. Although, the mechanisms responsible for cancer development is still discussed, the results of the present study suggest that there is an association between night shift work and breast cancer development. Further studies are needed to confirm such association and to understand which biomolecular mechanisms may be involved in the pathogenesis of cancer diagnosed in patients with night shift work exposure [Leonardi *et al.*, 2012].

1.1. The Purpose, Contribution and Content of Thesis

The main purpose of this study is to investigate the effect of the biological clock disruption on carcinogenesis steps (apoptosis and invasion capacity) by considering the genetic background in a systematical manner. For this purpose, cell culture models were obtained and used instead of animal models. CRISPR Cas9 technique was utilized to obtain the desired gene mutations and then the apoptotic cell death following DNA damage and the invasion capacities of cells were studied.

Because, most cancers stem from epithelial cells, epithelial cell lines were preferred for experiments.

In this study, it was aimed to have a table which could predict the effect of the biological clock on cancer by the genetic background of cells and according to the initiation or metastasis stages of tumorigenesis. Also, it was expected to have more knowledge to target the circadian clock proteins for cancer therapeutic strategies.

2. LITERATURE REVIEW

2.1. Overview of the The Circadian Clock

The daily rhythmicity of the physiology and behaviour of organisms is controlled by the circadian rhythm [Levi, 2006]. Circadian rhythm examines 24-h oscillations in biological processes at the molecular, cellular, and behavioural levels. These processes have been associated with from basic cellular functions such as gene expression and protein translation to the daily rituals of human life [Wulund and Reddy, 2015].

In mammals, circadian clock is composed of a master clock in the Suprachiasmatic Nucleus (SCN) in brain and peripheral clocks in tissues and organs. The suprachiasmatic nucleus (SCN) is located in hypothalamus and serves as a circadian master clock. It controls biochemical, physiological and behavioral rhythms, received by light and other external signals [Sancar et al., 2014].

The master clock is synchronized by sun light daily through retinohypothalamic tract (RHT) from eyes, and then regulates the peripheral clocks, which are generated by the sum of molecular clocks. Molecular clocks include a core transcription–translation feedback loop (TTFL) [Reppert and Weaver, 2002]. The TTFL is highly conserved across species and present in almost all mammalian cell types. It consists of transcriptional activators *CLOCK* (Circadian locomoter output cycles protein kaput)-*BMAL1* (Brain and muscle Arnt-like 1) and transcriptional repressors *CRY* (cryptochrome) and *PER* (period) which are defined as “core clock genes”. By the help of transcriptional repressors and activators, molecular oscillations are generated at the cellular level [Ko and Takahashi, 2006].

The CLOCK:BMAL1 heterodimer induces E-box–mediated transcription of *Per* and *Cry*; their products are released in the cytoplasm. When PER and CRY proteins reach a critical concentration, they form heterodimers PER:CRY, phosphorylate and translocate into the nucleus and they inactivate the BMAL1:CLOCK E-box–mediated transcription; including transcription of their own genes. This inactivation also

reduces their levels and allow for the new transcription cycle [Andreani et al., 2015].

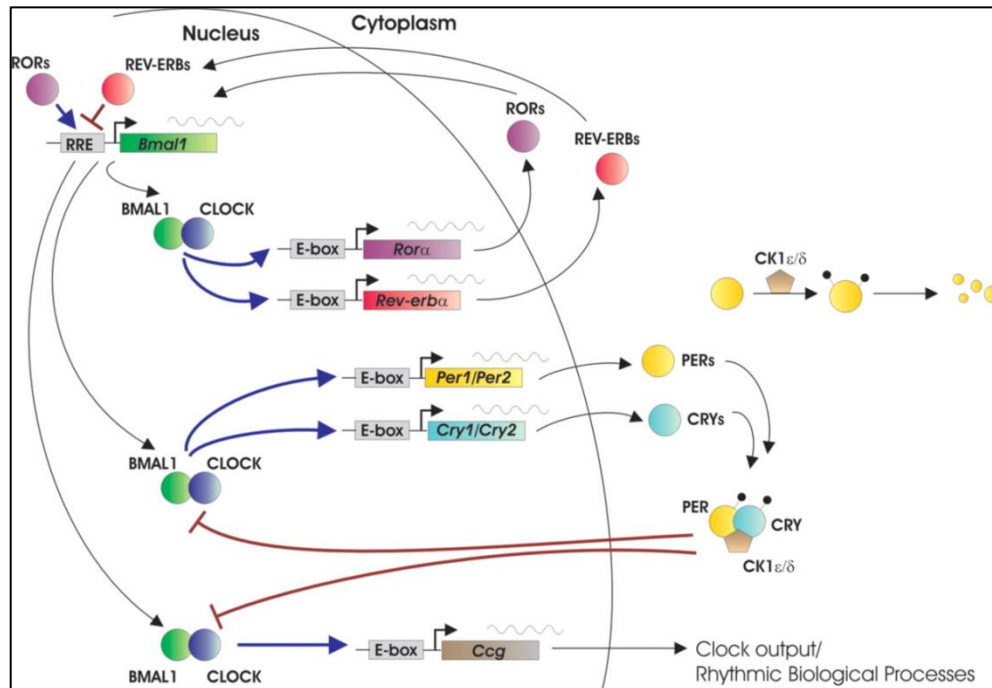


Figure 2.1: Molecular components of the mammalian circadian clock.

The circadian clock regulates ~10% of the transcriptome in any given cell. When second order genes and posttranslational modifications are considered, 20-30% of pathways are thought to be under control of the circadian clock [Partch et al., 2014]; including immune function [Keller et al., 2009], cell cycle progression/arrest, cell proliferation, apoptotic pathways, hormonal oscillations, body temperature, metabolism, DNA repair, response to anticancer drugs and so on [Grechez-Cassiau et al., 2008], [Lincoln and Wood., 2000], [Matsuo., 2003].

Because, circadian rhythm is involved in many biological processes, the disruption of the circadian clock has been associated with a variety of physiological states, ranging from metabolic disorders to cancer.

2.2. The Relationship Between circadian Clock and DNA Damage Response

2.2.1. Circadian Clock Regulation of DNA Repair

Direct repair, base excision repair, nucleotide excision repair, mismatch repair and recombination are pathways for DNA repair. From the pathways, nucleotide excision repair has been found to be directly regulated by circadian clock [Kang et al., 2011]. In addition, mismatch repair and double strand break repair are known to be affected by circadian clock, since it controls replication [Gaddameedhi et al., 2011], [Geyfman et al., 2012].

Nucleotide excision repair mechanism is involved in the elimination of bulky DNA adducts caused by UV, chemical carcinogenetic agents and anticancer drugs such as cisplatin. Human excision repair mechanism is achieved by the functions of RPA, XPA, XPC, TFIIH, XPG, and XPF-ERCC1 [Hu et al., 2013]. From the six main repair factors, only XPA protein was found out to be a clock controlled gene product.

The XPA protein has a damage recognition role on repair mechanism and its daily oscillation is achieved by HERC2 ubiquitin ligase that targets XPA for degradation and by the clock. When CLOCK and BMAL1 make a heterodimer, bind E boxes in the promoters, they activate the transcription of clock controlled gene *Xpa*, as well as *Cry* and *Per*. After dimerizing, CRY-PER complex enter the nucleus. In nucleus, CRY dissociates from PER and CLOCK-BMAL1 mediated transcription is inhibited and oscillation of *Xpa* is achieved. XPA protein has a short half life between ½ to 3 hours. Then it is ubiquitinated by HERC2 E3 ligase and degraded by ubiquitin proteasome system.

Transcription and protein expression analysis of XPA indicates that it is expressed during the day; at night, it is downregulated. In correlation with XPA, excision repair activity shows a daily rhythm; increases during the late afternoon and decreases during the early morning in mouse [Sancar et al., 2015].

2.2.2. DNA Damage Checkpoints

The DNA damage checkpoints maintain cell survival by preventing cells passing through a next cell cycle phase in the case of uncontrolled cell division or DNA damage. In mammals, two DNA damage checkpoint pathways have been found. The two pathways are directed by ATM (Ataxia Telangiectasia-Mutated) and ATR (ATM and Rad3-related) protein kinases [Sancar et al., 2004]. DNA damage caused by ionizing radiation (IR) or double-strand breaks trigger the ATM pathway. DNA damage caused by UV or UV mimetic agents trigger the ATR pathway. Both ATM and ATR damage checkpoints have been found to be in association with circadian clock.

2.2.2.1. Circadian Clock and the ATR–Chk1 DNA Damage Signaling System

In mammals, *Tim* gene has an important role in growth, differentiation as well as replication fork stability. *Tim* gene is suggested to be involved in both cell cycle and circadian regulation. Experimental analysis established that downregulation of *TIM* reduces PER2 protein and causes circadian rhythm disruption in SCN [Barnes et al., 2003]. In addition, *TIM* downregulation decreases Chk1 phosphorylation level in replication inhibition, showing that *TIM* has role in both circadian clock and DNA damage checkpoint [Sancar et al., 2015].

2.2.2.2. Circadian Clock and ATM-Chk2 DNA Damage Signalling System

PER1 is also found to have role in ATM → Chk2 signaling by directly interacting with both ATM and Chk2, downregulation of *PER1* is associated with IR-induced phosphorylation of Chk2 by ATM and reduced the level of apoptosis by agents that induce double strand breaks [Gery et al., 2006].

2.2.3. Circadian Clock and Apoptosis

Apoptosis is found to be related to both intrinsic (initiated by DNA damaging agents) and extrinsic apoptosis (initiated by cytokines).

2.2.3.1. Circadian Clock and Intrinsic Apoptosis

In the intrinsic pathway, cellular damage leads to an increased level of expression of pro-apoptotic members (Bax and Bak) of the Bcl-2 family. Tumor suppressor *p53* have an important role in intrinsic apoptosis pathway by upregulating the expression of Bax and Bak. [Hotchkiss et al., 2009]. However, almost half of the cancers have *p53* mutations.

It was experimentally found that *p53*^{-/-}*Cry1/2*^{-/-} cells were more sensitive to genotoxic agents than *p53*^{-/-} cells [Ozturk et al., 2009]. It suggests a *p53*-independent apoptosis pathway activated by absence of *CRY*s. By further analysis, in this pathway, involvement of another tumor suppressor, *p73* was discovered. *p73* upregulation following DNA damage is directed by transcriptional induction. The *p73* promoter has binding sites for transcriptional activators *Egr1* (Early growth response 1) and *E2F1*; and transcriptional repressor *C-EBPα*. Transcriptional activator *Egr1* has an E-box in its promoter that means it is a clock controlled gene [Sancar et al., 2015]. Following DNA damage by UV or UV-mimetic agents, *C-EBPα* is phosphorylated, released from the *p73* promoter. In addition, *Egr1* is triggered by UV or UV-mimetic agents, as a consequence, *p73* transcription is induced. *Egr1* was found to be regulated by *Cry* and in presence of inactive *Cry*, *Egr1* is up-regulated, UV or UV mimetic agent induced *p73* synthesis and apoptosis are enhanced [Lee et al., 2011a].

2.2.3.2. Circadian Clock and Extrinsic Apoptosis

It was found that circadian rhythm disruption by *CRY* mutation triggers extrinsic apoptosis in two ways. First pathway is achieved by increasing the

production of cytokines that are pro-apoptotic such as TNF α . In the absence of *CRY*, by TNF α overexpression, extrinsic apoptosis is triggered [Hashiramoto et al., 2010]. In second pathway, absence of *CRY* causes GSK3 β hyperphosphorylation that results in prevention of NF- κ B phosphorylation. Not being phosphorylated, NF- κ B has weakened anti-apoptotic function [Lee et al., 2011b].

2.3. Circadian Clock and Cancer

2.3.1. Circadian Clock and Carcinogenesis

Circadian clock is linked to almost all metabolic pathways; so its disruption is expected to have an effect on cancer development and progression [Yu et al., 2011]. To establish the relation between clock disruption and cancer risk, epidemiologic analysis in human, studies by using animal models and experiments in molecular level were performed [Sancar et al., 2015].

Some epidemiologic studies suggest the possibility that women working at night and exposing to light at night that may cause circadian disruption are in risk of having breast and colorectal cancer. In October 2007, the International Agency for Research on Cancer (IARC) classified shift work with circadian disruption as a probable human carcinogen [Stevens et al., 2009]. On the other hand, by a study with Chinese women, it was established that breast cancer incidence of night shift and rotating shift workers was lower than the women that have conventional working times [Pronk et al., 2010]. Because of the conflicting data from epidemiological studies, relation between cancer risk and circadian clock disruption could not be established.

To test relation between circadian clock disruption and carcinogenesis on animal model, following the SCN disruption surgically, tumors were inserted to mice and tumor growth was screened by comparing with mice that have normal SCN. Both pancreatic carcinoma and Glasgow osteosarcoma tumor growth were found to be faster at SCN-disrupted-mice. However, it is unknown whether the tumors

injected were directed by their own clock or by the mice circadian clock [Filipski et al., 2002].

By the studies at molecular level, it was reported that *Per2* mutant mice showed increased tumor development with decrease in apoptosis after gamma radiation [Fu et al., 2002]. However the studies performed by *Cry*, the other member of negative arm, did not support this data. *Cry* KO mice were found to be indistinguishable from wild type mice [Gauger et al., 2005]. In another experiment, neither *CLOCK* nor *BMAL1* null mutant mice were found to have elevated incidence of cancers [Khapre et al., 2014].

Because of the conflicting data obtained from studies, relation between circadian clock disruption and cancer is needed to be found out by further studies.

2.3.2. Circadian Control and Cancer Treatment

Chronotherapy is the timing of drug delivery with the appropriate phase of the circadian rhythm to achieve optimal efficacy. Chronotherapy based therapeutic applications for many diseases as well as cancer are attempted to be developed [Paschos et al., 2010].

One of the approaches is based on excision repair known to be controlled by circadian clock. Excision repair is the sole mechanism by which predominant DNA adducts induced by the anticancer drugs such as cisplatin could be removed. To decide the time of delivery of these group of drugs, it is important to determine whether the tumor is rhythmic and /or in phase with normal tissue. If the tumor has rhythmicity in phase with the normal tissue, delivery of drug may be appropriate when excision repair is in the descending phase with a less toxic dose. If the tumor has rhythmicity but out of phase with the normal tissue, the drug delivery may be appropriate when repair is ascending in normal tissue. If the cancerous tissue is lack of rhythmicity, delivery of the drug may be appropriate in the ascending phase of the excision repair rate in the normal tissue [Sancar et al., 2015].

Preferential killing of cancer cells relative to normal cells is the main objective in all therapeutic approaches. A major mechanism responsible for drug-induced cell killing is apoptosis. The other approach in chronotherapy aims to

increase the rate of apoptosis in cancer cells. Experimental analysis revealed that in the absence of *p53*, *p73* becomes the major driver of apoptosis and *p73* is highly upregulated as a second-order clock-controlled gene after DNA damage. When considered that the *p53* gene is mutated in ~50% of cancers, identification of small molecule *CRY* inhibitors would suggest that *CRY* inhibition might be applicable to cancer chemotherapy [Chun et al., 2014].

In addition, in the absence of *CRY*, tumors have found to be more sensitive to TNF α -induced apoptosis. TNF α is toxic and its usage for cancer treatment is not practical; but by the use of small molecule *CRY* inhibitors, this cytokine would be overproduced and the cells would be sensitized to TNF α -induced apoptosis [Sancar et al., 2015].

2.4. Cell Invasion and Epithelial-to-Mesenchymal Transition (EMT)

2.4.1. Cell Invasion and Cancer

Invasive growth is the main challenge of tumor progression because cell invasion is a trigger and a key factor for further cancer progression and metastasis in distant organs and tissues [Krakhmal et al., 2015]. Invasion of the primary tumor into surrounding tissues requires many steps: detachment of malignant cells from the tumor by having ability of motility, migration through blood vessels, intravasation (Entry of tumor cells into a blood vessel and their activation by platelet-activating factor), survival and circulation via blood vessel, extravasation (Attachment of activated tumor cells to the vessel wall and exit from blood vessel) and the formation of metastasis [Guan, 2015].

Although the growth limiting factors including the basal membrane, components of the surrounding stroma, limited oxygen levels, the presence of active oxygen forms and immune system cells, some tumor cells maintain the ability of metastatic progression [Guan, 2015]. Because being a complex process, invasion involves various molecular and cellular mechanisms as endothelial

mesenchymal transition (EMT). Cell invasion during cancer progression is thought to be associated with the obtaining of EMT features [Kang and Massague, 2004].

2.4.2. Endothelial Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a biological process required for normal embryonic development [Son and Moon, 2006]. In this process, a non-motile epithelial cell changes to a mesenchymal state with migratory capacities. However, EMT is known to be associated with pathological processes such as carcinogenesis, invasion, metastasis and cancer reoccurrence [Garcia et al., 2017].

During EMT, epithelial cells lose epithelial cell-specific specialities and have features of mesenchymal cells. The amount of tight- and adherens-junction proteins such as E-cadherin and α -catenin are decreased and mesenchymal cell specific proteins including N-cadherin, vimentin, and fibronectin are increased [Grunert et al., 2003]. Epithelial cells lose cell-cell adhesions and obtain cell motility and invasiveness. Then, epithelial cells undergo EMT digest and migrate along the extracellular matrix (ECM) by the ability of motility and invasiveness that endows cancer cells with tumor forming potential [Son and Moon, 2006].

2.4.3. The Role of EMT in Cell Invasion

The association between EMT and cell invasion has been demonstrated in cancer progression. Matrix metalloproteinases (MMPs) take part in invasion and metastasis. Increased enzymatic degradation of extracellular matrix components by MMPs induces cell invasion and tumor spread. MMPs are implicated in EMT during cancer progression via these ways: a) increased MMPs in the tumor microenvironment can directly induce EMT in epithelial cells, b) cancer cells undergoing EMT can produce more MMPs and then facilitate cell invasion [Son and Moon, 2006]. Because cell invasion is a crucial step in metastasis, to find out whether circadian rhythm is involved in this pathway could be a new approach to cancer therapy.

2.5. CRISPR/Cas9 System

Disruption of a gene to understand the effect of the gene or the gene product on an organism's phenotype is an important tool in molecular biology. Genomic sequencing technologies combined with in genome editing techniques has increased the possibilities of genetic manipulations in numerous organisms [Thurtle et al., 2018].

The adaptive immune system of bacteria and archaea, Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR-associate (CRISPR/Cas), has been adapted as a gene editing tool due to its ease of use and cost-effectiveness. By the help of well-designed single-guide RNA (sgRNA) which are developed by bioinformatic tools, efficient CRISPR/Cas performance could be introduced [Cui et al., 2018].

CRISPR/Cas system consists of three components: an endonuclease (Cas9), a CRISPR RNA (crRNA), and a transactivating crRNA (tracrRNA). The crRNA and tracrRNA molecules form a duplex structure called the guide RNA (sgRNA). The sgRNA contains a unique 20 base-pair-sequence and it is designed to recognise the target site by Watson-Crick base pairing. It has to be followed by a short DNA sequence, "protospacer-adjacent motif" (PAM), which is essential for compatibility with the Cas9 protein used. After the sgRNA and the Cas9 nuclease are expressed in the cell, they form a complex. In this complex, sgRNA is responsible for guiding Cas9 to target DNA site by Watson-Crick base-pairing. Then, Cas9 cleaves the DNA to produce a DSB. Cells then repair this DSB by homology-directed repair (HDR) or non-homologous end joining. Repair mechanism is decided by cells depending on the cell state and the presence of a repair template [Lage et al., 2018].

The CRISPR/Cas9 system is a specific, efficient and simple system and has opened new possibilities extending from genome editing to transcriptional regulation, in vivo imaging and more importantly, it brings the promise of new diagnostic and therapeutic interventions.

3. MATERIALS

3.1. Kits

All parental and subclone lines used in the thesis were tested for mycoplasma contamination using the e-Myco plus Mycoplasma PCR Detection Kit (Boca Scientific Inc., USA). Plasmid isolation and gel extraction were performed using Nucleospin Plasmid Kit and PCR clean-up Gel extraction Kit (Macherey Nagel, Germany) respectively. PCR products were column purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Total RNA was isolated by using Nucleospin RNA Kit (Macherey Nagel, Germany). Total RNA was converted into cDNA using cDNA synthesis kit (New England Biolabs, USA). Total DNA was isolated by using Nucleospin tissue kit (Macherey Nagel, Germany). All transfections were done using FuGENE® Transfection Reagent (Promega, USA) and Effectene® Transfection Reagent (QIAGEN, USA). Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, USA) were utilized to inhibit general degradation of proteins in cell extracts by endogenous proteases. In order to determine protein concentration, Bradford Protein Assay Dye (Bio-Rad, USA) and Pierce™ BCA Protein Assay Kit (Thermo Scientific, US) were used. Protein Molecular Weight Marker (New England Biolabs, USA) was used to monitor the progress of electrophoretic run and analysis conditions of proteins. For DNA analysis, DNA Ladder and DNA Loading Dye (New England Biolabs, USA) were used. Imaging and detection of proteins by Western Blotting was succeeded by using Chemiluminescent HRP Substrate (Advansta, USA) and SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Scientific, US). Invasion assays were performed using the CytoSelect 24-Well Cell Migration Assay kit (Cell Biolabs, USA).

3.2. Enzymes

BsmBI (R0580S, New England Biolabs), EcoRI (ER0275, Thermo Fisher), NheI (ER0971, Thermo Fisher), Taq DNA Polymerase (M0320S, NEB) T4 DNA ligase

(1U/μl) Invitrogen (15224017) and T7 Endonuclease I (M0302L, NEB) enzymes were obtained commercially.

3.3. Antibodies

Primary antibodies; Anti-HA tag Ab (1:10000, Abcam, ab13834), Anti c-Parp Ab (1:2000, Cell Signaling Technology (CST), 9541s), Anti Actin Ab (1:5000, CST, 4967) , Anti Bmal1 Ab (1:2000, Bethyl labs, A302-616A) , Anti Cry1 Ab (1:2000, Bethyl labs, A302-614A), Anti Cry2 Ab (1:2000, Bethyl labs, A302-615A), Anti Clock Ab (1:2000, Bethyl labs, A302-618A), Anti Per1 Ab (1:5000, Novus, NBP2-24589SS), Anti Per2 Ab (1:2000, Bethyl labs, A303-109A), Anti p53 Ab (1:5000, CST 1c12), Anti Calnexin Ab (1:10000, Santa Cruz, sc11397) and secondary antibodies; Anti-mouse HRP (1:3000, CST, 7076), Anti-Rabbit HRP (1:3000, CST, 7074) were commercially obtained and used in this study. Anti β -catenin Ab was obtained as supernatant from Prof. Dr. Tamer YAĞCI, Molecular Cell Biology Lab.

3.4. Chemicals

Methanol (947.046, Sigma), ethanol (920.026, Sigma), Agarose (351010, Peqlab), HEPES (P05-01100, PAN), LB Broth Medium (LBP03, Caisson Labs), LB Agar (LBP04, Caisson Labs), 40% Ac-BisAcrilamide (161-0146, Biorad), 2-Propanol (1.00995, Merck), Skim milk (non fat powder, SKI400, Biochop), TRIS (AC228030051, Fisher Scientific), Dimethyl Sulfoxide (BP231, Fisher Scientific), Glycine (G45, Fisher Scientific), NaCl (S271, Fisher Scientific), Sodium Dodecyl Sulfate (BP166, Fisher Scientific), Tween 20 (BP337, fisher Scientific), TEMED (Tetramethylethylenediamine, A1148, Applichem), 2-Mercaptoethanol (A1108, Applichem), Sodium Hydroxide (106498, Merck), Hydrochloric acid (A144, Fisher Scientific), IGEPAL CA-630 (I8896, Sigma), SYBR[®] Gold Nucleic Acid Gel Stain (S11494, Invitrogen), Cisplatin (P4394, Sigma), Doxorubicin (D1515, Sigma), Brilliant Blue G (27815, Sigma), Puromycin dihydrochloride (P8833, Sigma), Amphotericin (59349, Sigma), Polybrene (134220, Santa Cruz Biotechnology), Bromophenol Blue

(BP115, Fisher Scientific), Boric Acid (B7901, Sigma), Ethylene diamine tetra acetic acid (EDTA, BP118, Fisher Scientific), NP 40 Cell Lysis Buffer (FNN0021, Thermo Fisher), 1× NEB buffer 2 solution (New England Biolabs,U.K.) were used in this study.

3.5. Cell Culture Supplements and Solutions

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

3.6. Buffers and Solutions

Buffers and solutions used in this study are 10X TBE (108g Tris base, 55g Boric acid, 40 ml 0.5M EDTA (pH 8.0), 10X SDS Running Buffer (250 mM Tris-Base (pH: 8.3), 1.90 M Glycine, 1% SDS), 10X TBS (200 mM Tris-Cl (pH: 7.6), 1.5 M NaCl), 1X Transfer Buffer (10% 10X SDS Running Buffer, 20% Methanol), 1X TBST (1X TBS, 0.1% Tween-20), 5% Blocking Solution (5% non-fat dry milk in 1X TBS-T), 4X Protein Loading Dye (250 mM Tris-Cl (pH: 6.8), 8% SDS, 40% Glycerol, 20% β -mercaptoethanol, 0.008% Bromophenol Blue), Cell Lysis Buffer RIPA (750 mM NaCl, 250 mM Tris (pH: 8.0), 0.1% SDS, 1.0% NP-40).

SDS-PAGE Gels were prepared by following the instructions at Table 3.1, 3.2 and 3.3.

Table 3.1: SDS PAGE 8% separating gel preparation.

8% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.65	5.3	7.95
40% Acryl: Bis	1	2	3
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.003	0.006	0.009

Table 3.2: SDS PAGE 12% separating gel preparation.

12% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.15	4.3	6.45
40% Acryl:Bis	1.5	3	4.5
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006

Table 3.3: SDS PAGE 5% stacking gel preparation.

5% Stacking Gel	2 ml	4 ml	6 ml	8 ml
ddH ₂ O	1.46	2.92	4.38	5.84
40% Acryl:Bis	0.25	0.5	0.75	1
1 M Tris pH 6.8	0.25	0.5	0.75	1
10% SDS	0.02	0.04	0.06	0.08
10% APS	0.02	0.04	0.06	0.08
TEMED	0.002	0.004	0.006	0.008

8% SDS gels were used in immunoblotting of Bmal1, Cry, C-Parp and Actin.

12% gels were used in immunoblotting of HA.

Solution used in cell culture studies is cell freezing solution (10% FBS, 10% DMSO, 80% cell suspension in appropriate growth media).

3.7. Equipments

Agarose Gel Electrophoresis (1704406, Bio-Rad, USA), Centrifuges (521-1647 refrigerated, Micro Star 17R, VWR; 521-1646 Micro Star 17, VWR; 521-2844 Mini Star, VWR; NF400-Nuve, 02-1827), Chemiluminescence Imaging System (ChemiDoc XRS+ System, Bio-Rad), Incubator (CO₂) (Thermo-Scientific), Electrophoresis System (1658004, Mini-PROTEAN® Bio-Rad), Laminar Flow Cabinet (Hera-Safe Class II Cabinet, Thermo Scientific), Microplate Reader (Varioskan Flash, Thermo Scientific), Power Supply (1645050, Bio-Rad), PCR Thermal Cycler (41798, Bio-Rad), TransBlot Transfer System (1704155, Bio-Rad), NanoDrop (206-26300-48, Shimadzu Biotech) Standart Shaker (VWR) Vortex (Scientific Industries), Water Bath (Wisd), Synergy H1 microplate reader (BioTek Instruments, Inc. USA,) were used in this thesis.

3.8. Cell Lines and Growth Media

MCF10A, MDA-MB-231, and HEK293T cell lines were from American Type Culture Collection (ATCC; Rockville, MD) were used, and maintained in a humidified incubator at 37°C under 5% CO₂.

For initial growth and stocking purposes of MCF10A, Mammary Epithelial Basal Medium (MEBM; Lonza, Walkersville, MD, USA) supplemented with SingleQuots (MEGM BulletKit; Lonza) and 100 ng/mL cholera toxin (Lonza) was used.

For experimental procedures of MCF10A, Dulbecco's Modified Eagle Medium (DMEM)-based medium (Thermo Fisher Scientific) containing 20 ng/mL epidermal growth factor (Sigma), 0,5 mg/mL hydrocortisone (Sigma), 10 µg/mL insulin (Sigma), 1% nonessential amino acids (Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific), and 10% fetal bovine serum was used (FBS; Thermo Fisher Scientific).

For growth and experimental procedures of MDA-MB-231 and HEK293T, DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% non-essential amino acids was used (all from Thermo Fisher Scientific).

E. coli DH5α cells for transformation step needed for obtaining HEK293T KO cell lines and *E. coli Stb13* cells for transformation step needed for obtaining MDA MB 231 and MCF10A KO cell lines were used in this study.

The vectors given below were used in this study.

Figure 3.1. pSpCas9n(BB)-2A-Puro (PX462) V2.0 vector map.

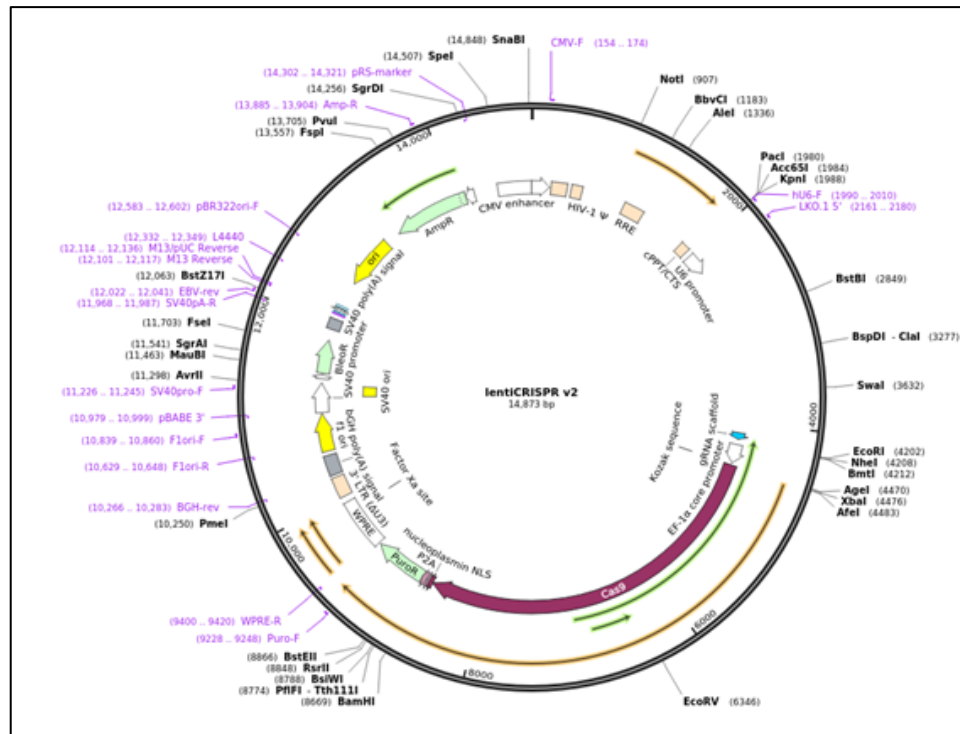


Figure 3.2. lentiCRISPRv2 vector map.

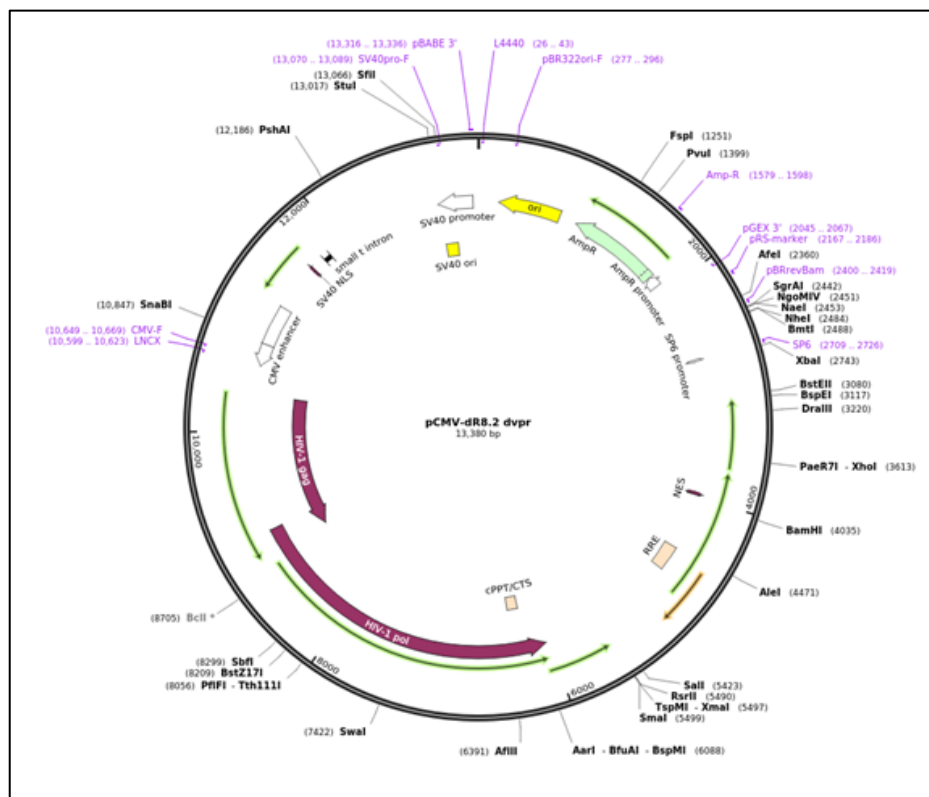


Figure 3.3. pCMV-dR8.2 dvpr lentiviral packing vector map.

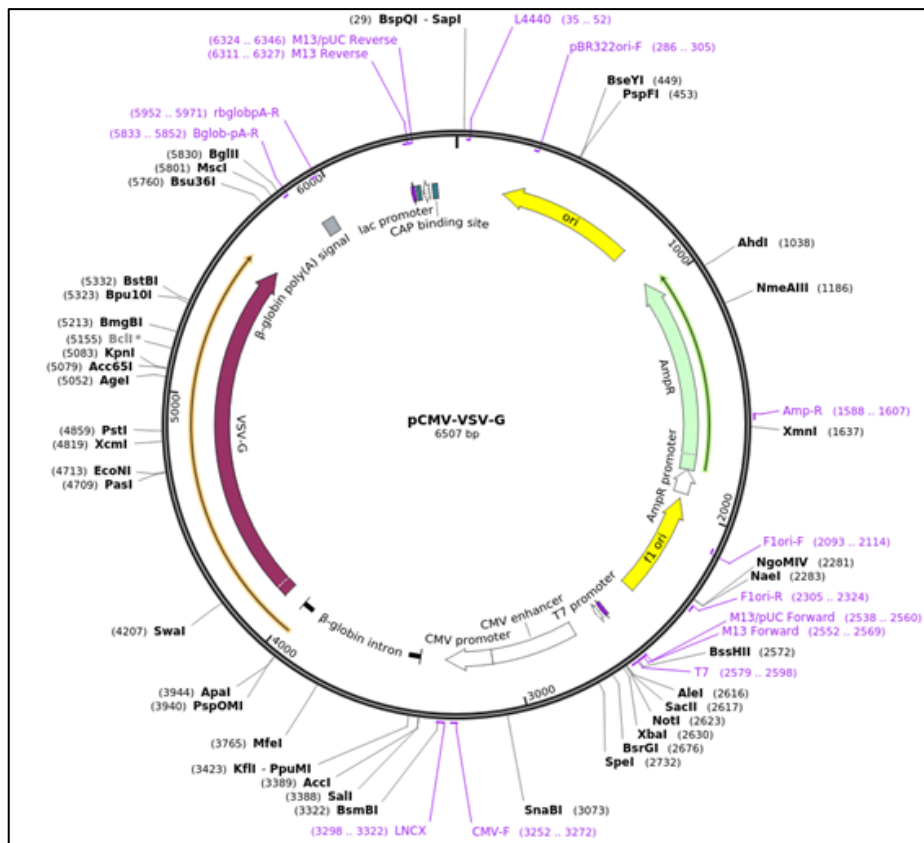


Figure 3.4. pCMV-VSV-G lentiviral envelope vector map.

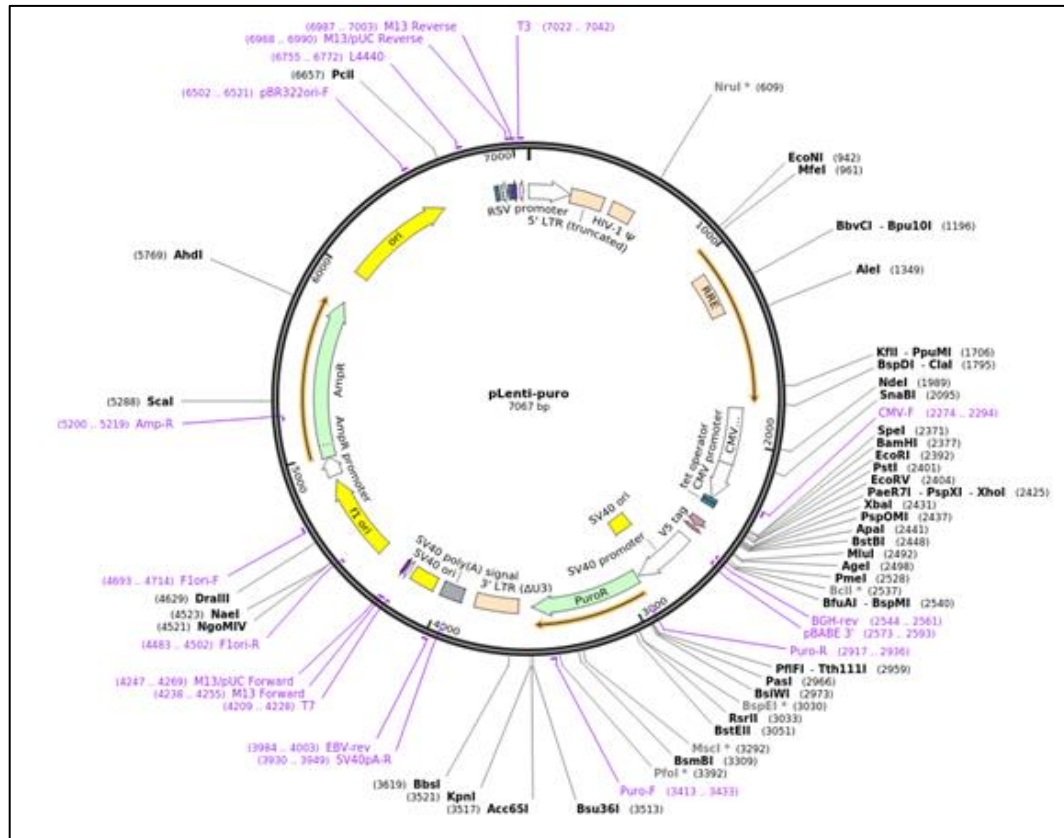


Figure 3.5. pLenti expression vector map.

3.11. Oligonucleotides

Double stranded oligos were purchased from Invitrogen, Thermo Fisher Scientific.

4. METHODS

4.1. Cell Culture

4.1.1. Maintenance of Cell Lines

MCF10A, MDA-MB-231, and HEK293T cell lines were maintained in a humidified incubator at 37°C under 5% CO₂. MCF10A cell line, was initially grown in MEM supplemented with SingleQuots (MEGM) and 100 ng/mL cholera toxin for initial growing and stocking purposes. For experimental procedures, MCF10A was grown and cultured in a DMEM based medium containing 20 ng/mL epidermal growth factor, 0,5 mg/mL hydrocortisone, 10 µg/mL insulin, 1% nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS.

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

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

For cell freezing, cell suspension at a high concentration and at as low a passage number as possible, 800uL of cell suspension was dispensed into storage vials. 100 uL of FBS and 100 uL of DMSO were added respectively to storage vials.

After a few hours of storage at -20°C , storage vials were transferred to -80°C or to liquid nitrogen.

4.2. Establishment of Knockout Cell Lines

4.2.1. Designing of Oligos

HEK293T, MCF10A and MDA-MB-231 cells were genome-edited by CRISPR method using an online CRISPR Design Tool single-guide RNA (sgRNA) design program that takes a genomic sequence of interest and identifies suitable target sites. By utilizing the program, the most efficient sgRNAs for the target genes were selected [Ran, 2013]. The double stranded guide sequences recommended by program with low off-target activity are given at Table 4.1.

Table 4.1: Oligo pairs designed to introduce desired mutations to HEK293T.

CRY1-P1-L-Sense	5' CACCGTAGACGCAGCGGATGGTGT 3'
Antisense	5' AAACACACCATCCGCTGCGTCTAC 3'
CRY1-P1-R-Sense	5' CACCGTTCGCCGGCTCCTCCAATGT 3'
Antisense	5' AAACACATTGGAGGAGCCGGCGAAC 3'
CRY2-P1-L-Sense	5' CACCGCCAGCAACGCCGGGTGTGTCG 3'
Antisense	5' AAACCGACAACCCGGCGTTGCTGGC 3'
CRY2-P1-R-Sense	5' CACCGCGTTTACATTCTCGACCCG 3'
Antisense	5' AAACCGGGTCGAGAATGTAAACGC 3'
Bmal-P1-L-Sense	5' CACCGACCCTGATTTCCCCGTTTAC 3'
Antisense	5' AAACGTGAACGGGGAAATCAGGGTC 3'
Bmal-P1-R-Sense	5' CACCGTTTCTCGGCACGCGATAGA 3'
Antisense	5' AAACCTATCGCGTGCCGAGAAAC 3'
Clock-P1-L-Sense	5' CACCGACCTCTGAATGTTCCCCTTC 3'
Antisense	5' AAACGAAGGGGAACATTCAGAGGTC 3'
Clock-P1-R-Sense	5' CACCGAATACTCTCTACTCATCTGC 3'
Antisense	5' AAACGCAGATGAGTAGAGAGTATTC 3'
Per1-P1-L-Sense	5' CACCGTGGCATCGGTGTCATCGGCC 3'
Antisense	5' AAACGGCCGATGACACCGATGCCAC 3'
Per1-P1-R-Sense	5' CACCGTCCAACGGGCATGAGTCTAG 3'
Antisense	5' AAACCTAGACTCATGCCCGTTGGAC 3'
Per2-P1-L-Sense	5' CACCGCACAACTTGGCATCGCTGA 3'
Antisense	5' AAACCTAGCGATGCCAAGTTTGTGC 3'
Per2-P1-R-Sense	5' CACCGTTCCTGGCGCCTCACGATG 3'
Antisense	5' AAACCATCGTGAGGCGCCAGGAAC 3'

Oligo pairs to simultaneously introduce single stranded nicks on both strands of desired circadian clock genes MCF10A and MDA-MB-231 are given at Table 4.2.

Table 4.2: Oligo pairs designed to introduce desired mutations to HEK293T.

<i>BMAL1</i> T1 Sense	5'CACCGTGTCTGTATATTCTAACCT 3'
Antisense	5' AAACAGGTTAGAATATACAGAACAC 3'
<i>BMAL1</i> T2 Sense	5'CACCGTAGATAAACTTACTGTGCTA 3'
Antisense	5' AAACTAGCACAGTAAGTTTATCTAC 3'
<i>CRY1</i> Sense	5' CACCGCCTTCAGGGCGGGGTTGTCG 3'
Antisense	5' AAACCGACAACCCCGCCCTGAAGGC 3'
<i>CRY2</i> Sense	5'CACCGCTGCGACTCCACGACAACC 3'
Antisense	5' AAACGGTTGTCGTGGAGTCGCAGC 3'

4.2.2. Preparation of sgRNA Expression Constructs and Delivery

4.2.2.1. sgRNA Expression Constructs at HEK293T

In order to generate the sgRNA expression construct, a plasmid-based (pX462.V2) procedure was used [Ran Fa et al., 2013]. Construction of an expression plasmid for sgRNA involves a single cloning step with a pair of partially complementary oligonucleotides. The oligo pairs were annealed and ligated into a plasmid (pX462.V2) bearing both Cas9 and the remainder of the sgRNA as an invariant scaffold immediately following the oligo cloning site.

For annealing of single guide oligos (sgRNAs), the top and bottom strands of oligos for each sgRNA design were resuspended to a final concentration of 100 μ M and following mixture were prepared for annealing. Oligos were annealed in microcentrifuge tubes containing in 1 $\times$ NEB buffer 2 solution by heating to 95°C for 5 minutes and then cooling to ambient temperature. The annealing reaction contains 2 μ l of each oligos (diluted to 1 μ g/ μ l), 5 μ l of 10X NEB Buffer 2 (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 7.9 at 25°C) and 41 μ l of ddH₂O.

To clone sgRNA into the pX462.V2 vector for co-expression with Cas9, a ligation reaction for each sgRNA was set up (Ligation components were pX462.V2 (Figure 3.1), 100ng; Diluted oligo complex; FastDigest BbsI; T7 ligase, and ddH₂O).

Also a reaction with no-insert, pX462.V2 only negative control for ligation was set up. The ligation reaction was incubated for 1 h.

Escherichia coli DH5 α competent strain was used for transformations. Competent cells were taken from -80°C freezer and thawed on ice. 5 μ l of ligated plasmids were added on competent cells and were incubated for 30 minutes on ice before heat shock at 42°C for 1 minute. Samples were then cooled on ice for 1,5 minutes, ~700 μ l Luria broth (LB) was added on tubes and they were incubated in shaker at 37°C for 1 hour to give them enough time to express the antibiotic resistance gene. 200 μ l of the samples were spread at ampicillin containing LB agar plates and incubated overnight. From each plate, two or three colonies were picked to check for the correct insertion of sgRNA. Sterile pipette tip was used to inoculate a single colony into a 5ml culture of LB medium with 100 μ g/ml ampicillin. The culture was incubated at 37 °C overnight by shaking for plasmid isolation.

4.2.2.2. sgRNA Expression Constructs at MCF10A and MDA-MB-231

For annealing of single guide oligos (sgRNAs), the top and bottom strands of oligos for each sgRNA design were resuspended to a final concentration of 100 μ M and following mixture were prepared for annealing. Oligos were annealed in microcentrifuge tubes containing in 1 $\times$ NEB buffer 2 solution by heating to 95°C for 5 minutes and then cooling to ambient temperature. The annealing reaction contains 2 μ l of each oligos (diluted to 1 μ g/ μ l), 5 μ l of 10X NEB Buffer 2 (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 7.9 at 25°C) and 41 μ l of ddH₂O.

In separate tubes, 50 ng of LentiCRISPRv2 (Figure 3.2) and the oligos were digested with BsmBI enzyme (recognizes CGTCTC (1/5) sites) in Tango buffer (Thermo Fisher). The mixture was set up for each digesting reaction took place in 50 μ l reaction volume: 2 μ l DNA, 2 μ l 10x restriction enzyme buffer, 1 μ l restriction enzyme(s), ddH₂O to a final volume of 50 μ l. The reaction was performed at 37°C for 1-2 hours.

In order to isolate the digested vector from the digestion solution, 1% agarose gel was prepared. 0.3 gr of agarose was dissolved in 1X TBE and 1 μ l of sybersafe was added. The entire digested vector was mixed with 6X DNA loading

dye of which final concentration was 1X, loaded to gel and in run horizontal electrophoresis tank filled with 1X TBE. 100-1000 bp DNA ladder was used as marker. Then, the sample was extracted from gel. For extraction, gel was cut from the borders of digested vector with a scalpel under UV light and Macherey-Nagel Nucleospin Gel and PCR Clean-up kit was used according to manufacturer's instructions.

Then, 100 ng of annealed oligos were ligated into 50 ng of LentiCRISPRv2 (Figure 3.3) digested with BsmBI. In an eppendorf tube, 100 ng of annealed oligos, 50 ng of LentiCRISPRv2, ligase buffer (1ul in 10ul reaction for 10X buffer), 1 ul of T4 DNA ligase and H₂O to a total of 10ul were mixed. Mix was incubated at room temperature for 2 hours.

After that, ligation products were transformed into ultracompetent Stbl3 *Escherichia coli* cells. Half of the ligation products were mixed with 100 µL competent cells, and then kept on ice for 30 min. The cells were exposed to a heat shock at 42°C for 60 s and transferred back into ice for 2 min. Approximately 900 µL of Luria–Bertani (LB) medium was added to the cells, which were then incubated for 1 h at 37°C with rotating. To select single colonies, 100 µL of the transformation products was plated on LB-Agar plates containing 100 µg/mL ampicillin and incubated overnight at 37 °C. After incubation, selected single colonies were taken into 5 ml ampicillin containing LB medium by micropipette tip and were incubated overnight at 37 °C for plasmid isolation.

4.2.2.3. Plasmid Isolation

To isolate the plasmid, Macherey-Nagel Nucleospin Plasmid Isolation kit protocol was followed (New England Biolabs). This protocol begins with centrifuging the lysate of bacteria pellet from the overnight solution, followed by binding the DNA to a filter column. The filter was then washed and DNA eluted. To quantify concentration and purity of the isolated plasmid, NanoDrop spectrophotometer was used and OD260 and OD280 values were read. Isolated plasmids were confirmed by DNA sequencing using the primer U6-F (5'-GAGGGCCTATTTCCCATGATT-3').

4.2.3. Delivery of sgRNA Constructs

4.2.3.1. Transfection of HEK293T Cells

For preparation of cells for transfection, well-dissociated MDA-MB-231 and MCF10A cells were plated onto 12-well plates in appropriate growth media without antibiotics 16–24 h before transfection. (The cells were seeded approximately to 60% confluency). Cell lines were transfected in 12-well plates using Fugene HD transfection agent. Suggested volumes for both transfection reagents with various multiwell formats were indicated at manual instructions of kits.

4.2.3.2. Infection of MCF10A and MDA-MB-231 Cells

LentiCRISPRv2-based CRISPR constructs were co-transfected with pCMV-dR8.2 dvpr (Figure 3.4) and pCMV410 VSVG (Figure 3.5) packing plasmids into HEK293T cells to produce lentiviral particles.

4.2.3.2.1. Production of Lentiviral Stocks

Lentiviral stocks were produced in HEK293T cells using the following optimized transfection conditions. HEK293T cells were plated in 10 cm tissue culture plate to a confluency that they should be 90-95 on the day of transfection. 10cm dish at 95% confluence was subcultured to two 10 cm dishes to obtain 95% confluent cells. On the day of transfection, a transfection mix was set up:

In a sterile 15 ml culture tube, 15 µg of Lenti-Combo Mix and 10 µg of pLenti expression plasmid DNA (Figure 3.5) were diluted in 1.0 ml of medium without serum and mixed gently. In a separate sterile 15 ml tube, 80 µl of Lentifectin was diluted in 1.0 ml of medium without serum, mixed gently and incubated for 5 minutes at room temperature. After the 5 minutes of incubation, diluted DNA was combined with the diluted Lentifectin and mixed gently. The mix was incubated for 20 minutes at room temperature to allow the Lentifectin/DNA complexes to form. 4.5 ml serum-free medium was added to the complexes followed by gently mixing.

The medium was removed from the cells, and then Lentifectin/ DNA complexes was carefully added to culture dishes without dislodging cells. The cells were incubated for 5-8 hours at 37 °C in a humidified 5% CO₂ incubator. 0.65 ml serum was added to each transfected culture dish and they were incubated overnight. The following day, the medium containing the Lentifectin/ DNA complexes was removed and replaced with 10 ml complete culture medium. Dishes were incubated at 37 °C in a humidified 5% CO₂ incubator. Virus-containing supernatants were harvested by 48-72hrs post-transfection by collecting medium into to a 15 ml sterile, capped, conical tube. Supernatants were centrifuged at 3000 rpm for 15 min. at 4°C to pellet debris. Viral supernatants were aliquot into cryovials in 1.0ml portions and stored as viral stocks at -80 °C.

4.2.3.2.2. Transduction Prosedure

Target cells (MDA-MB-231 and MCF10A cells) were plated in a 24-well-plate for 24 hours prior to viral infection at a density of 0.5×10^5 cells per well. 0.5 ml of complete appropriate medium was added and cells were incubated at 37 °C with 5% CO₂ overnight. A mixture of complete media with Polybrene at a concentration of 2 µg/ml was prepared. The media were removed from the wells and replaced with 0.5 ml of the Polybrene/media mixture per well (for 24-well plate). Target cells were infected by adding several different amounts of viral stock (example: 1µl, 5 µl, 10 µl, and 100 µl of virus). Cells were incubated at 37 °C with 5% CO₂ overnight. The culture medium was removed and replaced with 1 ml of complete medium. Cells were incubated at 37 °C with 5% CO₂ overnight. The following day, the cells were split depending on the growth rate of the cells, and continued incubating for 48 hours in appropriate medium. The infected target cells were selected for stable expression using puromycin as selection marker.

4.2.4. Clonal Isolation of Cell Lines

Infected cells were selected with puromycin (0.5 mg/mL for MCF10A and 1.5 mg/mL for MDA-MB-231) for 3–4 days by changing the medium of cells with

puromycin including medium. By following 3-4 day incubation, the cells were transferred to 96-well plate from 12-well-plates by serial dilution:

100 µl medium was added to each well of plate. 100 µl of cell suspension was added to the first column. 100 µl of diluted cells was multichannel-pipetted to each well of a 96-well plate thereby diluting in a ratio of 1:2. This procedure was repeated for each consecutive column and finally 100 µl from the last column was discarded. Cells were fed at every 2–3 days with fresh puromycin including medium. The colonies were inspected for a clonal appearance ~1 week after plating (rounded colonies radiating from a central point). Then single cell-derived clones were picked and grown in six-well plates in duplicate, and one was used for immunoblotting analysis to select candidate knockouts for further confirmation and one for maintenance of candidate knockout cell lines.

The *BMAL1* gene was targeted at two genomic locations separately to reduce nonspecific off-target effects of random integration or selection. Two single cell-derived cell lines were chosen to be used in further experiments for each targeting sgRNA according to Western Blotting results. Mutations caused by CRISPR-Cas9 T1 and T2 sgRNAs were described by T7 Endonuclease assay and Sanger sequencing from PCR products.

4.2.5. Confirmation of Mutations

4.2.5.1. Western Blotting Analysis of Candidate Knockouts

Candidate knockout cells were seed at 24 well tissue culture plates up to 80% confluency. Then, the medium was removed, the cells attached to monolayer were washed with 1X PBS, and lysed directly in 1X Lysis Buffer which contains 1X Laemmli Buffer added 1% Beta-Mercaptoethanol. The amount of 1 X Lysis Buffer used for lysis was determined according to cell confluency. After incubation 10 min at room temperature with shaking, cells were transferred into eppendorf tubes from plate and screened by Western Blotting analysis.

4.2.5.2. T7 Endonuclease Assay

Genomic DNA was extracted from approximately 10^6 cells using the NucleoSpin DNA isolation kit (Macherey-Nagel, GmbH & Co. KG, Germany) following the manufacturer's instructions and the genomic sequences spanning the target sequences of sgRNA's were PCR-amplified by Taq DNA Polymerase (New England Biolabs, Hertfordshire, U.K.) using specific forward and reverse primers, of which sequences are given below. The PCR conditions were a denaturation step at 95°C for 5 minutes; then 35 cycles at 95 °C for 30 seconds, 60 °C for 20 seconds, and 68 °C for 30 seconds; and a 2-minute final extension at 68 °C. PCR products were column purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). 200 ng from wild-type and mutated cleaned PCR samples were denatured and reannealed for heteroduplex formation in 1x NEB buffer 2 (New England Biolabs) by heating to 95 °C for minutes and allowing the samples to cool down after turning the heat block off. Finally, 10 µl of heteroduplex formation reaction product was incubated with 2.5 unit of T7 Endonuclease (NEB) at 37°C for 2 hours, and the digested products were analyzed with the agarose gel electrophoresis. Sequences of forward and reverse primers for T7 Endonuclease assay are BMAL1-T1_F and R: 5' GACAAAGATGACCCTCATGGA 3' and 5' AGCCAGTATGGAGAGCATGG 3' BMAL1-T2_F and R: 5' CCTGGCAGTGAGACCATTTT 3' and 5' TCACTTCATT TGGCATCACG 3'.

4.2.5.3. Confirmation of Knockouts by Sanger Sequencing

In order to confirm disruption of *BMAL1* knockouts by Crispr/Cas9, full-length *BMAL1* cDNAs from wild type and their T1 and T2 sgRNA targeted knockouts of both MCF10A and MDA-MB-231 were amplified. For this purpose, total RNA was converted into cDNA using ProtoScript II reverse transcriptase and oligo-dT by following the manufacturer's protocol (New England Biolabs). Full-length cDNAs (which encompasses the expected Cas9 cut sites at ~180 bp and ~342 bp following the first base of start codon for T1 and T2 respectively) were amplified using the primers with NheI and EcoRI sites to aid directional cloning. Forward and reverse

oligo sequences used to amplify full-length cDNAs from our cell lines are NheI-BMAL_F 5'CAAGCTAGCGCCACCATGGCAGACCAGAGAATGGACA 3' and EcoRI-BMAL_R 5' AAAGAATTCCAGCGGCCATGGCAAGTCACT 3'.

For T1 mutations, PCR products from cDNAs of knockout or wild type cells were sequenced using NheI-BMAL1_F oligo. Because using NheI-BMAL1_F oligo, clear sequencing for T2 sites could not be obtained, T2 mutation from gDNA after amplifying with T7 Endonuclease oligos were used. PCR products were sequenced using the BMAL1-T2_R oligo. Alignments of mutations read by Sanger sequencing were done using an online tool (CRISP-ID: Detecting CRISPR mediated indels by Sanger sequencing tool).

4.3. Population Doubling (PD) assay

50×10^3 cells were seeded onto 6-well plates, and counted manually using a hemocytometer after 48 hours. PD was calculated by the formula: $PD = \frac{\log(N_f/N_i)}{\log 2}$, where N_f the number of cells counted and N_i the number of cells seeded.

4.4. Analysis of Mycoplasma Status of Cell Lines

The contamination with mycoplasma in cells was tested using polymerase chain reaction (PCR) based e-Myco Plus Mycoplasma PCR Detection Kit (Boca Scientific Inc., Boca Raton, FL, USA) according to the procedure recommended by the manufacturer. Shortly, genomic DNAs (50 ng) of cell lines, which were kept in the same culture medium for 5 days, were added to the tubes containing the reconstituted PCR mix. Primers were included in the mix for a housekeeping gene and for common species of mycoplasma.

4.5. Apoptosis Assay

Apoptosis was induced by treating cells with a bulky-DNA adduct forming agent, cisplatin, or a double strand break forming agent, doxorubicin (Sigma).

Cisplatin (Sigma) stock at 5 mM was dissolved in 0.9% (w/v) NaCl solution. Doxorubicin was prepared at 5 mM in double distilled sterile water.

4.5.1. Apoptosis Assay at MDA-MB-231 Cells

For apoptosis assay, cells were counted by using a hemocytometer and 300 000 cells were seed in 60 mm dishes. The following day, the cells were treated with 0, 20 and 40 μ M of cisplatin; or 0, 2.5 and 5 μ M of doxorubicin for 16 h. After incubation, apoptotic response was checked by Western Blotting by detecting cleaved PARP, and was normalized to actin, served as a loading control.

4.5.2. Apoptosis Assay at MCF10A Cells

For apoptosis assay, cells were counted by using a hemocytometer and 300 000 cells were seed in 60 mm dishes. The following day, the cells were treated with 0, 10 and 20 μ M of cisplatin; or 0, 0.5 and 1 μ M of doxorubicin for 16 h. After incubation, apoptotic response was checked by Western Blotting by detecting cleaved PARP, and was normalized to actin, served as a loading control.

4.6. Western Blotting

4.6.1. Sample Preparation

Medium was discarded of the culture dish or plate and appropriate amount of ice-cold 1X PBS was added. Adherent cells were scraped off the dish using a cold cell scraper and the cell suspension was gently transferred into a pre-cooled microcentrifuge tube. After centrifugation at 5000 rpm for 3 minutes, the supernatant was discarded. Appropriate amount of ice-cold RIPA lysis buffer, prepared shortly before using, added to the pellet, it was homogenized by pipetting (RIPA lysis Buffer) and incubated on ice for 20 minutes. For each 5 minute, the pellet was dissolved by gently pipetting. After incubation, the tubes were centrifuged for 15 min, at 13000rpm, +4⁰C. The supernatant was transferred to a fresh tube and

kept on ice (or freeze at -20°C). With a small volume ($2\ \mu\text{l}$) of supernatant, Bradford assay was performed (Biorad Protein Assay Dye Reagent Concentrate, Cat: 500-0006). For determination of the concentrations of proteins by Bradford assay, protein standard was prepared by using different concentrations of BSA stock. Bradford reagent added ($250\ \mu\text{l}$) on to each BSA standard and each of the $2\ \mu\text{l}$ -samples in 96 well plate. Absorbance is measured at 595nm . Using obtained absorbance values, protein concentrations were determined by Microsoft EXCEL. The concentrations of lysates were equalized by diluting with the appropriate amounts of RIPA lysis buffer. $30\ \mu\text{g}$ of each sample was introduced with 4X Laemmli buffer that includes 2.5 % volume of beta Mercaptoethanol (Final concentration of laemmli buffer should be 1X in samples). Samples were boiled at 95°C for 10 minutes and shortly spinned.

4.6.2. Protein Separation by Gel Electrophoresis

Equal volumes of the samples were loaded into the wells of SDS-PAGE gel, density of which was determined according to the size of protein expected to be detected by Western Immunoblotting. One of the wells was loaded by appropriate protein marker. All empty wells were loaded with 1X Laemmli Buffer. The samples were run for 15 min. at 80 V, then the voltage was increased up to 120-150V. 1X SDS Running Buffer, prepared as 10X Stock Solution and diluted to 1X with distilled water was used for running.

4.6.3. Transfer of Proteins to the Membrane

Trans-blot Turbo Transfer System (Biorad Transblot Turbo Transfer System, Serial No: 690BR011760) was used for transferring of proteins to nitrocellulose membrane. 4 blotting papers and 1 nitrocellulose membrane with the same dimensions of SDS PAGE gel were wet with 1X transfer buffer and transfer sandwich was created (1X Transfer Buffer Preparation: 10X SDS Running Buffer (Final concentration of SDS Running Buffer should be 1X) and 20% methanol are completed to final volume with ddH_2O).

Transfer Sandwich was assembled by the components on the cassette in order. Then, air bubbles were removed from the sandwich by a roller. Transfer was performed at 12V constant voltage for 60 min for low molecular weight proteins and 90 min for high molecular weight proteins at transblot transfer system.

4.6.4. Blotting and Antibody Incubation

After transfer, the membrane was blotted in 5 % nonfat milk powder dissolved at 1X TBS-T, at room temperature, for 1 hour and incubated in the primary antibody solution overnight at 4 °C or 2 hours at room temperature (Primary antibody was added to 5 % nonfat milk powder dissolved at 1X TBS-T, 0.05% sodium azide was added to the solution to re-use primary antibody solution). The membrane was rinsed for 4 times (5-15-5-5 minutes respectively) with 1X TBS-T on an orbital shaker and then incubated in horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBST as recommended in its datasheet) for 1 hour at room temperature. Then, the membrane was rinsed for 4 times (5-15-5-5 minutes respectively) with 1X TBS-T on an orbital shaker.

4.6.5. Imaging and Data Analysis

The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation:

In our laboratory, Western Blotting Detection Kit Advansta Cat: K-12043-D10 and SuperSignal West Femto Chemiluminescent Substrate Cat: 34095 were used.

Western Blotting Detection Kit Advansta includes Western Bright Peroxide (Component 1) and Western Bright Sirius (Component 2). Working solution mix is prepared with equal aliquot of component 1 and 2. The membrane is exposed to the mix for 2 minutes.

SuperSignal West Femto Chemiluminescent Substrate includes Stable Peroxide Solution and the Luminol/Enhancer Solution B working solution mix is

prepared with equal aliquot of two reagents. The membrane is exposed to the mix for 5 minutes.

The chemiluminescent signals were captured by using an imager (BIORAD Molecular Imager ChemiDoc XRS+with Image Lab Software, Universal Hood II, Serial No 721BR04545).

4.7. Invasion Assay

Invasion assays were performed using the CytoSelect 24-Well Cell Migration Assay kit following the manufacturer's instructions. The CytoSelect Cell Invasion Assay Kit contains polycarbonate membrane inserts (8 μm pore size) in a 24-well plate. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. This basement membrane layer serves as a barrier to discriminate invasive cells from non-invasive cells. Invasive cells are able to degrade the matrix proteins in the layer, and ultimately pass through the pores of the polycarbonate membrane. Finally, the cells are removed from the top of the membrane and the invaded cells are stained and quantified.

Under sterile conditions, the invasion chamber plate was allowed to warm up at room temperature for 10 minutes. The basement membrane layer of the cell culture inserts were rehydrated by adding 300 μL of warm, serum-free media to the inner compartment and incubated at room temperature for 1 hour. MDA-MB-231 and its knockout clones were trypsinized and washed once with DMEM containing 10% FBS, and then washed twice with serum free-DMEM. The cells were resuspended in DMEM containing 0.1% Bovine Serum Albumin at a density of 5×10^5 cells/mL. Carefully rehydration medium was removed from the inserts without disturbing the basement membrane layer. 500 μL of media containing 10% fetal bovine serum was added to the lower well of the invasion plate. 300 μL of the cell suspension solution was added to the inside of each insert and incubated for 36 hours at 37°C in 5% CO_2 atmosphere. Carefully, the media was aspirated from the inside of the insert. The ends of 2-3 cotton-tipped swabs were wet with water, the ends of the swabs were flatten by pressing against a clean hard surface, and gently

the interior of the inserts were swabbed to remove non-invasive cells. Insert was transferred to a clean well containing 400 μ L of Cell Stain Solution and incubated for 10 minutes at room temperature. Gently the stained inserts were washed several times in a beaker of water. The inserts were allowed to air dry. Each insert was transferred to an empty well, adding 200 μ L of Extraction Solution per well, then incubating 10 minutes on an orbital shaker. 100 μ L from each sample were transferred to a 96-well microtiter plate and measured the OD 560nm using a Synergy H1 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Invasion was also checked under a microscope by focusing to the lower part of the Matrigel to control the invasion potential of the cell lines. Invasive potential was normalized to that of wild-type MDA-MB-231 parental cells.

4.8. Statistical Analysis

Values are shown as mean $\pm$ SD of at least three experiments. Results were statistically analysed by (two-way or one-way) ANOVA followed by Tukey's post hoc tests for pairwise comparisons using GraphPad Prism 7.04 (GraphPad Software, Inc., San Diego, USA). Types of the tests were indicated in the figure legends.

5. RESULTS

The main purpose of this thesis is to investigate the relationship between circadian clock core genes and cancer development. For this purpose, core clock genes (*Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1* and *Per2*) were initially targeted and knocked out at HEK293T cell line using plasmid-based CRISPR method to test the efficiency of the oligos designed by utilizing the online CRISPR Design Tool single-guide RNA (sgRNA) design program. Knockout candidates were screened with immunoblotting using specific antibodies against the circadian clock proteins targeted (Figure 5.1).

Then, *BMAL1* gene in a nearly normal cell line (MCF10A) and an invasive cell line (MDA-MB-231) was knocked out to study two cancer-related pathways, apoptosis and invasion. In contrast to other core clock genes, *BMAL1* is the only gene whose loss alone leads to complete loss of rhythmicity [Bunger et al., 2000]. Therefore *BMAL1* gene was targeted at both cell lines. To obtain *BMAL1* knockouts, *BMAL1* gene was targeted at two different genomic sites separately. After confirmation of gene knockout, first, the effect of *BMAL1* gene deletion on the apoptosis in response to genotoxic agents was investigated in MCF10A cells. Then, the effect of *BMAL1* knockout mutation on invasion process was established on invasive MDA-MB-231 cell line. Later, apoptotic response of MDA-MB-231 cells were tested to find out whether the increased apoptotic response in untransformed MCF10A is also observed in a transformed and invasive cancer cell line.

5.1. Knockout of Core Clock Genes in HEK293T Cell Line

Core clock genes were targeted at HEK293T cell line using plasmid-based CRISPR method. Knockout candidates were screened with immunoblotting using specific antibodies against the core clock proteins targeted. Immunoblot of *BMAL1*, *CLOCK*, *CRY1*, *CRY2* and *PER2* proteins in the HEK293T cells confirmed that the knockouts were successful and *PER1* expression was decreased when compared to wild type cell line. One knockout cell line for *BMAL1*, *CLOCK* and *CRY1* and two knockout cell lines for *CRY2* and *PER2* were obtained (Figure 5.1.).

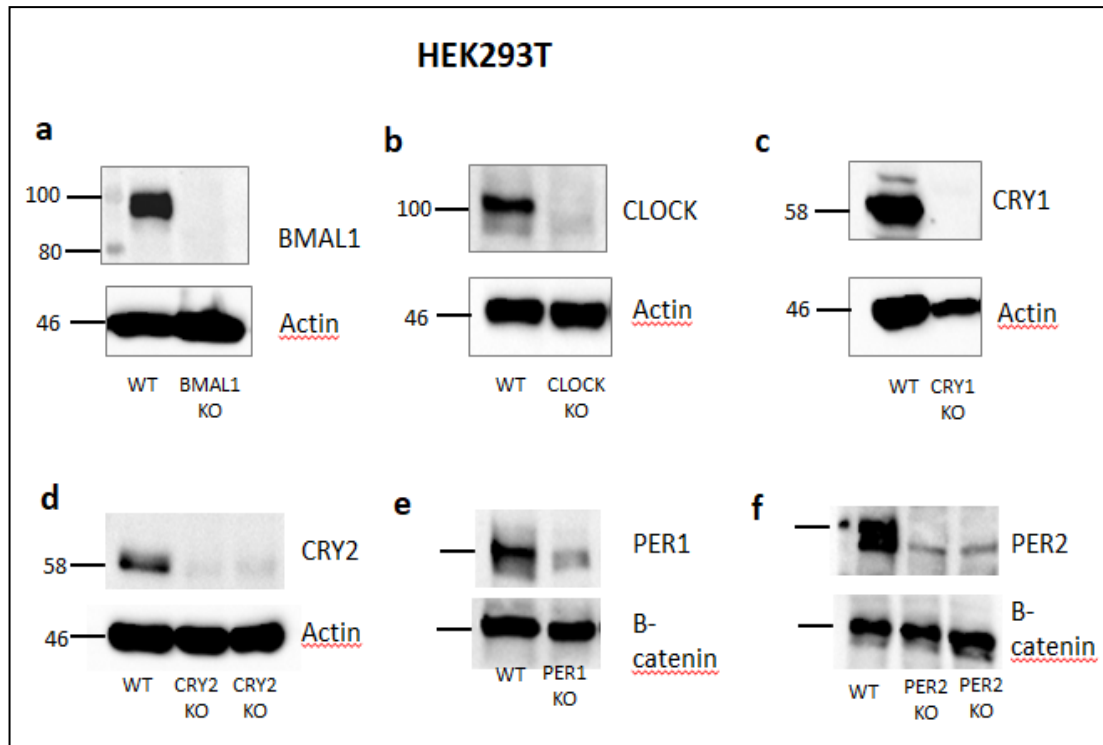


Figure 5.1. Confirmation of core clock gene knockouts at HEK293T. a) Immunoblot of BMAL1 protein. b) Immunoblot of CLOCK protein. c) Immunoblot of CRY1 protein. d) Immunoblot of CRY2 protein. e) Immunoblot of PER1 protein. f) Immunoblot of PER2 protein. Actin (in a, b,c and d) and β -catenin (in e and f) were blotted as loading control.

5.2. Knockout of *BMAL1* Gene in MDA-MB-231 and MCF10A Cell Lines

5.2.1. Confirmation of Mutations by Western Blotting Analysis

BMAL1 was targeted at two different locations at both MCF10A and MDA-MB-231 cell lines. Two knockout subclones were obtained. Knockouts were screened with immunoblotting using a specific anti-BMAL1 antibody against the C terminus of the protein (Figure 5.2). *CRY1* and *CRY2* (*CRY*-DKO) were also knocked out in both cell lines as a control. In MCF10A, *CRY*-DKO mutant control was used. For MDA-MB-231 cell line, a mock control transfected with *BMAL1*-targeting lentivirus particles but escaped the knockout process was used to compare the knockouts. Although a *CRY* DKO MDA-MB-231 cell line was obtained, it showed sensitivity to cisplatin-induced apoptosis because of a *p53* mutation in this cell line, which was established

by previous findings [Ozturk et al., 2009]. In Figure 5.2, T1 and T2 indicate that *BMAL1* was disrupted by two different target (T) sgRNAs, and Line 1 (L1) and line 2 (L2) are different subclones for T1 (sgRNA) or different subclones of T2 (sgRNA). The same nomenclature was also used for MDA-MB-231 cell line knockouts. Actin was used as a loading control. WT represents parental cell lines. At first line, immunoblot of *BMAL1*, *CRY1*, and *CRY2* proteins in the MCF10A clones confirmed that the knockouts were successful. In the second and third lines, immunoblot of *BMAL1*, *CRY1* and *CRY2* proteins in the MDA-MB-231 clones confirmed that the knockouts were successful.

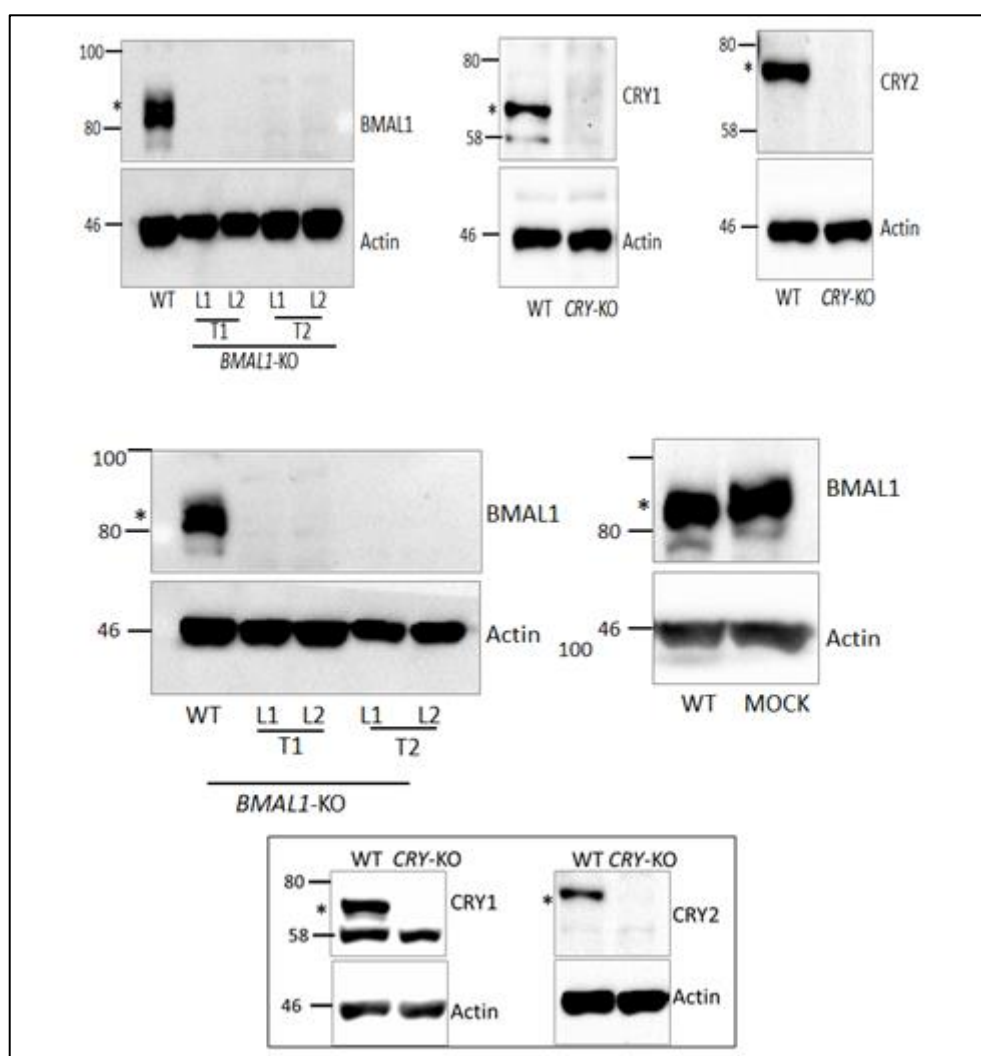


Figure 5.2. Confirmation of *BMAL1* knockouts at MDA-MB-231 and MCF10A cell lines. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa.

5.2.2. Confirmation of Mutations by T7 Endonuclease Assay

T7 endonuclease assay was used to confirm genome editing from genomic DNA. The genomic DNA from wildtype and mutated samples were amplified by PCR, using primers spanning sgRNA target sites, and cleaned by PCR cleaning columns. Then, these samples from wild-type and mutants were mixed and denatured and re-annealed. Mismatches between wildtype and mutant PCR products are recognized by T7 endonuclease which generated double-strand breaks. Therefore mutations are presented by the fragmentation of PCR products compared to PCR products only from wildtype samples.

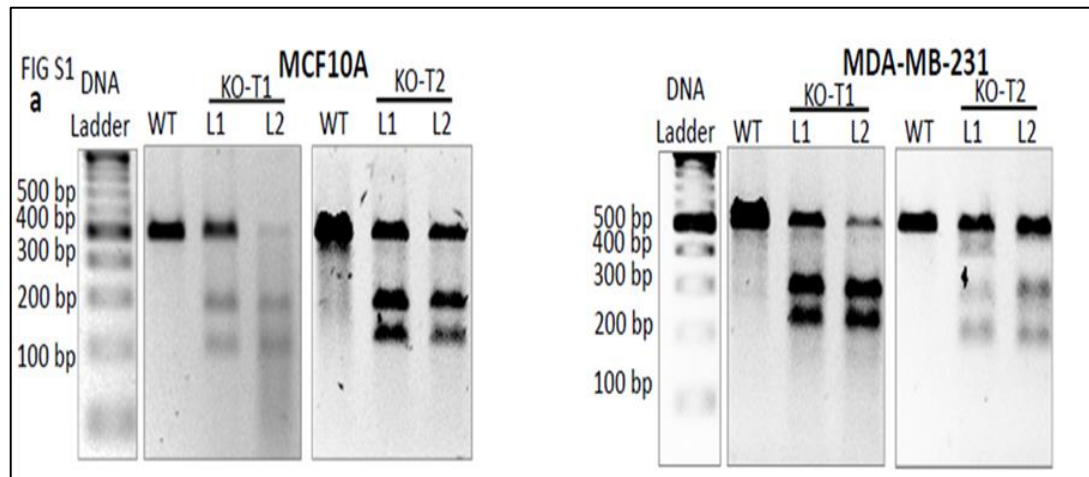


Figure 5.3: Confirmation of CRISPR-Cas9 mediated mutations by T7 endonuclease assay.

In addition, full-length cDNAs from wildtype cell lines and knockout clones were amplified and cloned into an expression vector (pcDNA3.3xHA in frame) for sequencing and expression analysis. These vectors were transfected into HEK293T cells, and expression of wild-type and mutant *BMAL1* from our knockouts were analysed with anti-HA tag blotting. T1 and T2 cDNAs expressed short proteins at very low level.

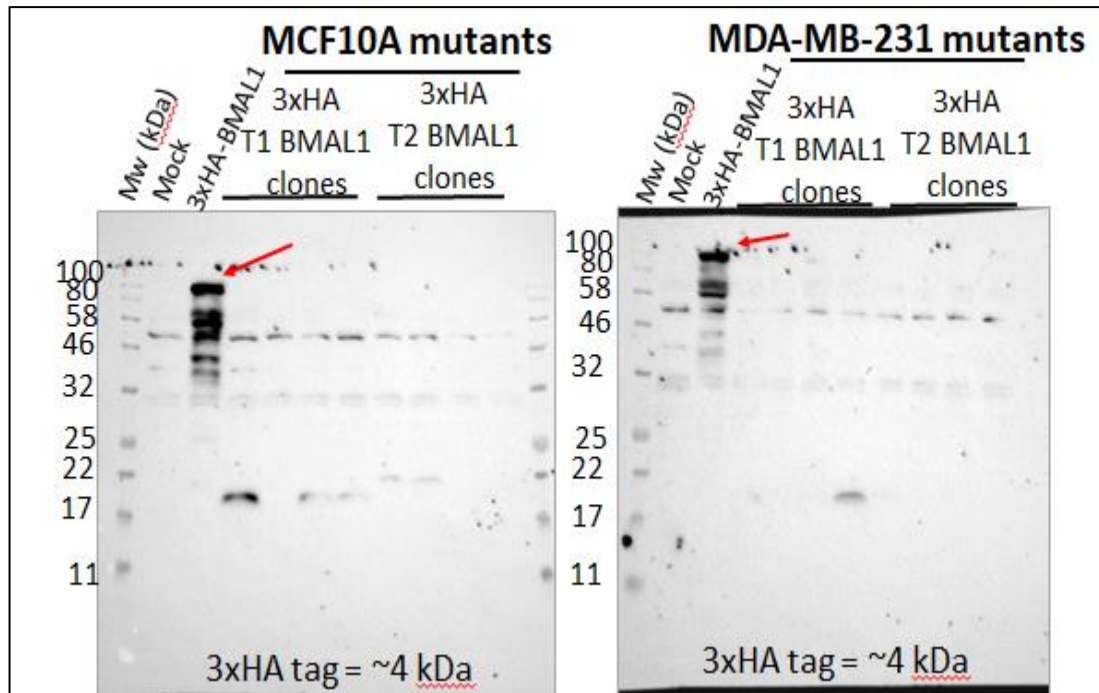


Figure 5.4. Expression Analysis of *Bmal1* WT and knockout cell lines with anti-HA blotting. Full-length BMAL1 protein from wildtype cells were shown by red arrows. T1 and T2 cDNAs expressed short proteins at very low level.

5.2.3. Confirmation of Knockouts by Sanger Sequencing

In order to understand the nature of the mutations induced by CRISPR in *BMAL1* knockouts, PCR products from cDNAs or gDNAs for T1 and T2 induced knockouts were sequenced (Figures 5.6, 5.7, 5.8 and 5.9 respectively). Then, the Sanger sequencing results were analyzed to detect the indels by CRISP-ID method [Dehairs et al., 2016]. All mutations in knockouts were found to generate early stop codons before the end of the basic helix loop helix (bHLH) domain (Figure 5.5).

These truncated parts of BMAL1 protein are far from being a functional BMAL1 because they do not have a full bHLH domain as well as PAS domains which are important for dimerization [Huang et al., 2012]. Moreover, the truncations with T1 sgRNA generated proteins (stops at codon 58-64) just before the start of the first helix (which starts at codon 62) which would not fold into a functional helix structure.

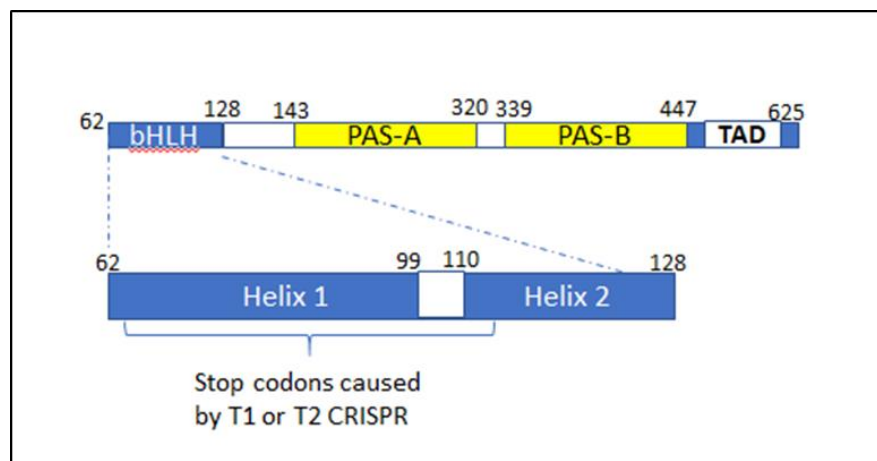


Figure 5.5: Identification of CRISPR/Cas9 induced mutations in *BMAL1* gene by CRISP-ID method. Premature stop codons truncated proteins in the bHLH, and PAS domain was not expressed in the knockouts.

MCF10A T1 (L2 line)	
CRISP-ID alignment from amplified cDNA sanger sequencing	
1 CCGGCCCCACCGACCTGCTTTCCAGCTCTCTTGGTACCACTGGTGTGGATTGCAACCGCAAACGGAAAGGCAGCTCCACTGACTACCAAGAAAGCATGG	100
1...GGGCCCCACCGACCTGCTTTCCAGCTCTCTTGGTACCACTGGTGTGGATTGCAACCGCAAACGGAAAGGCAGCTCCACTGACTACCAAGAAAGCATGG	98
1...GGGCCCCACCGACCTGCTTTCCAGCTCTCTTGGTACCACTGGTGTGGATTGCAACCGCAAACGGAAAGGCAGCTCCACTGACTACCAAGAAAGCATGG	98
101 ACACAGACAAAGATGACCTCATGAGGTTAGAAATACAGAACACCAAGGAAGGATAAAAAATGCAAGGGAAGCTCACAGTCAGATTGAAAAGCGGCG	200
99 ACACAGACAAAGATGACCTCAGGGAAGG-----AATATACAGAACACCAAGGAAGGATAAAAAATGCAAGGGAAGCTCACAGTCAGATTGAAAAGCGGCG	193
99 ACACAGACAAAGATGACCTCATGGAAGG-----ATACACAACACCCGGAGGGAGAAAAATGCGCGGGAAACTCACAGTCACATTGAAAAGCGGCG	191
201 TCGGGATAAAATGAACAGTTTATAGATGAATTGGCTTCTTTGGTACCAACATGCAACGCAATGTCCAGGAAATTAGATAAACTTACTGTGCTAAGGATG	300
194 TCGGGATAAAATGAACAGTTTATAGATGAATTGGCTTCTTTGGTACCAACATGCAACGCAATGTCCAGGAAATTAGATAAACTTACTGTGCTAAGGATG	293
192 TCGGGATAAAATGAACAGTTTATAGATGAATTGGCTTCTTTGGTACCAACATGCGACGCACTGTCCAGGAAATTATATAAACTTACTGTGCTAAGGAGG	291
Corresponding Protein Sequences and calculated MWs:	
Wildtype Protein	
..GPTDLLSSSLGTSGVDCNRKRKGSSTDYQESMDTDKDDPHGRLEYTEHQGRIKNAREAHSQIEKRRRDKMNSFIDELASLV....	
MW: 68.69 kDa	
Protein 1	
...GPTDLLSSSLGTSGVDCNRKRKGSSTDYQESMDTDKDDPQGRNIQNTKEG	
MW: 8.26 kDa (Codon 60**)	
Protein 2	
...GPTDLLSSSLGTSGVDCNRKRKGSSTDYQESMEQTKMTLMEGYTTPREKKCAGNSQSH	
MW: 9.34 (Codon 58**)	

Figure 5.6. Alignment of mutation in MCF10A *BMAL1* knockout T1 L2. Green highlight: PAM Sequences, Yellow highlight: sgRNA target sequence, - premature stop codon caused by frameshift. MW: Molecular Weight in kDa; ** indicates the codon number where truncated proteins lose any significant homology with wild type protein. MW calculation is based on the expressed proteins including frame shift caused peptides (maximum 10 a.a.) before the early stop codons.

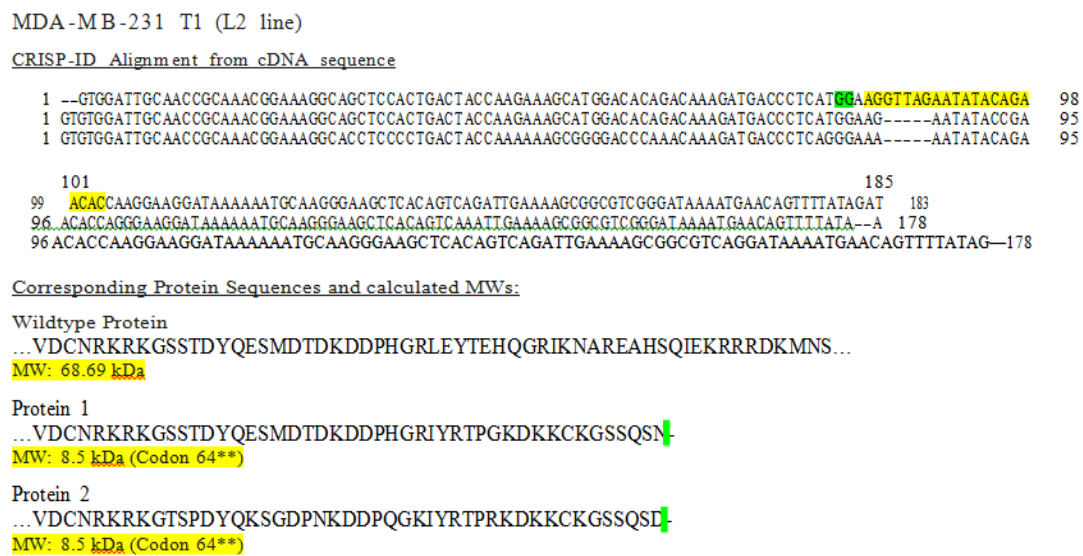


Figure 5.7. Alignment of mutation in MDA-MB-231 *BMAL1* knockout T1 L2. Green highlight: PAM Sequences, Yellow highlight: sgRNA target sequence, - premature stop codon caused by frameshift. MW: Molecular Weight in kDa; ** indicates the codon number where truncated proteins loose any significant homology with wild type protein. MW calculation is based on the expressed proteins including frame shift caused peptides (maximum 10 a.a.) before the early stop codons.

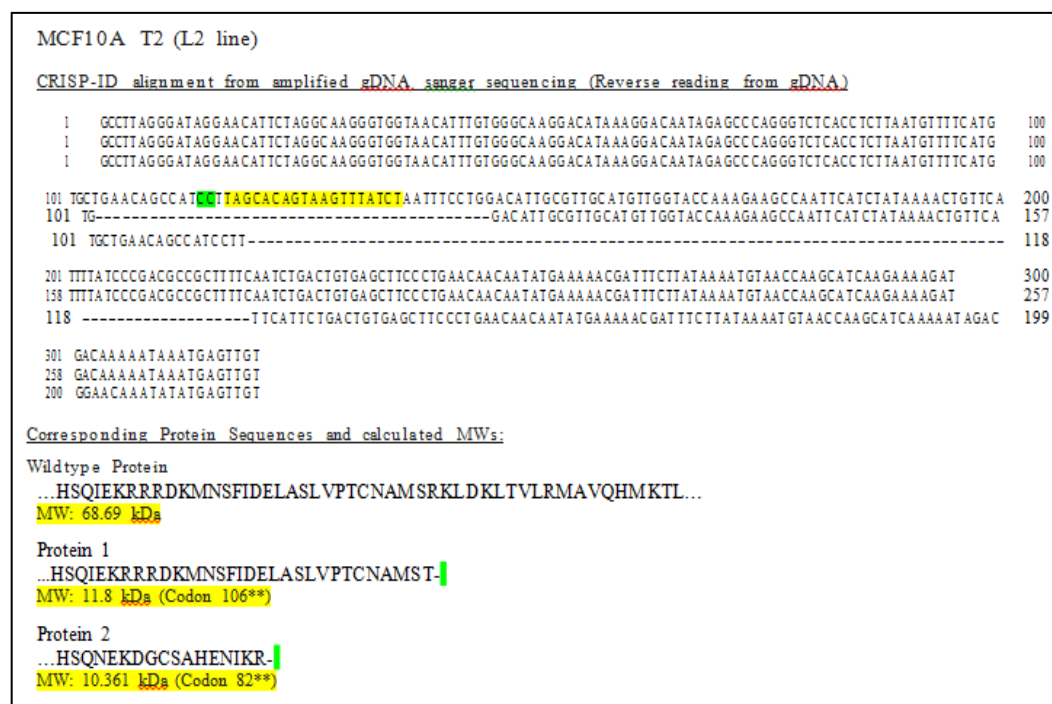


Figure 5.8. Alignment of mutation in MCF10A *BMAL1* knockout T2 L2. Green highlight: PAM Sequences, Yellow highlight: sgRNA target sequence, - premature stop codon caused by frameshift. MW: Molecular Weight in kDa; ** indicates the codon number where truncated proteins loose any significant homology with wild type protein. MW calculation is based on the expressed proteins including frame shift caused peptides (maximum 10 a.a.) before the early stop codons.

MDA-MB-231 T2 (L2 line)		
<u>CRISP-ID alignment from amplified gDNA, sanger sequencing (Reverse reading from gDNA)</u>		
1	GCCTTAGGGATAGGAACATTTCTAGGCAAGGGTGGTAACATTTGTGGCAAGGACATAAAGGACAATAGAGCCAGGGTCTCACCTCTTAATGTTTTCATG	100
1	--CTTAGGGATAGGAACATTTCTAGGCAAGGGTGGTAACATTTGTGGCAAGGACATAAAGGACAATAGAGCCAGGGTCTCACCTCTTAATGTTTTCATG	98
1	--CTTAGGGATAGGAACATTTCTAGGCAAGGGTGGTAACATTTGTGGCAAGGACATAAAGGACAATAGAGCCAGGGTCTCACCTCTTAATGTTTTCATG	98
101	TGCTGAACAGCCATCTTAGCACAGTAAGTTTATCTAATTTCTGGACATTGCGTTGCATGTTGGTACCAAGAGCCAATTCATCTATAAACTGTTCA	200
99	TG-----GACAT TGC GT TGC ATGTTGGT ACC AAG AAG CCA ATT C AT C TAT AAA ACT GTT CA	155
99	TGCAGA-----T TGC CCT G C C T G T T C G T ACC G A C T G T G A C C A T T C C T C T A T A A C A C T G T G A A	155
201	T-TTTATCCCGACGCCGCTTTTCAATCTGACTGTGAGCTTCCCTGAACAACAATATGAAAAACGATTTCCTTATAAAATGTAACCAAGCATCAAGAAAAGA	299
156	T-TTTATCCCGACGCCGCTTTTCAATCTGACTGTGAGCTTCCCTGAACAACAATATGAAAAACGATTTCCTTATAAAATGTAACCAAGCATCAAGAAAAGA	254
156	TAACAATCTC-ACACCACATGTCACCAGAATGTGAACCTCCCTGAACAACAATATGAAAAACGATTTCCTTATAAAATGTAACCAAGCATCAAGAAAAGA	254
300	TGACAAAAATAAATGAGTTGTAACGTGTTTCAATGTTTCATCTGACAAGGCAAAATTTAGAATTTGCAGAATGTTTAAATTA380	
255	TGACAAAAATAAATGAGTTGTAACGTGTTTCAATGTTTCATCTGACAAGGCAAAATTTAGAATTTGCAGAATGTTTAAATTA335	
255	AGACTGCAAAAAGTGAATTGTAAAGTTTCAATGTTTCATCTGACAAGGCAAAATTTACAATTTGCAGAATGTTTAAATTA335	
<u>Corresponding Protein Sequences and calculated MWs:</u>		
Wildtype Protein		
...HSQIEKRRRDKMNSFIDELASLVPTCNAMSRKLDKLTVLRMAVOHMKTL...		
MW: 68.69 kDa		
Protein 1		
...HSQIEKRRRDKMNSFIDELASLVPTCNAMST-		
MW: 11.8 kDa (Codon 106**)		
Protein 2		
...HSVIEEWSQSVRTGRAICT-		
MW: 10.56 kDa (Codon 81**)		

Figure 5.9. Alignment of mutation in MDA-MB-231 *BMAL1* knockout T2 L2. Green highlight: PAM Sequences, Yellow highlight: sgRNA target sequence, -premature stop codon caused by frameshift. MW: Molecular Weight in kDa; ** indicates the codon number where truncated proteins loose any significant homology with wild type protein. MW calculation is based on the expressed proteins including frame shift caused peptides (maximum 10 a.a.) before the early stop codons.

5.3. Population Doubling (PD) assay

After obtaining knockout cell lines, population doubling times of cell lines were established. A significant change in population doubling times in knockouts was not observed when compared to parental or control clones (Figures 5.10 and 5.11).

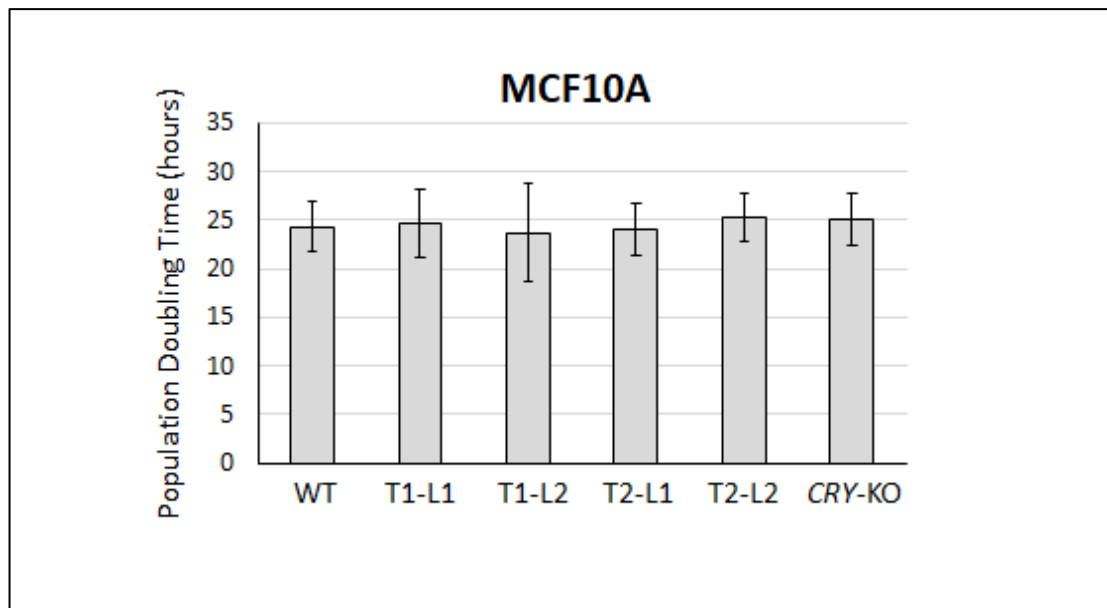


Figure 5.10. Population Doubling (PD) Time of MCF10A cell lines. PD times were calculated and compared to parental cell lines. PD times were not significantly altered by *BMAL1* or *CRY* mutations. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

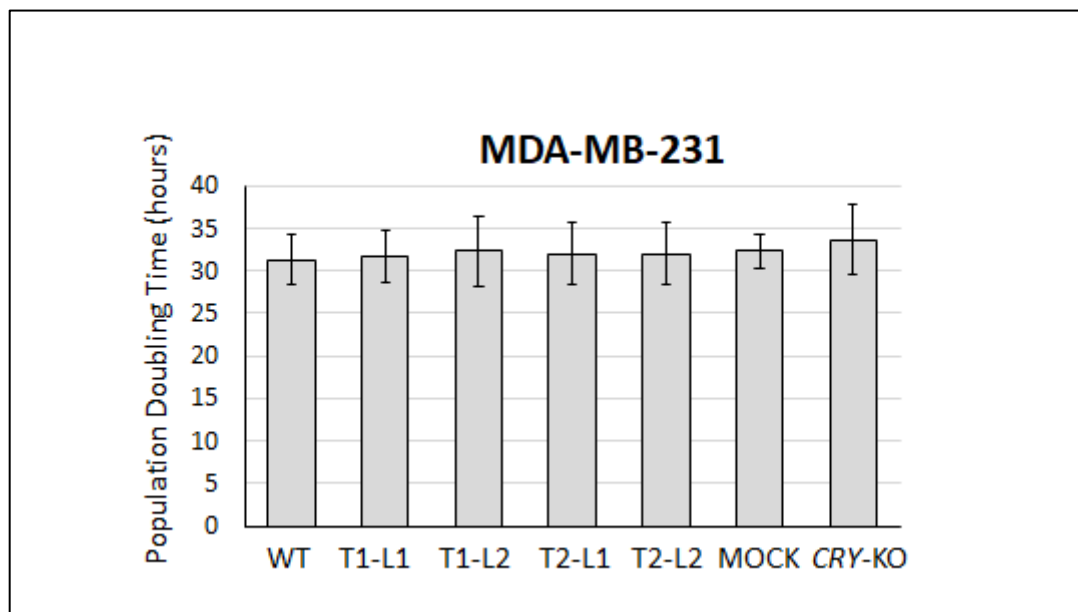


Figure 5.11. Population Doubling (PD) Time of MDA-MB-231 cell lines. PD times were calculated and compared to parental cell lines. PD times were not significantly altered by *BMAL1* or *CRY* mutations and by mock cell line. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

5.4. Analysis of Mycoplasma Status of Cell Lines

All parental cell lines and knockout cell lines were tested for mycoplasma contamination using the e-Myco plus Mycoplasma PCR Detection Kit (Boca Scientific Inc., Boca Raton, FL, USA). No mycoplasma contamination was seen in the cell lines used for further experiments (Figures 5.12 and 5.13).

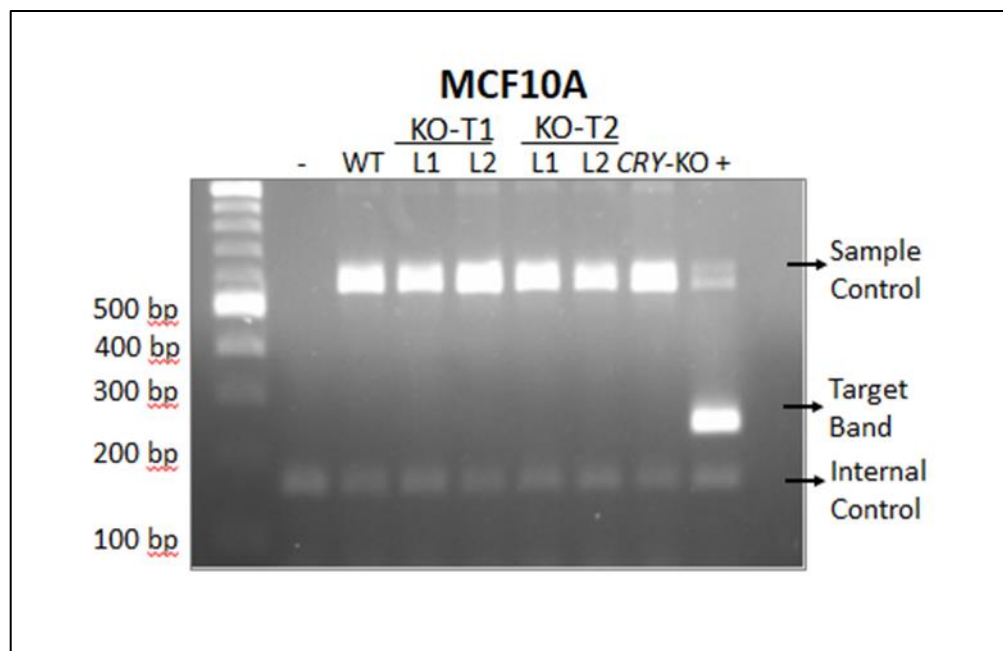


Figure 5.12. Analysis of mycoplasma status of MCF10A cell lines. No mycoplasma were contaminated in the cell lines used in this study. KO indicates *BMAL1* or *CRY* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in bp.

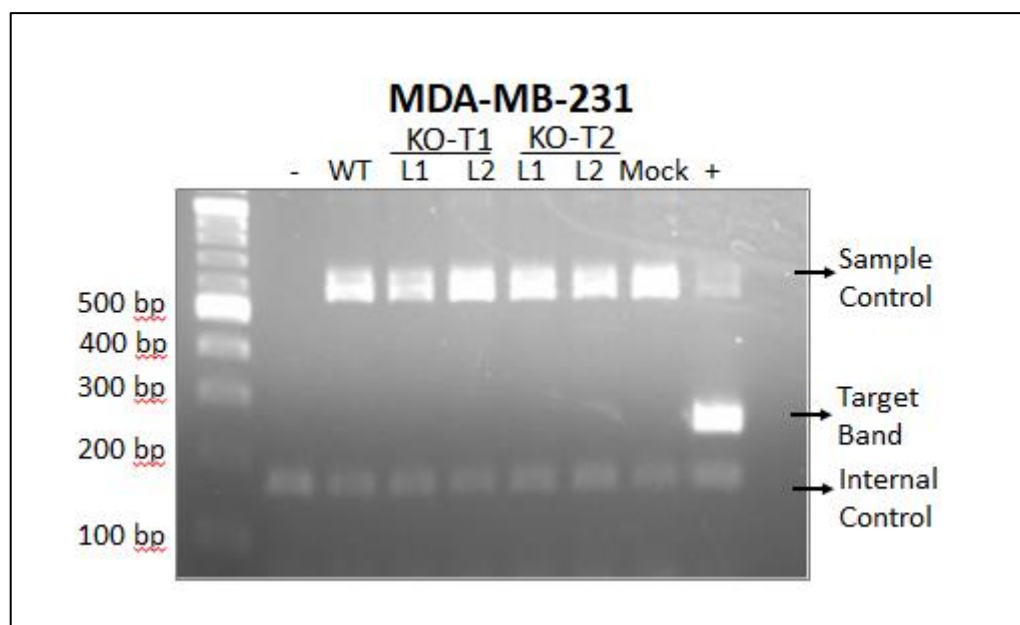


Figure 5.13. Analysis of mycoplasma status of MDA-MB-231 cell lines. No mycoplasma were contaminated in the cell lines used in this study. KO indicates *BMAL1* or *CRY* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in bp.

5.5. The Effect of *BMAL1* Knockout Mutation on Apoptosis

To understand the effect of the disruption of the *BMAL1* gene on DNA damage-induced apoptosis, the effect of cisplatin, which causes bulky DNA-adducts and the effect of doxorubicin, which causes double strand breaks, were analysed. Different doses of cisplatin and doxorubicin were used. Doses were decided based on the linear response in wild-type MCF10A after probing for apoptosis by measuring PARP cleavage [Ozturk et al., 2009] over a period of 16 hours from the start of drug treatment (Figures 5.14 and 5.15).

The literature was searched to find out suitable dose range of cisplatin and doxorubicin for the apoptotic responses of MCF10A cells to these genotoxic agents. Then the apoptotic response was analyzed by quantitating the c-PARP signal over actin signal and by plotting this ratio over time. 16 hour-treatment was selected for apoptosis assays on the fact that 16 hour-treatment showed a linear response. However, doses of drugs were further adjusted to get a linear response also with

highly sensitized *BMAL1* knockout cells while also seeing a response with parental cells.

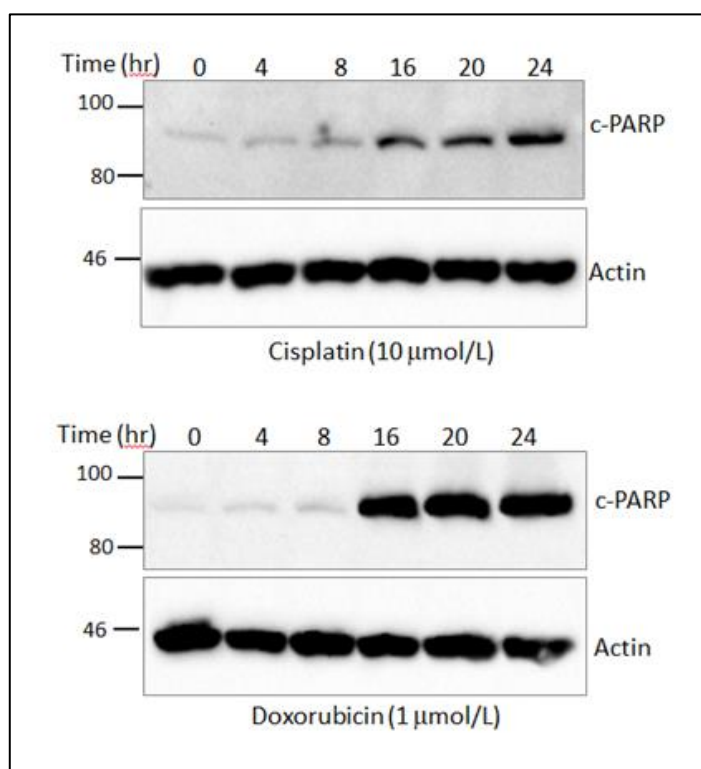


Figure 5.14. Determination of time kinetic of c-PARP in MCF10A. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa.

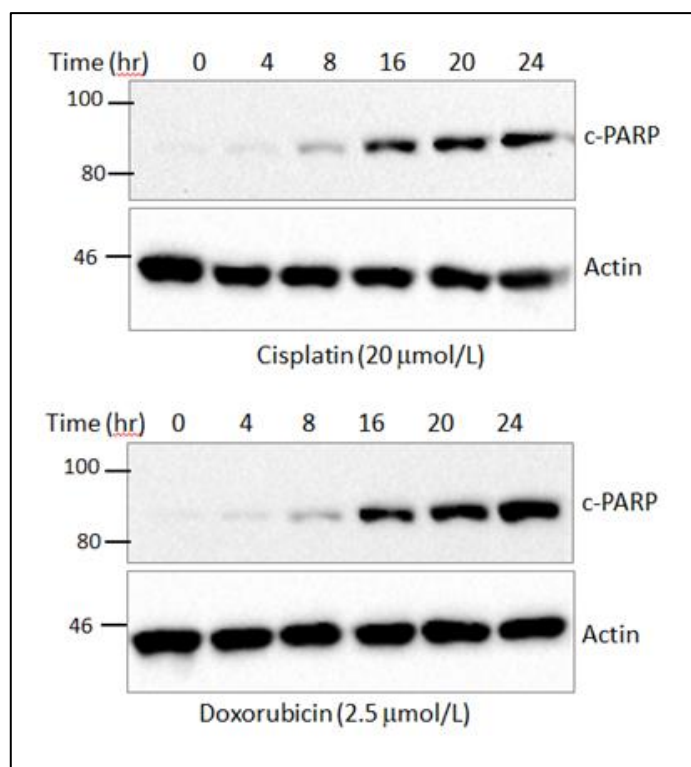


Figure 5.15. Determination of time kinetic of c-PARP in MDA-MB-231. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa.

5.5.1. Cisplatin Induced Apoptosis at MCF10A and MDA-MB-231

After selecting the appropriate doses and the time, the effect of cisplatin on apoptosis at *BMAL1* knockout MCF10A and MDA-MB-231 were analysed by measuring PARP cleavage over a period of 16 hours from the start of drug treatment. MCF10A cells were treated with 0, 10, and 20 μmol/L cisplatin and MDA MB 231 cells were treated with 0, 20, and 40 μmol/L cisplatin. Cell lysates were probed for cleaved PARP (c-PARP) by immunoblotting. Actin was used as a loading control. Quantifications of the c-PARP levels normalized to actin are plotted, which show that *BMAL1* mutations made these cells sensitive to cisplatin-induced apoptosis. The data are the mean of three independent experiments $\pm$ SD. P-values are given for the apoptosis induced by 20 μmol/L cisplatin in *BMAL1* knockouts relative to the parental cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting. Relative c-PARP was analyzed by two-way ANOVA followed

by Tukey's post hoc tests for pairwise comparisons. ****p <0.0001, ns; not significant.

The experiments were done for L1s and L2s for each sgRNA, respectively. For MCF10A cells, *CRY*-DKO cells were used as a control, as these cells do not have cisplatin-induced apoptosis on a *p53* wild-type background. Because MDA-MB-231 cells have *p53* knockout background, for these knockouts, MDA-MB-231 WT cell line was used as control.

It is found that *BMAL1* mutation sensitized both MCF10A (Figure 5.16 and Figure 5.17) and MDA-MB-231 (Figure 5.18 and Figure 5.19) cells to cisplatin induced apoptosis, and a very similar pattern was obtained with four different cell lines.

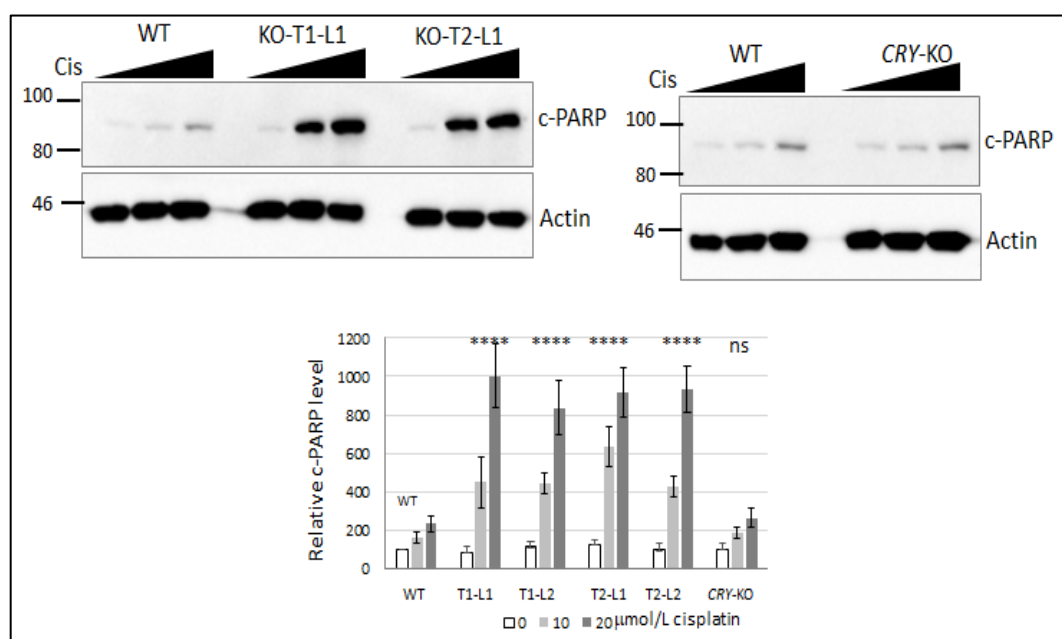


Figure 5.16. Effect of *BMAL1* mutations on apoptosis induced by cisplatin in MCF10A cells.

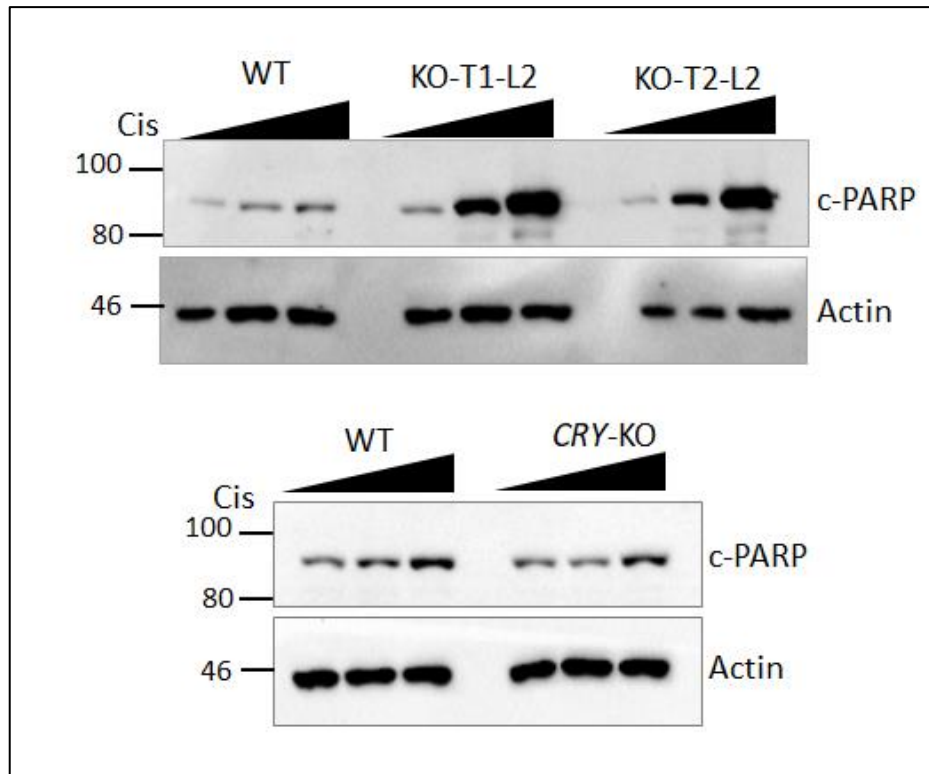


Figure 5.17. Effect of *BMAL1* mutations on apoptosis induced by cisplatin in MCF10A cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

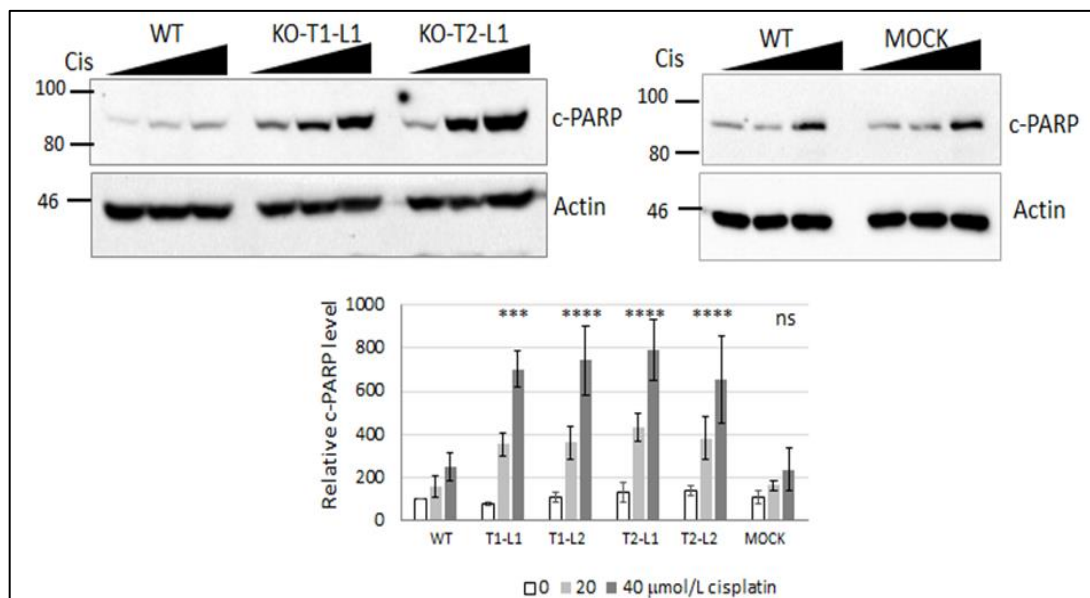


Figure 5.18. Effect of *BMAL1* mutations on apoptosis induced by cisplatin in *p53* mutant cell line MDA-MB-231. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa. Relative c-PARP was analysed by two-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons. *** $P < 0.001$, **** $p < 0.0001$, ns; not significant.

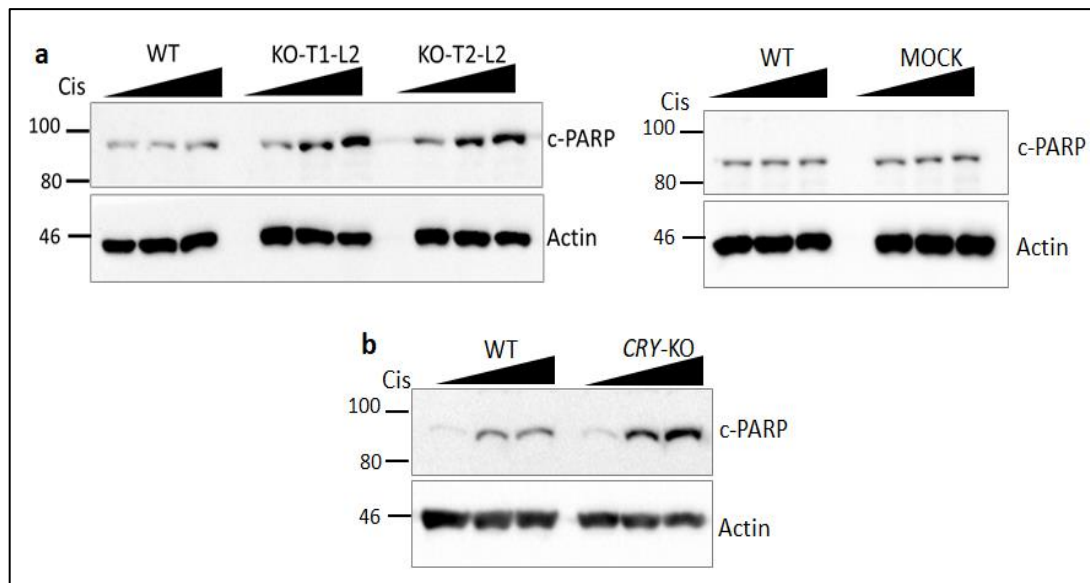


Figure 5.19. Effect of *BMAL1* mutations on apoptosis induced by cisplatin in MDA-MB-231 cells. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

5.5.2. Doxorubicin Induced Apoptosis at MCF10A and MDA-MB-231

After selecting the appropriate doses and the time, the effect of doxorubicin on apoptosis at *BMAL1* knockout MCF10A and MDA-MB-231 were analysed by measuring PARP cleavage over a period of 16 hours from the start of drug treatment. MCF10A cells were treated with 0, 0.5, and 1 $\mu\text{mol/L}$ doxorubicin. Cell lysates were probed for cleaved PARP (c-PARP) by immunoblotting. The experiments were done for L1s and L2s for each sgRNA, respectively. For MCF10A cells, *CRY*-DKO cells were used as a control, as these cells do not have doxorubicin-induced apoptosis on a *p53* wild-type background. Actin was used as a loading control. Quantifications of the c-PARP levels normalized to actin are plotted, which show that *BMAL1* mutations sensitized these cells to doxorubicin-induced apoptosis. The data are the mean of three independent experiments $\pm$ SD. P-values are given for the apoptosis induced by 1 $\mu\text{mol/L}$ doxorubicin in *BMAL1* knockouts relative to the parental cells.

MDA MB 231 cells were treated with 0, 2.5, and 5 $\mu\text{mol/L}$ doxorubicin. Cell lysates were probed for cleaved PARP (c-PARP) by immunoblotting. MDA-MB-231

WT cell line was used as control. Actin was used as a loading control. Quantifications of the c-PARP levels normalized to actin are plotted, which show that *BMAL1* mutations made these cells sensitive to doxorubicin-induced apoptosis. The data are the mean of three independent experiments $\pm$ SD. P-values are given for the apoptosis induced by 1 μ mol/L doxorubicin in *BMAL1* knockouts relative to the parental cells.

It is found that *BMAL1* mutation made both MCF10A (Figure 5.20 and 5.21) and MDA-MB-231 (Figure 5.22 and 5.23) cells sensitive to doxorubicin-induced apoptosis as much as cisplatin-induced apoptosis and a very similar pattern was obtained with four different cell lines.

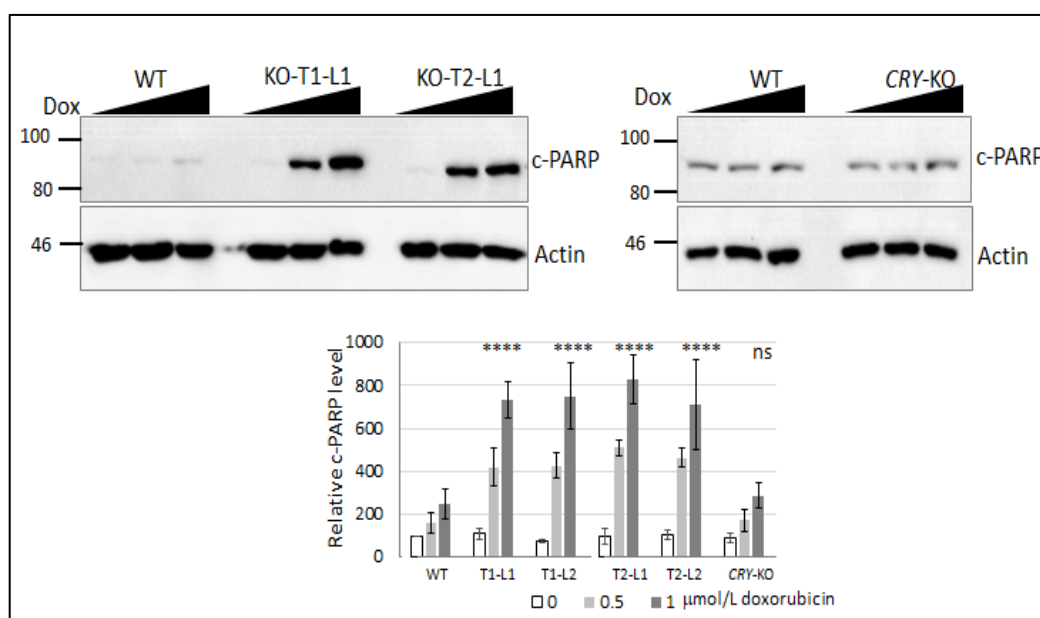


Figure 5.20. Effect of *BMAL1* mutations on apoptosis induced by doxorubicin in MCF10A cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting. Relative c-PARP was analyzed by two-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons. **** p <0.0001, ns; not significant.

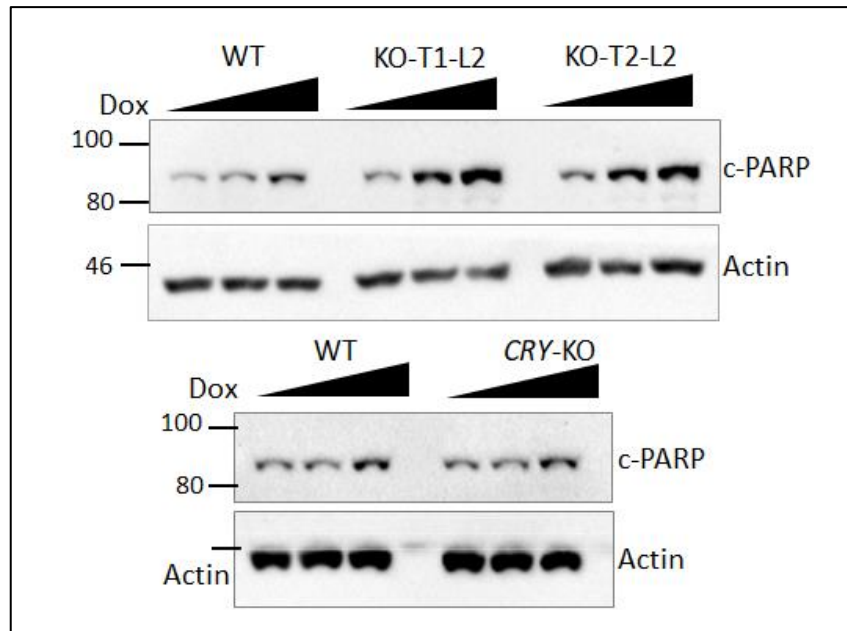


Figure 5.21. Effect of *BMAL1* mutations on apoptosis induced by doxorubicin in MCF10A cells. Cells were treated with 0, 0.5, and 1 $\mu\text{mol/L}$ doxorubicin. Cell lysates were probed for cleaved PARP (c-PARP) by immunoblotting. Actin was used as a loading control. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

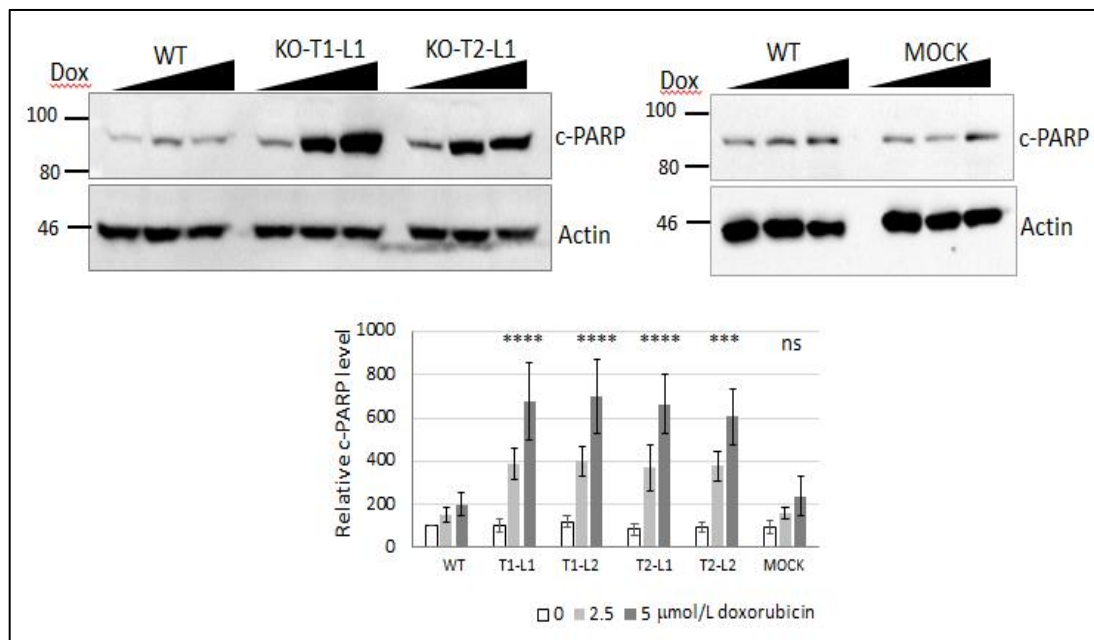


Figure 5.22. Effect of *BMAL1* mutations on apoptosis induced by doxorubicin in MDA-MB-231 cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. Relative c-PARP was analyzed by two-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons. **** $p < 0.0001$, ns; not significant.

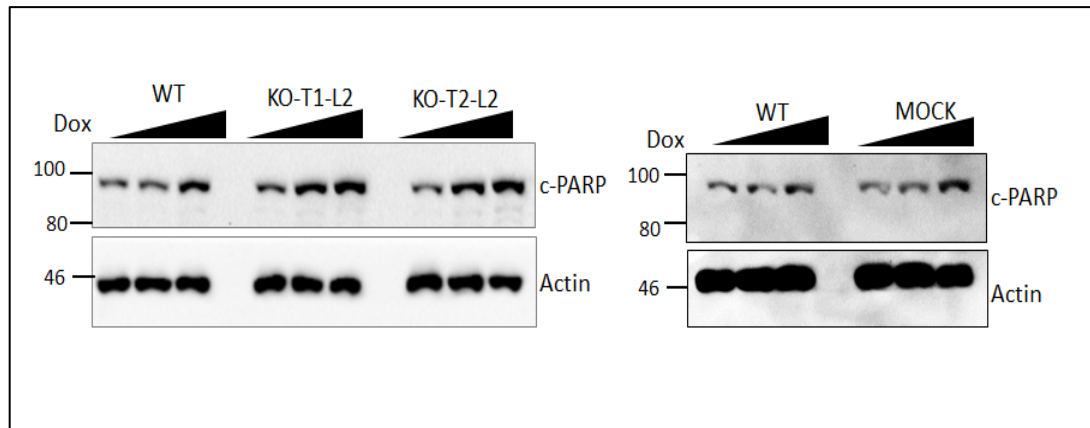


Figure 5.23. Effect of *BMAL1* mutations on apoptosis induced by doxorubicin in MDA-MB-231 cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

5.5.3. Enhancement of DNA Damage-Induced Apoptosis

BMAL1 deletion amplified the both cisplatin and doxorubicin induced apoptosis in both MCF10A and MDA-MB-231 cells. It is considered whether the increase in apoptosis in the presence of *BMAL1* knockout mutation may be caused by a different mechanism than that of *Cry* knockout mutation in *p53* null cells. A similar increase in apoptosis by *BMAL1* mutations in untransformed (MCF10A) and transformed (MDA-MB-231) suggests that this event is independent of the transformation status of the cells.

MCF10A and MDA-MB-231 cells express wildtype and mutant *p53* proteins, respectively. *BMAL1* mutation causes an amplification on apoptosis in both cell lines while wildtype *p53* accumulates at similar levels in wildtype and *BMAL1* (as well as *CRY*) knockouts in MCF10A clones, which suggest that *p53* does not explain the amplification of apoptosis in *BMAL1* knockouts (Figure 5.24 and Figure 5.25). Moreover, *p53* is accumulated in MDA-MB-231 cells and its clones because of mutant *p53* (Figure 5.26 and Figure 5.27). A mock (transduced with *BMAL1* targeting lentivirus but had a wildtype *BMAL1*) subclone of MDA-MB-231 was used as a control instead of *CRY*-DKO, because *CRY*-DKO in *p53* mutant background

caused an amplification of cisplatin induced apoptosis in MDA-MB-231 cells. This result is consistent with previous reports [Ozturk et al., 2009].

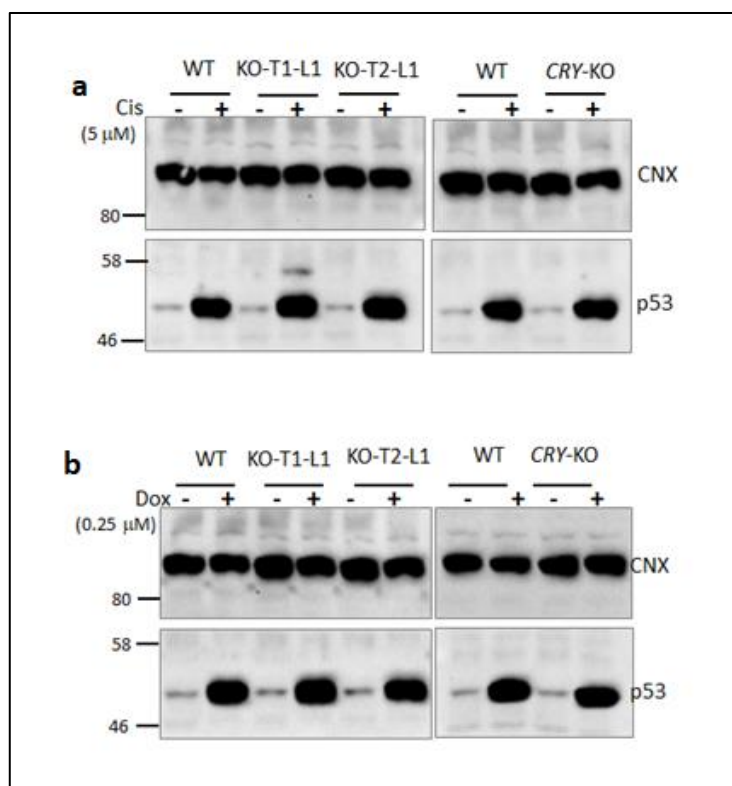


Figure 5.24. The effect of *BMAL1* knockout mutation on p53 protein accumulation following cisplatin a) or doxorubicin b) treatment at L1 subclones of MCF10A cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa.

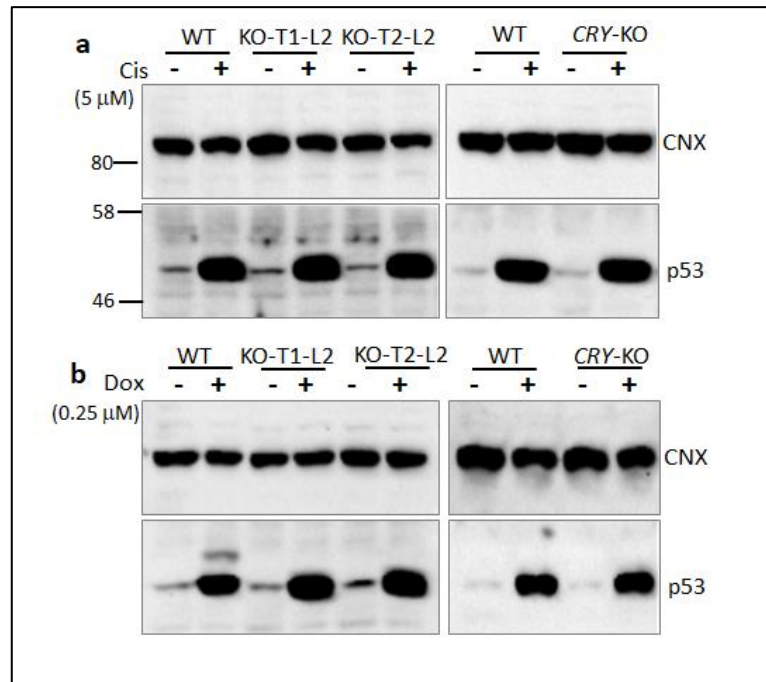


Figure 5.25. The effect of *BMAL1* knockout mutation on p53 protein accumulation following cisplatin a) or doxorubicin b) treatment at L2 subclones of MCF10A cells. Wild-type p53 accumulates at similar levels in wild-type and *BMAL1* (as well as *CRY*-DKO) knockouts in MCF10A clones. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa.

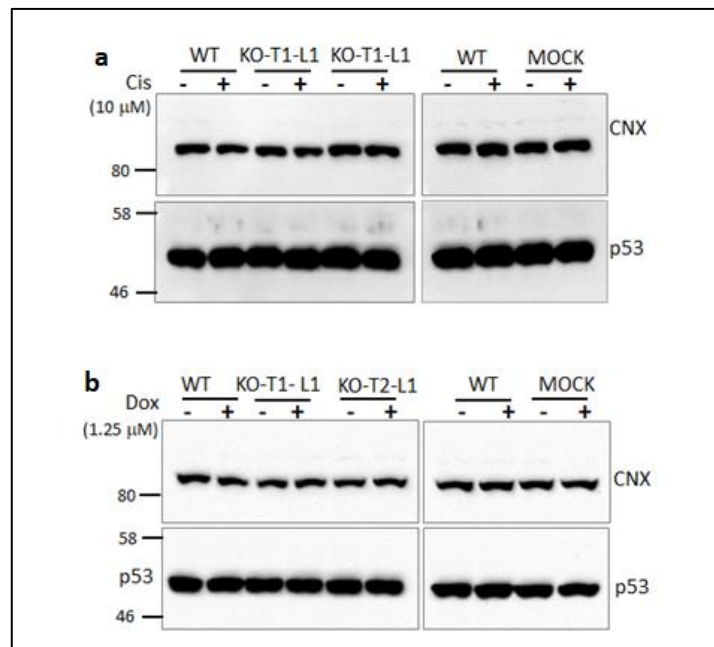


Figure 5.26: The effect of *BMAL1* knockout mutation on p53 protein accumulation following cisplatin a) or doxorubicin b) treatment at L1 subclones of MDA-MB-231 cells. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L1 indicates subclone for each sgRNA targeting.

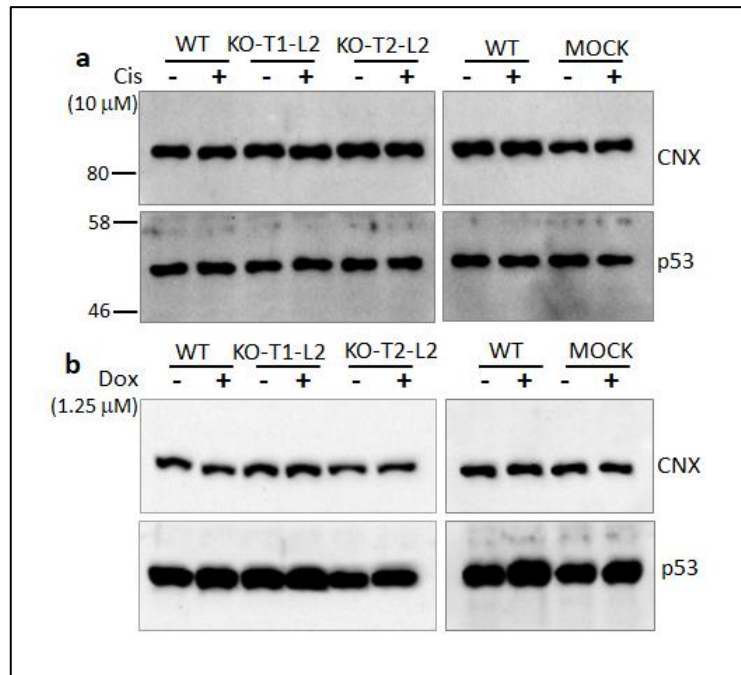


Figure 5.27: The effect of *BMAL1* knockout mutation on p53 protein accumulation following cisplatin a) or doxorubicin b) treatment at L2 subclones of MDA-MB-231 cells. Numbers and marks on the left of each figure indicate the positions of the corresponding molecular size markers in kDa. KO indicates *BMAL1* knockouts generated by T1 or T2 sgRNA targeting, L2 indicates subclone for each sgRNA targeting.

5.6. Invasive Capacity of *BMAL1* Knockout Cell Lines

After establishing the relation between *BMAL1* disruption and apoptosis, it was thought that deletion might have an anti-tumor effect in certain types of cells. However, induction of apoptosis before transformation or during eradication of cancer cells for therapeutic purposes should be considered in overall effect of *BMAL1* mutations. Another pathway which is important for therapeutic intervention of cancer spread is invasion potential of cancer cells. Therefore, the effect of *BMAL1* deletion on the invasion capacity of MDA-MB-231 cells was analysed. Tumor cells need to disrupt the basement membrane to form a malignant phenotype or (micro) metastases. During this process, cells obtain invasive properties which can be measured *in vitro*.

Matrigel invasion assay of MDA-MB-231 cells and its knockout subclones were performed using matrigel-coated invasion plates. Cells were seeded at 150,000 cells/well and allowed to invade toward 10% FBS for 36 hours. Invasive cells on the

bottom of the invasion membrane were stained and quantified at OD 560 nm after extraction.

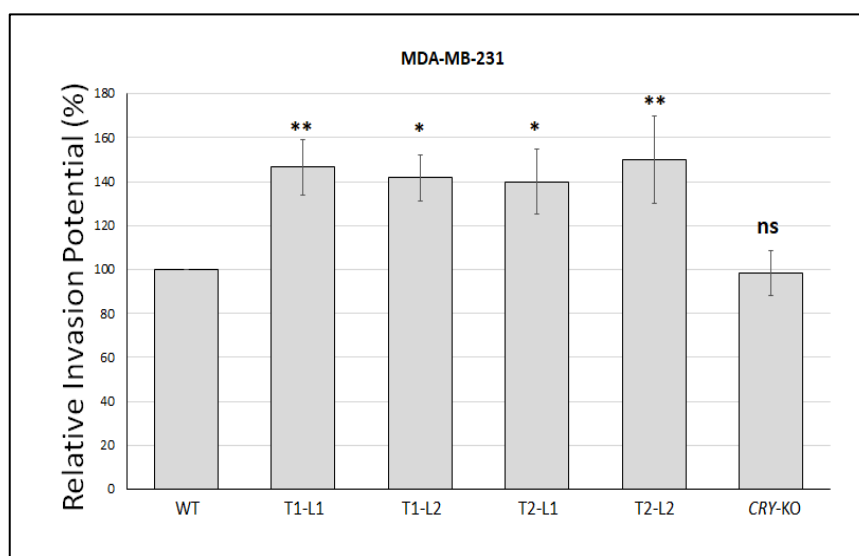


Figure 5.28: Effect of *BMAL1* mutations on MDA-MB-231 cell invasion. The results of invasion are presented as means $\pm$ SD of three independent experiments; SD is denoted by error bars. Invasion was analysed by one-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons. * $P < 0.05$, ** $P < 0.01$, ns; Not significant. KO indicates *BMAL1* knockout mutations generated by T1 or T2 sgRNA targeting, L indicates subclone for each sgRNA targeting.

BMAL1 knockout mutation increased the invasion into the basement membrane compared to parental MDA-MB-231 cells (Figure 5.28). Moreover, another control was used to eliminate any off-target effects induced by CRISPR by testing *CRY* mutant MDA-MB-231 cells in parallel.

5.7. Statistical Analysis

Statistical methods used for the analyses of apoptosis and invasion assays were detailed in Table 5.1 and Table 5.2.

5.7.1. Statistical Analyses for Apoptosis Assays

Statistical methods used for the analyses of apoptosis in Figure 5.16, Figure 5.18, Figure 5.20 and 5.22 were detailed in Table 5.1, Table 5.2 and Table 5.3.

Table 5.1. Statistical analysis for apoptosis assays. Relative c-PARP signal was analyzed by two-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons using GraphPad Prism 7.04 (GraphPad Software, Inc., San Diego, USA). The table shows results of comparisons between means of c-PARP level in wild-type and knockout cells exposed to different doses of cisplatin or doxorubicin.
*P<0.05, **P<0.01 ***P<0.001, ****P<0.001, Ns: Not significant.

Two-way ANOVA Table for Variance Analysis						
Source of Variation	% of Total Variation	SS	DF	MS	F value	P value
MCF10A Cells: 0μM-10μM-20μM Cisplatin						
Interaction	17.18	990565	10	99057	14.56	<0.0001****
Between Cells	55.09	3177281	2	1588641	233.6	<0.0001****
Doses of Drug	23.49	1354536	5	270907	39.83	<0.0001****
Residual		244866	36	6802		
MCF10A Cells: 0μM-0.5μM-1.0μM Doxorubicin						
Interaction	13.18	498907	10	49891	7.572	<0.0001****
Between Cells	58.45	2212493	2	1106246	167.9	<0.0001****
Doses of Drug	22.1	836654	5	167331	25.4	<0.0001****
Residual		237200	36	6589		
MDA-MB-231 Cells: 0μM-20μM-40μM Cisplatin						
Interaction	14.15	465530	10	46553	6.122	<0.0001****
Between Cells	56.09	1845848	2	922924	121.4	<0.0001****
Doses of Drug	21.45	705965	5	141193	18.57	<0.0001****
Residual		273733	36	7604		
MDA-MB-231 Cells: 0μM-2.5μM-5.0μM Doxorubicin						
Interaction	13.98	397807	10	39781	5.024	0.0001***
Between Cells	54.32	1545881	2	772941	97.61	<0.0001****
Doses of Drug	21.68	617009	5	123402	15.58	<0.0001****
Residual		285067	36	7919		

Table 5.2. Tukey's post hoc tests for pairwise comparisons of MCF10A.

P (Tukey's post hoc tests for pairwise comparisons) MCF10A					
Drugs	WT vs. T1-L1	WT vs. T1-L2	WT vs. T2-L1	WT vs. T2-L2	WT vs. CRY DKO
0μM-10μM-20μM Cisplatin	****	****	****	****	Ns
0μM-0.5μM-1.0μM Doxorubicin	****	****	****	****	Ns

Table 5.3. Tukey's post hoc tests for pairwise comparisons of MDA MB 231.

P (Tukey's post hoc tests for pairwise comparisons) MDA-MB-231					
Drugs	WT vs. T1-L1	WT vs. T1- L2	WT vs. T2-L1	WT vs. T2- L2	WT vs. Mock
0μM-20μM-40μM Cisplatin	***	****	****	****	Ns
0μM-2.5μM-5.0μM Doxorubicin	****	****	****	***	Ns

5.7.2. Statistical Analysis for Invasion Assay

Statistical methods used for the analyses of apoptosis in Figure 5.28 were detailed in Table 5.4 and 5.5.

Table 5.4. Statistical analysis for invasion assay. Invasion was analyzed by one-way ANOVA followed by Tukey's post hoc tests for pairwise comparisons using GraphPad Prism 7.04 (GraphPad Software, Inc., San Diego, USA). The table shows results of comparisons between means of invasion level in wild-type and knockout cells.

*P<0.05, **P<0.01 ***P<0.001, ****P<0.001, Ns: Not significant.

One-way ANOVA Table for Variance Analysis					
Source of Variation	SS	DF	MS	F value	P value
MDA-MB-231 Cells					
Between Cells	8444	5	1689	10.13	0.0006***
Residual	2000	12	166.7		
Total	10444	17			

Table 5.5. Tukey's post hoc tests for pairwise comparisons.

P (Tukey's post hoc tests for pairwise comparisons)					
	WT vs. T1-L1	WT vs. T1-L2	WT vs. T2-L1	WT vs. T2-L2	WT vs. CRY DKO
Invasion	**	*	*	**	Ns

6. CONCLUSION

It has been reported that *Cry* mutation renders the Ras-transformed *p53*-null cells, but not cells with wild-type *p53*, more susceptible to killing by agents that activate either the intrinsic or the extrinsic apoptosis pathways [Lee and Sancar, 2011], [Lee and Sancar, 2004]. It has also been shown that the deletion of other clock genes does not affect apoptosis following treatment with a variety of DNA damaging agents including UV-mimetics and double strand break-forming agents in fibroblasts isolated from different clock gene knockout mice [Gaddameedhi et al., 2012]. Moreover, spontaneous and/or ionizing radiation induced tumorigenesis has been performed using *Cry*, *Clock*, *Per* or *BMAL1* knockout mice in different laboratories. For *Per*, it has been reported that *Per2* deficiency increased irradiation induced tumor incidence [Fu et al., 2002], although another group did not obtain increased tumor incidence with *Per2* (or *Per1*) knockout [Antoch et al., 2013]. In another study, it was shown that loss of *Per2* function accelerated lung cancer formation in mutant mice [Papagiannakopoulos et al., 2016]. These findings with *Per2* and carcinogenesis suggest that the effect of *Per2* mutation in tumorigenesis should be further studied. Even though in early studies, *BMAL1* knockout mice were not a good model for studying cancer because these animals age early, following the accumulation of reactive oxygen species in tissues [Kondratov et al., 2006], recently an inducible *BMAL1* knockout mouse model, which expresses the gene during embryogenesis but not in after birth, was generated to eliminate early aging phenotype [Yang et al., 2016]. *Clock* knockout mice did not have an increased incidence of cancer [Antoch et al., 2008], [Kondratov et al., 2006] while whole-body knockout of *BMAL1* in mouse was recently associated with increased accelerated lung cancer [Papagiannakopoulos et al., 2016]. In a similar organ specific targeting approach, Kettner *et al.* reported that hepatocyte-specific loss of *BMAL1* enhances the formation of spontaneous hepatocellular carcinoma [Kettner et al., 2016]. On the other hand, a study by Puram *et al.* suggested that genetic deletion of *BMAL1* in established leukemias results in a competitive disadvantage and suppressed leukemogenesis [Puram et al., 2016]. In respect to the effect of *Cry* on

carcinogenesis, it was reported that *Cry* DKO mice were not distinguishable from wild-type ones [Gauger and Sancar, 2005], while *Cry2* deletion increased the cancer risk [Huber et al., 2016]. In opposite to *Cry* DKO, the deletion of *Cry* genes in *p53* null background had a protective effect against the spontaneous development of lymphomas [Ozturk et al., 2009]. Considering the findings with different outputs from animal models, in this study, it was aimed to test the effect of circadian clock genes in specific cancer-related events in order to form a platform where we can add more factors quickly and study molecular events which might difficult or time-consuming at whole-organism level. It was decided to knockout *BMAL1* gene in the cell lines chosen, because the deletion of *BMAL1* can completely disrupt the circadian clock while requirement of deletion of two genes for *Cry* or *Per* genes to disrupt the circadian clock completely is needed. Carcinogenic events (such as apoptosis and invasion) using cell lines as models following knockout of *BMAL1* with CRISPR was tested. Epithelial cells were chosen by considering that most tumors originate from these cells. As a result, by the use of appropriate cell lines, it could be possible to test events such as apoptosis, which has a protective effect against cancer formation by eliminating abnormally behaving but still non-transformed cells, and invasion capacity in transformed cells, which has a cancer promoting effect by increasing metastasis or micro-metastasis. Epidemiological data suggest that disruption of the circadian clock in night-shift nurses or flight attendants increases breast cancer risk two fold [Truong et al. 2014], suggesting that breast epithelial cell lines are an appropriate cell model. MCF10A is a nearly normal (nontumorigenic) cell line with wild-type *p53* and a resettable circadian clock in culture. Therefore, its apoptotic response after treating parental cells and *BMAL1* knockout clones with bulky DNA adduct forming or double strand break forming agents, cisplatin and doxorubicin, respectively were studied. First, *BMAL1* knockout sensitized the cells to cisplatin-induced apoptosis. Similar results were obtained by targeting the *BMAL1* gene with two different sgRNAs. A *CRY*-null knockout (*CRY*-DKO) cell line was also used as a control to eliminate the possibility that lentiviral infection might sensitize cells to apoptosis. It was found that *CRY*-DKO did not have an altered apoptotic response to cisplatin or doxorubicin in MCF10A cell line which expresses wildtype *p53*. It was reported that *CRY1* and *CRY2* play different roles in

tumorigenesis [Huber et al. 2016], therefore double knockout (DKO) of *CRY1* and *CRY2* were preferred to be used as the control because experiences of us with *CRY*-DKO cell lines showed that DNA-damage induced apoptosis is not altered in *p53* wildtype background. The increase in apoptosis by *BMAL1* knockout in MCF10A cells did not require a *p53* null background. This was different from the amplification of apoptosis by *Cry* mutation which can be observed only in *p53* mutant background [Ozturk et al. 2009]. More strikingly, it was also observed strong amplification of the apoptotic response by *BMAL1* knockout following double strand break induction with doxorubicin. This increase in both UV-mimetic and double strand break damage induced apoptosis in the same cell line was not observed with any other circadian clock protein mutations. Our finding that *BMAL1* deletion increases sensitivity to chemotherapeutic agents suggests that interfering with *BMAL1* expression might have a protective or preventive function against tumor initiation following repeated DNA damage insults. It has been reported that *BMAL1* alters cell growth and can control *p53* activation [Jiang et al., 2016]. A significant change in population doubling times in knockouts compared to those of parental or control clones was not observed (Figure 5.10 and Figure 5.11). More importantly, on DNA damage-induced apoptosis, a significant sensitization in breast epithelial cells was detected. It has also been reported that *BMAL1* overexpression sensitized tongue squamous cell carcinoma cells to apoptosis induced by an antineoplastic agent, paclitaxel [Tang et al., 2017]. However, DNA damage-induced and paclitaxel-induced apoptosis are different events, and this data does not conflict with the results obtained from the study. It has also been reported that *BMAL1* overexpression in colon cancer cell lines sensitized these cells to oxaliplatin [Zeng et al. 2014]. Alternatively, *BMAL1* knockdown was found to suppress proliferation, and anchorage-dependent and independent clonal growth of malignant pleural mesothelioma cells [Elshazley et al. 2012]. Differentially from this work, it was shown that knockdown of *BMAL1* in murine colon cancer cells (C26) and fibroblast cells (L929) decreased apoptosis induced by etoposid (VP16) and decreased DNA damage induced by cisplatin [Zeng et al., 2010]. One possibility is that low level of *BMAL1* protein in shRNA treated cells might be enough to sustain a functional circadian clock while complete knockout of *BMAL1* gene disrupt the circadian clock.

This difference between the full blown disruption or attenuation the circadian clock may change the effect of *BMAL1* on the sensitivity to double-strand break or bulky-adduct forming drugs. It is also possible that the modulation of cells to DNA damages by *BMAL1* gene can be cell or tissue specific. These reports with different findings suggest that overexpression in the presence of endogenous *BMAL1* and depletion of *BMAL1* through small interfering RNA or genome editing may result in different outputs depending on the origin of the cells and type of chemotherapeutic drugs. Then, it was checked if *BMAL1* mutation had a protective effect by decreasing the invasion of transformed cells. In fact, it has been reported that the knockdown of *BMAL1* by RNA interference promoted cancer cell invasion independent of *p53* status in lung cancer and glioma cells [Jung et al. 2013]. On the other hand, it has been reported that *BMAL1* inhibits invasion in tongue squamous cell carcinoma cells and telomerase reverse transcriptase gene (*hTERT*) mediates *BMAL1*-driven sensitivity to an antineoplastic agent paclitaxel [Tang et al., 2017]. *BMAL1* also promoted migration and invasion in an immortalized human extravillous trophoblast cell line HTR-8/SVneo [Li et al., 2017]. In the present study, an invasive breast cancer cell line, MDA-MB-231 was used, to analyse the effect of *BMAL1* mutation on invasion. *BMAL1* knockout mutation increased invasive capacity more than ~40%, which was statistically significant. Additionally, for the invasion assay, similar results were obtained from *BMAL1* knockout cells which were generated by targeting the gene with different sgRNAs. The parental cell line and a *CRY*-DKO of the MDA-MB-231 cell line were used as a reference. *CRY* knockout mutation did not change invasive capacity significantly under the same conditions. This might suggest the effect of *BMAL1* on the invasion might be circadian clock-independent because deletion of *CRY* genes did not have the same phenotype. On the other hand, these effects may be cell type specific even though one transformed and one untransformed cell line were used to reduce this risk. This cell type specific phenotype might be the result of a combination of circadian-dependent and -independent functions with the genetic make-up of the cell lines. Actually, this is also true for the tissue specific effects in whole-body knockouts depending on the circadian-dependent and independent functions of *BMAL1* genes in different organs. For example, in *BMAL1* knockouts, accumulations of reactive

oxygen species are not the same in all cell types [Kondratov et al., 2006]. Keeping in mind that platforms or models with cell lines have technical difficulties to reflect *in vivo* events, it may offer a very easy modifiable, cheaper, and much quicker working material to study molecular events. For example, testing the relationship between *BMAL1* and another gene for example with *p53* would be much easier just by knocking out *p53* in *BMAL1* null cells.

Altogether, there are different findings from different laboratories in respect to the relationship between cancer and circadian clock as discussed above. Based on the finding obtained from this study, it was observed that the same knockout mutation had both protective and promoting functions in two breast epithelial cell lines. *BMAL1* gene was chosen for knocking out for the following reason: *BMAL1* is the only gene whose deletion alone leads to complete loss of rhythmicity. Other than *BMAL1* gene, the disruption of the circadian rhythm requires knockouts of at least two genes such as *CRY1* and *CRY2* or *PER1* and *PER2*, which would increase off-targets by two different sgRNAs. However, it is also known that *BMAL1* has also non-circadian functions. For example, germline *BMAL1* knockout is associated with early aging while inducible knockout mouse does not have such gross effects while both are deficient in the circadian clock [Yang et al., 2016]. Moreover, *Cry1* and *Cry2* or *Per1* and *Per2* double knockouts do not have aging properties or some other metabolic abnormalities observed in *BMAL1* knockout mice [Dierickx et al., 2018]. In fact, the increase in the invasion properties in *BMAL1* knockout MDA-MB-231 cells was not observable in *CRY* DKO MDA-MB231 cells (Figure 5.28), this suggests that this might operate through non-circadian functions of *BMAL1* gene. In opposite view, using two sgRNAs to knockout *CRY1* and *CRY2* might have induced an unforeseen off target effect which might have cancelled the increased invasions properties. In any case, the deletion of *BMAL1* gene sensitized an untransformed and a transformed breast epithelial cell line to cisplatin or doxorubicin-induced apoptosis while it enhanced invasion capacity of transformed MDA-MB-231 cell line either a circadian-dependent or -independent way.

In summary, in this study it was found that knockout of *BMAL1* gene in two mammalian cells affects the apoptotic response and invasion properties differently in respect to the contribution to carcinogenesis. Although *in vitro* circadian systems

based on cell lines have been thought as poor models to study the circadian clock's effect on carcinogenesis, this study suggests that appropriate modelling of events *in vitro* might reveal new findings which were not detectable in animal models. On the other hand, it is wished to emphasize that even though cell-based systems such as in this work are useful for studying molecular events of basic circadian clock, they have limitations to reflect circadian clock-associated phenotypes observed *in vivo*. Therefore, findings from the study should be interpreted from the view that circadian clock gene *BMAL1* affects the molecular events associated with carcinogenesis differentially but these findings do not validate or invalidate the findings from *in vivo* animal models because there are more factors than what investigated in this study.

REFERENCES

- [1] Andreani T. S., Itoh T. Q., Yildirim E., Hwangbo D., Allada R., (2015), "Genetics of Circadian Rhythms", *Sleep Medicine Clinics*, 10(4); 413–421.
- [2] Antoch M. P., Gorbacheva V. Y., Vykhovanets O., Toshkov I. A., Kondratov R.V., Kondratova A. A., Lee C., Nikitin A. Y., (2007), "Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis", *Cell Cycle*, 7, 1197-1204.
- [3] Antoch M. P., Toshkov I., Kuropatwinski K. K., Jackson M., (2013), "Deficiency in PER proteins has no effect on the rate of spontaneous and radiation-induced carcinogenesis", *Cell Cycle*, 12, 3673-3680.
- [4] Barnes J. W., Tischkau S. A., Barnes J. A., Mitchell J. W., Burgoon P. W., Hickok J. R., Gillette M. U., (2003), "Requirement of mammalian Timeless for circadian rhythmicity", *Science*, 302, 439–442.
- [5] Bunger M. K., Wilsbacher L. D., Moran S. M., Clendenin C., Radcliffe L. A., Hogenesch J. B., Simon M. C., Takahashi J. S., Bradfield C. A., (2000), "Mop3 is an essential component of the master circadian pacemaker in mammals", *Cell* 103, 1009-1017.
- [6] Chun S. K., Jang J., Chung S., Yun H., Kim N. J., Jung J. W., Son G. H., Suh Y. G., Kim K., (2014), "Identification and validation of cryptochrome inhibitors that modulate the molecular circadian clock", *ACS Chemical Biology*, 9, 703–710.
- [7] Cui Y., Xu J., Cheng M., Liao X., Peng S., (2018), "Review of CRISPR/Cas9 sgRNA Design Tools", *Interdisciplinary Sciences: Computational Life Sciences*, 10 (2), 455-465.
- [8] Dehairs J., Talebi A., Cherifi Y., Swinnen J. V., (2016), "CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing", *Scientific Reports*, 6, 28973.
- [9] Dierickx P., Van Laake L. W., Geijsen N., (2018), "Circadian clocks: from stem cells to tissue homeostasis and regeneration", *EMBO Reports*, 19, 18-28.
- [10] Elshazley M., Sato M., Yamashita R., Yoshida K., Toyokuni S., Ishiquro F., Osada H., Sekido Y., Yokoi K., Usami N., Shames D. S., Kondo M., Gazdar A. F., Minna J. D., hasegawa Y., (2012), "The circadian clock gene BMAL1 is a novel therapeutic target for malignant pleural mesothelioma", *International Journal of Cancer*, 131, 2820-2831.

- [11] Filipski E., King V. M., Li X., Granda T. G., Mormont M. C., Liu X., Claustrat B., Hastings M. H., Levi F., (2002), "Host circadian clock as a control point in tumor progression", *Journal of the National Cancer Institute*, 94, 690–697.
- [12] Fu L., Pelicano H., Liu J., Huang P., Lee C., (2002), "The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo", *Cell*, 111, 41-50.
- [13] Gaddameedhi S., Reardon J. T., Ye R., Ozturk N., Sancar A., (2012), "Effect of circadian clock mutations on DNA damage response in mammalian cells", *Cell Cycle*, 11, 3481-3491.
- [14] Gaddameedhi S., Selby C. P., Kaufmann W. K., Smart R. C., Sancar A., (2011), "Control of skin cancer by the circadian rhythm", *Proceedings of the National Academy of Science*, 108, 18790–18795.
- [15] Gauger M. A., Sancar A., (2005), "Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer", *Cancer Research*, 65, 6828–6834.
- [16] Gery S., Komatsu N., Baldjyan L., Yu A., Koo D., Koeffler H. P., (2006), "The circadian gene *Per1* plays an important role in cell growth and DNA damage control in human cancer cells", *Molecular Cell*, 22, 375–382.
- [17] Geyfman M., Kumar V. Liu, Q. Ruiz R., Gordon W., Espitia F., Cam E., Millar S. E., Smyth P., Ihler A., Takahashi J. S., Andersen B., (2012), "Brain and muscle Arnt-like protein-1 (BMAL1) controls circadian cell proliferation and susceptibility to UVB-induced DNA damage in the epidermis", *Proceedings of the National Academy of Science*, 109, 11758–11763.
- [18] Grechez-Cassiau A., Rayet B., Guillaumond F., Teboul M., Delaunay F., (2008), "The Circadian Clock Component BMAL1 is a Critical Regulator of p21WAF1/CIP1 Expression Hepatocyte Proliferation", *Journal of Biological Chemistry*, 283(8), 4535–4542.
- [19] Grunert S., Jechlinger M., Beug H. (2003), "Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis", *Natural Reviews Molecular Cell Biology*, 4, 657-665.
- [20] Guann X., (2015), "Cancer metastases: challenges and opportunities", *Acta Pharmaceutica Sinica*, 5(5), 402-418.
- [21] Hashiramoto A., Yamane T., Tsumiyama K., Yoshida K., Komai K., Yamada H., Yamazaki F., Doi M., Okamura H., Shiozawa S., (2010), "Mammalian Clock Gene Cryptochrome Regulates Arthritis via Proinflammatory Cytokine TNF- α .", *The Journal of Immunology*, 184, 1560–1565.

- [22] Hotchkiss R. S., Strasser A., McDunn J. E., Swanson P. E., (2009), "Cell death", *New England Journal of Medicine*, 361, 1570–1583.
- [23] Hu J., Choi J. H., Gaddameedhi S., Kemp M. G., Reardon J. T., Sancar A. (2013), "Nucleotide excision repair in human cells: Fate of the excised oligonucleotide carrying DNA damage in vivo, *Journal of Biological Chemistry*, 288, 20918–20926.
- [24] Huang N., Chelliah Y., Taylor C. A., Yoo S. H., Partch C., Green C. B., Zhang H., Takahashi J. S., (2012), "Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex", *Science* 337, 189-194.
- [25] Huber A. L., Papp S. J., Chan A. B., Henriksson E., Jordan S. D., Kriebs A., Nguyen M., Wallace M., Li Z., Metallo C. M., Lamia K. A., (2016), "CRY2 and FBXL3 Cooperatively Degrade c-MYC", *Mol Cell*, 64, 774-789.
- [26] Jiang W., Zhao S., Jiang X., Zhang E., Hu G., Hu B., Zheng P., Xiao J., Lu z., Lu Y., Ni J., Chen C., Wang X., Yang L., Wan R, (2016), "The circadian clock gene Bmal1 acts as a potential anti-oncogene in pancreatic cancer by activating the p53 tumor suppressor pathway", *Cancer Letters*, 371, 314-325.
- [27] Jung C. H., Kim E. M., Park J. K., Hwang S. G., Moon S. K., Kim W. J., Um H. D., (2013), "Bmal1 suppresses cancer cell invasion by blocking the phosphoinositide 3-kinase-Akt-MMP-2 signaling pathway", *Oncology Reports*, 29, 2109-2113.
- [28] Kang T. H., Reardon J. T., Sancar A., (2011), "Regulation of nucleotide excision repair activity by transcriptional and posttranscriptional control of the XPA protein", *Nucleic Acids Research*, 39, 3176–3187.
- [29] Kang Y., Massague J., (2004), "Epithelial-mesenchymal transitions: twist in development and metastasis", *Cell*, 118, 277-279.
- [30] Keller M., Mazuch J., Abraham U., Eom G. D., Herzog E. D., Volk H.-D., Kramer A., Maier, B., (2009), "A circadian clock in macrophages controls inflammatory immune responses", *Proceedings of the National Academy of Sciences of the United States of America*, 106(50), 21407–21412.
- [31] Kettner N. M., Voicu H., Finegold M. J., Coarfa C., Sreekumar A., Putluri N., Katchy C. A., Lee C., Fu L., (2016), "Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis", *Cancer Cell*, 30, 909-924.
- [32] Khapre R. V., Kondratova A. A., Patel S., Dubrovsky Y., Wrobel M., Antoch M. P., Kondratov R. V., (2014), "BMAL1- dependent regulation of the mTOR signaling pathway delays aging", *Aging*, 6, 48–57.

- [33] Ko C. H., Takahashi J. S., (2006), "Molecular components of the mammalian circadian clock", *Human Molecular Genetics*, 15(2), 271-277.
- [34] Kondratov R.V., Kondratova A. A., Gorbacheva V. Y., Vykhovanets O. V., Antoch M. P., (2006), "Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock", *Genes Development*, 20, 1868-1873.
- [35] Krakhmal M. V., Zavyalova E. V., Denisov E. V., Vtorushin S. V., Perelmuter V. M., (2015), "Cancer Invasion: Patterns and Mechanisms", *Acta Naturae*, 7 (25).
- [36] Lage M. M., Serra P. P., Menendez P., Ruiz R. T., Peralez S. R., (2018), "CRISPR/Cas9 for Cancer Therapy: Hopes and Challenges", *Biomedicines*, 6, 105.
- [37] Lee J. H., Sancar A., (2011a), "Circadian clock disruption improves the efficacy of chemotherapy through p73-mediated apoptosis", *Proceedings of the National Academy of Science*, 108, 10668–10672.
- [38] Lee J. H., Sancar A., (2011b), "Regulation of apoptosis by the circadian clock through NF- κ B signaling", *Proceedings of the National Academy of Science* 108, 12036–12041.
- [39] Leonardi G. C., Rapisarda V., Marconi A., Scalisi A., Catalano F., Proietti L., Travali S., Libra M., Fenga C., (2012), "Correlation of the risk of breast cancer and disruption of the circadian rhythm", *Oncology Reports*, 28, 418-428.
- [40] Levi F., (2006), "The circadian timing system, a coordinator of life processes. Implications for the rhythmic delivery of cancer therapeutics", 28th IEEE EMBS Annual International Conference, New York City, USA, August 30-September 3.
- [41] Li S., Zhai J., Liu J., Hong Y., Zhao W., Zhao A., Sun K., Du Y., Chen Z., (2017), "BMAL1 facilitates trophoblast migration and invasion via SP1-DNMT1/DAB2IP pathway in recurrent spontaneous abortion", *Oncotarget*, 8, 89451-89464.
- [42] Lincoln D. W., Hrushesky W. J., Wood P. A., (2000), "Circadian organization of thymidylate synthase activity in normal tissues: a possible basis for 5-fluorouracil chronotherapeutic advantage", *International Journal of Cancer*, 88(3), 479–85.
- [43] Matsuo T., (2003), "Control Mechanism of the Circadian Clock for Timing of Cell Division in Vivo", *Science*, 302(5643), 255–259.
- [44] Ozturk, N., Lee J. H., Gaddameedhi S., Sancar A., (2009), "Loss of cryptochrome reduces cancer risk in p53 mutant mice", *Proceedings of the National Academy of Science*, 106, 2841-2846.

- [45] Papagiannakopoulos T., Bauer M. R., Davidson M., Heimann M. K., Subbarai L., Bhutkar A. V., Bartlebaugh J. M. E., Heiden M. G. V., Jacks T. E., (2016), "Circadian Rhythm Disruption Promotes Lung Tumorigenesis", *Cell Metabolism*, 24, 324-331.
- [46] Partch C., Green C., Takahashi J., (2014), "Molecular architecture of the mammalian circadian clock", *Trends in Cell biology*, 24(2), 90–99.
- [47] Paschos G. K., Baggs J. E., Hogenesch J. B., FitzGerald G. A., (2010), "The role of clock genes in pharmacology", *Annual Review of Pharmacology and Toxicology*, 50, 187–214.
- [48] Prieto-García E., Díaz-García C. V., García-Ruiz I., Agulló-Ortuño M. T., (2017), "Epithelial-to-mesenchymal transition in tumor progression", *Medical Oncology*, 34 (7), 122.
- [49] Pronk A., Ji B. T., Shu X. O., Xue S., Yang G., Li H. L., Rothman N., Gao Y. T., Zheng W., Chow W. H., (2010), "Nightshift work and breast cancer risk in a cohort of Chinese women", *American Journal of Epidemiology*, 171, 953–959.
- [50] Puram R. V., Kowalczyk M. S., Boer C. G., Schneider R. K., Miller P. G., McConkey M., Tothova Z., Teiero H., Heckl D., Jaras M., Chen M. C., Li H., Tamavo A., Cowley G., S., Rosenblatt-Rosen O., Al-Shahrour F., Regey A., Ebert B. L., (2016), "Core Circadian Clock Genes Regulate Leukemia Stem Cells in AML", *Cell*, 165, 303-316.
- [51] Ran F. A., Hsu P. D., Wright J., Agarwala V., Scott D. A., Zhang F., (2013), "Genome engineering using the CRISPR-Cas9 system", *Nature Protocols*, 8 (11).
- [52] Reppert S. M., Weaver D. R., "Coordination of circadian timing in mammals", *Nature*, 418, 935-941.
- [53] Sahar S., Sassone Corsi P., (2009), "Metabolism and cancer: the circadian clock connection", *Nature Reviews Cancer*, 9(12), 886-96.
- [54] Sancar A., Lindsey-Boltz L. A., Unsal-Kacmaz K., Linn S., (2004), "Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints", *Annual Review of Biochemistry*, 73, 39–85.
- [55] Schernhammer E. S., Kroenke C. H., Laden F., Hankinson S. E., (2006), "Night work and risk of breast cancer", *Epidemiology*, 17, 108–111.
- [56] Son H., Moon A., (2010), Epithelial-mesenchymal Transition and Cell Invasion, *Toxicological Research*, 26(4), 245-252.

- [57] Stevens R. G., (2009), "Light-at-night, circadian disruption and breast cancer: Assessment of existing evidence", *International Journal of Epidemiology*, 38, 963–970.
- [58] Tang Q., Cheng B., Xie M., Chen Y., Zhao J., Zhou X., Chen L., (2017), "Circadian Clock Gene Bmal1 Inhibits Tumorigenesis and Increases Paclitaxel Sensitivity in Tongue Squamous Cell Carcinoma, *Cancer Research*, 77, 532-544.
- [59] Thurtle D. M., Lo S. T., (2018), "Molecular Biology at the Cutting Edge: A Review on CRISPR/CAS9 Gene Editing for Undergraduates", *Biochemistry and Molecular Biology Education*, 46 (2), 195-205.
- [60] Truong T., Liguët B., Menegaux F., Plancoulaine S., Laurent-Puig P., Mulot C., Cordina-Duverger E., Sanchez M., Arveux P., Kerbrat P., Richardson S., Guenel P., (2014), "Breast cancer risk, nightwork, and circadian clock gene polymorphisms", *Endocr Relat Cancer*, 21, 629-638.
- [61] Wulund L., Reddy A. B., (2015), "A brief history of circadian time: The emergence of redox oscillations as a novel component of biological rhythms", *Perspective in Science*, 6, 442-454.
- [62] Yang G., Chen L., Grant G. R., Paschos G., Song W. L., Musiek E. S., Lee V., McLoughlin S. C., Grosser T., Cotsarelis G., FitzGerald G. A., (2016), "Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival", *Science Translational Medicine*, 8 (324), 324ra316.
- [63] Yu E. A., Weaver D. R., (2011), "Disrupting the circadian clock: Gene-specific effects on aging, cancer, and other phenotypes", *Aging*, 3, 479–493.
- [64] Zeng Z. L., Luo H. Y., Yang J., Wu W. J., Chen D. L., Huang P., Xu R. H., (2014), "Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer", *Clinical Cancer Research*, 20, 1042-1052.
- [65] Zeng Z. L., Wu M. W., Sun Y. L., Cai Y., C., Huang Y. J., Xian L., J., (2010), "Effects of the biological clock gene Bmal1 on tumour growth and anti-cancer drug activity", *Journal of Biochemistry*, 148, 319-326.

BIOGRAPHY

Tuba Korkmaz was born in İzmit, Turkey, in August 13, 1987. She received her B. Sc. Degree in Fundamental and Industrial Microbiology from Ege University in 2009. Later, she joined the MSc. program in Molecular Biology and Genetics at Gebze Institute of Technology under the supervision of Assistant Prof. F. İnci ÖZDEMİR and was graduated from this programme in 2012. She has been a part of PhD programme of Molecular Biology and Genetics at Gebze Technical University since 2012 under the supervision of Associated Prof. Nuri ÖZTÜRK. She also worked as a research assistant between 2015-2018.

APPENDICES

APPENDIX A: Protein and DNA Markers

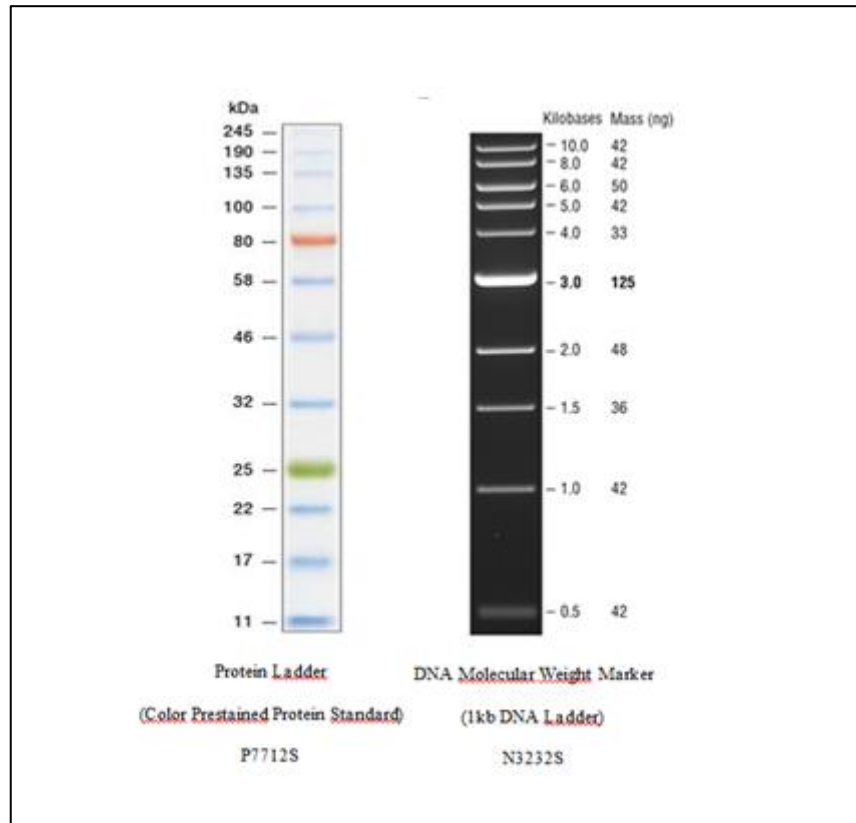


Figure A.1: Protein and DNA markers.

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

Korkmaz T., Aygenli F., Emisoglu H., Ozcelik G., Canturk A., Yilmaz S., and Ozturk N., (2018), Opposite carcinogenic effects of circadian clock gene Bmal1, Scientific Reports, 8, 16023.

APPENDIX C: Permissions for Copyrighted Materials

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Table C.1.1: Permissions for copyrighted materials.