

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

**CONNECTIONS BETWEEN THE CIRCADIAN CLOCK AND
CELLULAR STRESS IN CANCER CELLS**

HANDAN KÜLAHLI
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
PROF. DR. NURİ ÖZTÜRK

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HANDAN KÜLAHLI
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

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2021



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SUMMARY

The circadian clock is a mechanism that provides regulation at many levels, from gene expression to organism's behavior. Disruption of circadian rhythm affects many diseases including cancer and biological processes. It has been reported that disruption of *BMAL1* gene increases the sensitivity of breast cancer cells to apoptosis caused by DNA damaging agents and invasion capacity. In this study, it is aimed to reveal the mechanism of the phenotype previously observed in *BMAL1* gene deleted breast cancer cells with genome-level approach. For this purpose, *BMAL1* knockout (KO) and wild-type (WT) MDA-MB-231 cells were treated with cisplatin and doxorubicin. We analysed differentially expressed genes (DEGs) in drug-treated cells by using RNA sequencing technique. Gene set enrichment analyzes of transcripts showed that enrichments detected in many genes/pathways increased apoptosis by *Bmal1* gene deletion. Protein-protein interaction analysis from common DEGs revealed that *GSK3 β* , *NACC1* and *EGFR* genes are the main genes involved in regulation response of KO cells. DEG analysis suggested that non-coding transcripts RNAs (lncRNA, processed pseudogenes) may play roles in drug-induced apoptosis. Additionally, cells were treated with Endoplasmic Reticulum (ER) stress-inducing agent tunicamycin and it was found that response to tunicamycin-induced stress were minimally affected by *BMAL1* deletion. DEG analysis between untreated KO and WT cells showed that epithelial-mesenchymal transition genes were upregulated, a finding that supports the increase in invasion capacity of KO cells.

In summary, this study revealed a detailed transcriptome picture in which the opposite carcinogenic effects of *BMAL1* gene disruption is caused by regulation of many genes.

Key words: *BMAL1*, Circadian Clock, Apoptosis, Cancer, RNA-sequencing, Transcriptome

ÖZET

Sirkadiyen saat, gen ifadesinden organizmanın davranışına kadar birçok düzeyde düzenleme sağlayan bir mekanizmadır. Sirkadiyen ritmin bozulması, kanser gibi birçok hastalığı ve biyolojik süreçleri etkilemektedir. *BMAL1* geninin silinmesinin meme kanseri hücrelerinin DNA hasarına sebep olan ajanların indüklediği apoptoza duyarlılığını ve invazyon kapasitesini artırdığı bildirilmiştir. Bu çalışmada, *BMAL1* geni silinmiş meme kanseri hücrelerinde daha önce gözlemlenen fenotipin mekanizmasının genom düzeyinde bir yaklaşımla ortaya çıkarılması amaçlanmıştır. Bu amaçla, *BMAL1* knockout (KO) ve yabanıl tip (WT) MDA-MB-231 hücrelerine cisplatin ve doxorubicin ile muamele edilmiştir. İlaçla muamele edilmiş hücrelerde farklı şekilde ifade edilen genler (DEG'ler) RNA sekanslama tekniği kullanılarak analiz edilmiştir. Transkriptlerin gen seti zenginleştirme analizleri, birçok gen/yolakta tespit edilen zenginleşmelerin *Bmal1* gen delesyonunun apoptozu artırdığını göstermiştir. Ortak DEG'ler ile yapılan protein-protein etkileşim analizi, *GSK3β*, *NACC1* ve *EGFR* genlerinin, KO hücrelerinin yanıtının düzenlenmesinde rol oynayan temel genler olduğunu ortaya çıkarmıştır. DEG analiz sonuçları, kodlanmayan RNA'ların (lncRNA, işlenmiş psödojenler) ilaç-indüklü apoptozda rol oynayabileceğini öne sürmüştür. Hücrelere Endoplazmik Retikulum (ER) stres indükleyici ajan tunicamycin ile muamele edilmiş ve tunicamycin tarafından oluşturulan strese karşı cevabın *BMAL1* delesyonundan minimum düzeyde etkilendiği tespit edilmiştir. Ek olarak, ilaç ile muamele edilmemiş KO ve WT hücreleri arasındaki DEG analizi, KO hücrelerinin invazyon kapasitesindeki artışı destekleyen bir bulgu olan epitelyal-mezenkimal geçiş genlerinin upregüle edildiğini göstermiştir.

Özetle; bu çalışma, birçok genin düzenlenmesi üzerinden *BMAL1* gen bozulmasının farklı kanserojen etkilerinin olduğu ayrıntılı bir transkriptom tablosunu ortaya çıkarmıştır.

Anahtar kelimeler: *BMAL1*, Sirkadiyen Saat, Apoptoz; Kanser, RNA-sekanslama, Transkriptom

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TABLE of CONTENTS

	<u>Page</u>
SUMMARY	v
ÖZET	vi
ACKNOWLEDGEMENTS	vii
TABLE OF CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	xi
LIST OF FIGURES	xiii
LIST OF TABLES	xv
 1. INTRODUCTION	 1
1.1. The Purpose, Contribution and Content of Thesis	1
2. LITERATURE REVIEW	3
2.1. The Mechanism of the Circadian Clock	3
2.2. Circadian Dysfunction and Apoptosis	5
2.3. Circadian Dysfunction and Cancer	7
2.4. The mechanism of Cell Invasion and Epithel-Mezenchymal Transition (EMT) of Cancer Cells	8
2.5. The Mechanism of ER Stress	10
2.6. Transcriptome Analysis	11
3. MATERIALS	12
3.1. Chemicals	12
3.2. Cell Culture Supplements and Solutions	12
3.3. Kits	13
3.4. Antibodies	13
3.5. Cell line, Growth Media and Solutions for Cell Culture	14
3.6. Oligonucleotids Used for Quantitative Real-time PCR	14
3.7. Buffers and Solutions	15
3.8. Equipments	16
4. METHODS	18
4.1. Cell Culture	18
4.2. Drug Treatments	18

4.3. Apoptosis Assay	20
4.4. ER Stress Induction	20
4.5. Western Blotting	20
4.5.1. Sample Preparation (to extract proteins from adherent cells)	20
4.5.2. Gel Electrophoresis	21
4.5.3. Transfer of Proteins to the Membrane	21
4.5.4. Blotting and Antibody Incubation	22
4.5.5. Imaging and Data Analysis	23
4.6. Wound Healing Assay	23
4.7. Total RNA isolation	24
4.8. RNA-seq Library Preparation	24
4.9. Transcriptome Analysis	28
4.10. Identification of Differentially Expressed Genes	28
4.11. Gene Set and KEGG Pathway Enrichment Analysis	29
4.12. Protein-protein interaction network analysis	30
4.13. Validation of RNA-seq Data with qPCR	30
5. RESULTS	32
5.1. Confirmation of Mutations by Western Blotting Analysis of MDA-MB-231 WT and KO cells	32
5.2. Effect of <i>BMAL1</i> Knockout Mutation on DNA Damage-induced Apoptosis at MDA-MB-231 WT and KO cells	33
5.3. Effect of <i>BMAL1</i> Knockout Mutation on ER Stress Response at MDA-MB -231 WT and KO cells	35
5.4. Effect of <i>BMAL1</i> Knockout Mutation on Migration Rates of MDA-MB-231 WT and KO cells	36
5.5. RNA Extraction and RNA-seq Library Preparation	38
5.6. Mapping Reads to Reference Genome	39
5.7. Correlation Matrix of Reads from Biological Replicates	40
5.8. Identification of Differentially Expressed Genes in KO Cells Compared to WT Cells in Response to Treatments	40
5.9. Classification of Differentially Expressed Genes	45
5.10. Correction of Zero Division Errors Allows Gain of Information on Non coding Genes	47

5.11. Identified Pathways in Gene Set and KEGG Enrichment Analyses May Increase Apoptosis in KO Cells Upon Exposure to Cisplatin and Doxorubicin	63
5.12. Analysis of Transcriptome Reveals EMT Regulated Genes are Significantly Up-regulated in KO Cells	67
5.13. Protein-protein Interaction Network Analysis Helps Identify Key Protein Interactions Responsible for Sensitization of KO Cells to Cisplatin- and Doxorubicin -Induced Apoptosis	67
5.14. Validation of RNA-seq Data with qPCR	70
6. DISCUSSION	71
REFERENCES	75
BIOGRAPHY	82
APPENDICES	83

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
µg	: Microgram
µl	: Microliter
APS	: Ammonium persulfate
BMAL1	: Brain and muscle Arnt-like 1
CIS	: Cisplatin
CLOCK	: Circadian locomoter output cycles protein kaput
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
CRY1	: Cryptochrome 1
CRY2	: Cryptochrome 2
DEGs	: Differentially expressed genes
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
DOX	: Doxorubicin
ECM	: Extracellular matrix
EDTA	: Ethylenediaminetetraaceticacid
EGF	: Epithelial Growth Factor
EMT	: Epithelial Mesenchymal Transition
ER stress	: Endoplasmic Retikulum stress
FBS	: Fetal Bovine Serum
GSEA	: Gene set enrichment analysis
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid)
HRP	: Horseradish peroxidase
KO	: BMAL1 knockout
MMP	: Matrix metalloproteinases
NaCl	: Sodium chloride
PAS	: Period-Arnt-Single-minded
PBS	: Phosphate Buffered Saline
PCR	: Polimerase chain reaction
PER	: PERIOD
PPI	: Protein-protein interaction network

Rev-erba	: Retinoic acid-related orphan nuclear receptors
RIPA	: Radio Immuno Precipitation Assay
RNA seq	: RNA sequencing
ROR α	: Retinoid-related orphan receptor α
RT-qPCR	: Real-time quantative polymerase chain reaction
SCN	: Suprachiasmatic nucleus
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	: Tris/Borate/EDTA
TBS	: Tris Buffered Saline
TBS-T	: Tris buffered Saline with Tween 20
TEMED	: Tetramethylethylenediamine
TRIS	: Hydroxymethylaminomethane
TTFL	: Transcription translation feedback loop
TUN	: Tunicamycin
VC	: Vehicle
WT	: Wild-type

LIST OF FIGURES

<u>FIGURE NO:</u>	<u>Page</u>
2.1: The molecular circadian clock in mammalian cells.	4
2.2: Interactions between the clock and the mechanism of apoptosis.	6
2.3: The epithelial-mesenchymal transition (EMT) of primary epithelial tumor cells.	9
4.1: Experimental design, RNA-Seq analysis procedure.	19
4.2: RNA-sequencing library preparation protocol.	25
4.3: Illumina sequencing-data processing workflow.	27
5.1: Confirmation of <i>BMAL1</i> gene knockout at MDA-MB-231 MDA-MB-231 cells.	33
5.2: Effect of <i>BMAL1</i> mutation on apoptosis at MDA-MB-231 cells.	34
5.3: Effect of <i>BMAL1</i> mutation on ER stress response at MDA-MB-231 cells.	35
5.4: Effect of <i>BMAL1</i> mutation on Migration Rates of MDA-MB-231 WT and KO cells.	37
5.5: Electropherogram and gel images showing the quality and quantity controls of the RNA libraries obtained with the Bioanalyzer.	39
5.6: Correlation matrix of reads from biological replicates of each sample.	40
5.7: Analysis of methodology of the significantly differentially expressed genes.	42
5.8: Analysis of number of differentially analyzable genes and differentially expressed genes.	44
5.9: The classification of differentially expressed genes.	46
5.10: The classification of eliminated transcripts with zero FPKM causes.	62
5.11: Gene Set Enrichment (GSEA) and KEGG pathway analysis.	64
5.12: Gene Set Enrichment Analysis after TUN treatment.	65
5.13: Gene Set Enrichment Analysis and KEGG GO term of DEGS without treatment.	66

5.14:	Number of common DEGs in CIS, DOX, and TUN treatments were shown in Venn schema.	68
5.15:	STRING network analysis of common DEGs in CIS and DOX treatments.	69
5.16:	Validation of RNA-seq data with qPCR.	70

LIST OF TABLES

<u>Tablo No:</u>	<u>Page</u>
3.1: List of primers.	14
3.2: Buffers used in nucleic acid related studies.	15
3.3: Buffers used in Western Blot Analysis.	15
3.4: List of SDS-PAGE stacking and seperating gel recipe.	16
3.5: Equipments.	16
5.1: RNA quantity and purity measurements of drugs treated WT and KO cells.	38
5.2: Read numbers of each replicates from WT and KO cells treated with indicated agents.	39
5.3: The list of genes having 0 FPKM values for cisplatin treatment	47
5.4: The list of genes having 0 FPKM values for doxorubicin treatment	51
5.5: The list of genes having 0 FPKM values for tunicamycin treatment	58

1. INTRODUCTION

Circadian clock is known to play roles in regulating many biological mechanisms such as sleep-wake cycle, metabolism and acute responses to stresses. An important function of the circadian system is to ensure the harmony of an organism to its environment. Many conditions of diseases and dysfunction occur with the disruption of this synchronization [Antoch and Kondratov, 2013]. Epidemiological and clinical studies have suggested that disarrangement of rhythms cell can increase both tumor formation and cancer progression [Morgan et al., 2019].

Our knowledge of molecular interplay between circadian clock and stress response pathways is limited, new studies are needed to understand the involvement of circadian clock in associated diseases through identification of novel circadian clock associated proteins and genes. For the purpose of studying the relationship of the circadian clock and diseases such as cancer, the knocking-out is one of the circadian clock genes in animal models is a common strategy to understand the gene-phenotype relationship. Due to aging and death problems caused by rapid aging in *Bmal1* mutant mouse, it was difficult to study the circadian clock and cancer relationship in this mouse model [Kondratov et al., 2006].

The subject of this thesis is to analyse the relationship between the biological clock and cancer development steps such as apoptosis and invasion capacity in order to understand stress and circadian clock relationship in the *BMAL1* gene knockout breast cancer cells platform that was obtained using the CRISPR/Cas9 based genome editing technique.

1.1. The Purpose, Contribution and Content of Thesis

We have previously reported that deletion of the *BMAL1* gene in a nearly normal breast epithelial and an invasive breast cancer cell line makes the cells susceptible to apoptosis after treatment with a bulky-DNA adduct forming agent (cisplatin) and double-strand break forming agent (doxorubicin). Moreover, this sensitization phenotype has been shown to occur independently of p53 in invasive MDA-MB231 cells [Korkmaz et al., 2018]. In this thesis we used two *BMAL1* knockout cells to

explore the dynamic gene responses under stress conditions such as DNA-damage and Endoplasmic Reticulum (ER) stress. DNA damage related stress was generated using cisplatin and doxorubicin while ER stress was formed using tunicamycin. We used two different DNA damaging agents because their action mechanism are different and therefore generate different stresses. The main purposes of this thesis was to reveal 1) the mechanism of enhanced sensitization of *BMAL1* knockout cell line (MDA-MB-231) to DNA damage-induced stress by analysing differentially expressed genes (DEGs) in MDA-MB-231 cells by using RNA sequencing (RNA seq) analysis of transcriptome 2) the network analysis of common DEGs, to determine in which pathways the DEGs are concentrated and how the differentiation status of cells is affected.

In the content of this thesis, DEG analysis was performed using RNA seq analysis in cells treated with different drugs. We used only MDA-MB-231 *BMAL1* knockout model in RNA-seq study, confirmed expected phenotype such as sensitization to DNA damage induced apoptosis and increased invasion and motility capacity. ER stress was included as a control in our study in the way that *BMAL1* knockout cells show a selected sensitization to various stress conditions because *BMAL1* knockout and wild-type cells showed a similar response to tunicamycin-induced stress.

With this study, *BMAL1*-regulated genes and related pathways have been investigated especially in considering their involvement in migration and invasion potential. Transcriptome analysis of untreated and drug-treated cells helped us to find out genes which contributes the regulation of apoptosis in a circadian manner. This will enable new strategies to be targeted at different stages in the cancer process. Thereby, it will be possible to determine the molecular agents that will increase the effect of chemotherapeutic drugs by targeting the biological clock.

2. LITERATURE REVIEW

2.1. The Mechanism of the Circadian Clock

The circadian clock is a programme identified in almost all organisms from bacteria to humans. The circadian rhythm is a synchronization with the rotation of the earth that provides a 24-hour order to processes at many levels, from gene expression to the behavior of the organism. [Roenneberg and Merrow, 2016], [Sancar et al., 2014]. More than 40% of all protein-coding genes have 24-hour gene transcription rhythms [Wulund and Reddy, 2015].

The mammalian circadian timing system consists of a master clock and oscillators in the most peripheral cell tissues-organs. Master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Master clock accepts light signal from the retina that adjusts the internal clock timing to the external solar day [Nagoshi et al., 2004], [Partch et al., 2014]. This information is transformed a chemical version. The clock gene expression profiles change in SCN neurons and transferred to cells in other brain parts and peripheral organs [Dibner et al., 2009].

The molecular clocks in the cells that form the master and peripheral clocks consist of a transcription-translation feedback loop (TTFL) mechanism [Ko and Takahashi, 2006] (Figure 2.1), (Figure was modified from Shafi and Knudsen 2019). This loop is made of two parts, where the positive arm activates the transcription of the negative arm elements and the products of negative arm inhibits the transcription factors in the positive arm. In this way, activation and inhibition generates a cyclic transcriptional-expression pattern [Hurley et al., 2016].

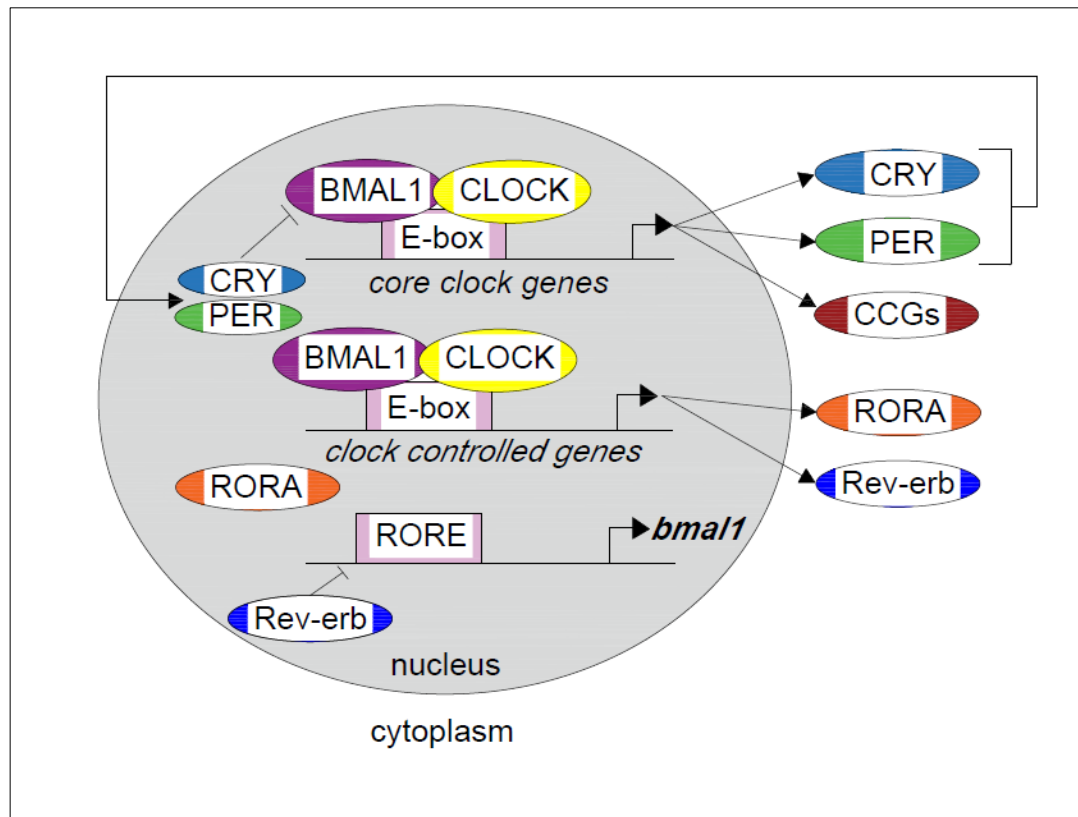


Figure 2.1: The molecular circadian clock in mammalian cells.

The core TTFL is directed by four integral clock proteins: the positive elements contain basic helix-loop-helix PAS (Period-Arnt-Single-minded) transcription factor family members, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle Arnt-like1); the negative elements include Cryptochrome (CRY) and Period (PER). CLOCK and BMAL1 heterodimerize, then activates *CRY* and *PER* genes expression by binding to E-box promoter elements. Subsequently, the synthesized CRY and PER proteins heterodimerize in the cytoplasm and migrate to the nucleus to associate with CLOCK:BMAL1, preventing more transcriptional activation [Kiyohara, 2006], [Ko and Takahashi, 2006], [Partch et al., 2014] (Figure 2.1). This activation and inhibition in molecular clock generates the oscillations in circadian clock-regulated genes (CCGs) which are adjusted approximately to 24h by the secondary loops and posttranslational modifications [Reppert and Weaver 2002].

There are more components such as *Rora*, *Rev-erba* and *Rev-erbβ* for controlling the expression of the *BMAL1* and *CLOCK* genes and these genes are also under the CLOCK: BMAL1 regulation. ROR elements stimulate *BMAL1* expression, while Rev-

erb elements suppress *BMAL1* expression, this cycles form a second positive-negative feedback loop [Shafi and Knudsen 2019].

2.2. Circadian Dysfunction and Apoptosis

Programmed cell death or apoptosis mechanism is generally defined by different morphological features and energy-dependent biochemical events. Apoptosis, which is a critical and important mechanism that preserves the balance between surviving and death in cells to prevent diseases such as cancer, includes two main pathways: extrinsic pathway via death receptors, and intrinsic pathway via mitochondria [Jan and Chaudri, 2019] [Elmore, 2007].

- The intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis is induced by DNA damaging agents, extrinsic apoptosis is induced by death ligands for instance tumor necrosis factor- α (TNF- α). It has been revealed that the circadian clock is associated with both mechanisms (Figure 2.2), (Figure was modified from Sancar et al., 2014). Bcl-2 family protein members have crucial roles to control and modulate apoptotic mitochondrial pathways. The tumor suppressor protein p53 has an critical function in Bcl-2 protein family regulation [Elmore, 2007].

CRY mutation effect on the phenotype of mouse prone to tumor development due to the *p53* mutation was examined. It was observed that the *CRY* mutation decreased the frequency of tumor formation and increased the tumor-free lifespan of mice one and a half times, contrary to expectations. In cytotoxic analysis with genotoxic agents, *p53*^{-/-} *Cry1/2*^{-/-} cells showed sensitivity compared to *p53*^{-/-} cells [Ozturk et al., 2009]. It was revealed that it activates a *p53* independent apoptosis pathway in the absence of Cryptochrome genes. In the following study, it was shown that in the absence of *p53*, the apoptotic response was partially restored by activation *p73* through *Egr1* [Lee and Sancar, 2011]. The upregulation of *p73* induced by DNA damage is mainly associated with transcriptional induction. Positive activators of *p73* are; *E2F1* and *Egr1* directed via circadian clock. The suppressing element is *C-EBPa*. UV or UV mimetic agents cause phosphorylation of C-EBPa. It facilitates *p73*

transcription by causing it to be released from *p73* promoter then removed from the nucleus. Circadian clock regulated *Egr1* is triggered by UV and UV-mimetic agent, oxaliplatin. So, this leads to an increase in *p73* transcript and apoptosis caused by DNA damaging agents [Lee et al., 2011a], [Sancar et al., 2014].

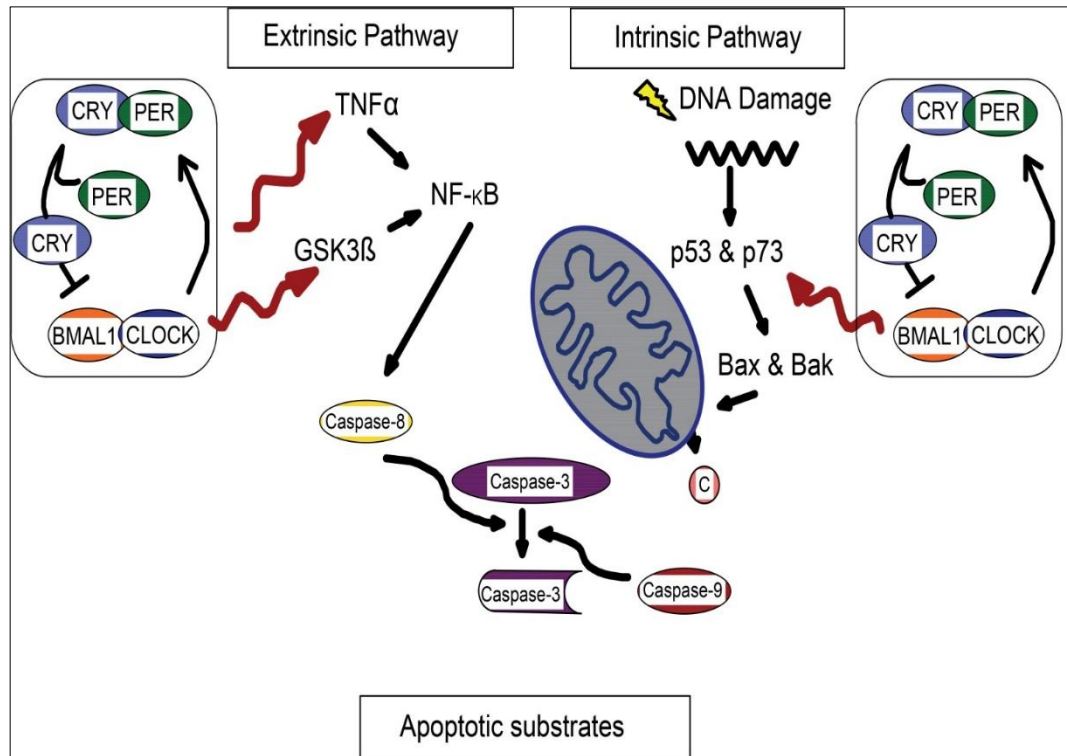


Figure 2.2: Interactions between the clock and the mechanism of apoptosis.

- The extrinsic pathway of apoptosis

The extrinsic pathway is induced via cytokine receptors, TNF (tumor necrosis factor) family members, such as the type 1 TNF receptor (TNFR1), Fas and TRAIL receptors and their ligands such as TNF, Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand). When ligand binds to the receptor, the adaptor molecule that has death domains such as FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain) forms a death-triggering signaling complex (DISC). This complex also contains procaspase-8 and -10, and the cellular FLICE inhibitory proteins. Active caspase 8 can activate caspase 3 cascade of apoptosis or activate the proapoptotic protein Bid to transport it to the mitochondria (Figure was modified from Sancar et al., 2014), [Sancar et al., 2014], [Jan and Chaudri, 2019], [Foo, 2010].

In a study conducted on the relationship between extrinsic apoptosis and circadian clock, it was reported that grade of inflammatory and proapoptotic cytokines such as TNF α were high in *Cry* mutant mice [Hashiramoto et al., 2010]. In another study, in *p53* mutant cells that have functional clock, cytokines trigger the anti-apoptotic genes transcription via GSK3 β phosphorylation regulated by the circadian clock. *p53* mutant and *Cry*-null mutant cells cause hyperphosphorylation and inactivation of GSK3 β . In this case, GSK3 β cannot phosphorylate *NF- κ B* gene anti-apoptotic activity, cells become more vulnerable to TNF α -triggered apoptosis. In summary; The *CRY* mutation induces extrinsic apoptosis in two different ways via causing excess production of proapoptotic cytokines like TNF α and hyperphosphorylation of GSK3 β [Lee et al., 2011b].

2.3. Circadian Dysfunction and Cancer

Circadian regulation is known to be highly involved in gene expression. The circadian clock regulates approximately 10% of all genes expressed in any cell. [Xie et al., 2019]. The complete disruption or deregulation of the biological clock has been linked to development of many diseases like cancer, diabetes, various metabolic and neurological diseases, and sleep disorders [Evans and Davidson, 2013].

There are several epidemiological studies revealing a moderate rise in the incidence of breast cancer and other cancers in night shift workers [Evans and Davidson, 2013]. In a study, the risk of rectal cancer increased with long-term shift work was reported [Papantaniou et al., 2018]. Based on raised cancer risk among shift workers, the International Agency for Research on Cancer (IARC) listed circadian disruption as a possible human carcinogen. However, ovarian cancer risk did not increase and melanoma which is triggered by excessive UV light exposure was decreased among shift workers. Shift work can be linked to factors like alcohol consumption, smoking habits, food preferences, radiation exposure, or other carcinogens. Therefore, in some reports there is no clear link among shift work and an raised risk of cancer [Sulli et al., 2019].

It was reported in a study with animal models, mice carrying *Per2* mutation became susceptible to cancer [Fu et al., 2002], while in another study, mutations in

PER2 didn't have effect on spontaneous and radiation-induced cancer formation [Antoch et al., 2013]. It was reported that, *Clock* mutant and *Bmal1* knockout mutant mice displayed an extensively sensitive response to an anticancer drug cyclophosphamide. Whereas, mice that have *Cryptochrome* (*Cry1*^{-/-}*Cry2*^{-/-}) mutations causing complete dysfunction were more resistant than wild-type mice [Gorbacheva et al., 2005]. In a different study *Bmal1*^{-/-} mice were reported to show several premature aging phenotypes [Khapre et al., 2014].

For the purpose of clarifying the connection between circadian clock and cancer, it is very crucial to increase the number of molecular-based, in vivo and epidemiological studies.

2.4. The mechanism of Cell Invasion and Epithel-Mezenchymal Transition (EMT) of Cancer Cells

Malignant cells have disorderly proliferation characteristics and significant properties such as metastasize [Jiang et al., 2015]. Cancer metastasis is the most crucial reason of cancer-related deaths and is a multi-stage process expressed as the advanced level of malignant tumors. The important feature of metastasis is the invasive capacity of cells that are guided through cell motility [Ritch et al., 2019], [Tahtamouni et al., 2019]. In epithelial tumor types, cells must first pass through the basement membrane between the epithelium and the stroma for invasion. Invasive cells cross this boundary, which consists of an extracellular matrix network, to reach the blood or lymph pathways and eventually aim to reach other organs [Clark and Vignjevic, 2015].

Epithel-Mezenchymal Transition (EMT) is a process that allows immobile epithelial cells to migrate and invade as separate cells. Epithelial cells have cell to cell connections via adhesion molecules such as E-cadherin and cytokeratin. In response to a variety of extracellular and tissue-specific EMT triggering signals, tumor cells become to have partially relax epithelial properties and develop molecular changes to allow the acquisition of a mesenchymal phenotype [Yeung and Yang, 2017]. Factors causing mesenchymal-to-epithelial transition in cancers are hypoxia, cytokines and growth factors, stroma crosstalk, metabolic changes, innate and adaptive immune responses and anticancer drug treatments [Roche, 2018].

The main cellular events seen in EMT are; disruption of cell-cell interactions, differences in cellular shape, structure and function, cytoskeleton rearrangement, and alterations in cell-matrix adhesion. Eventually, transformation into isolated, mobile and apoptosis-resistant cells are observed (Figure 2.3), (Figure was modified from Yeung and Yang, 2016).

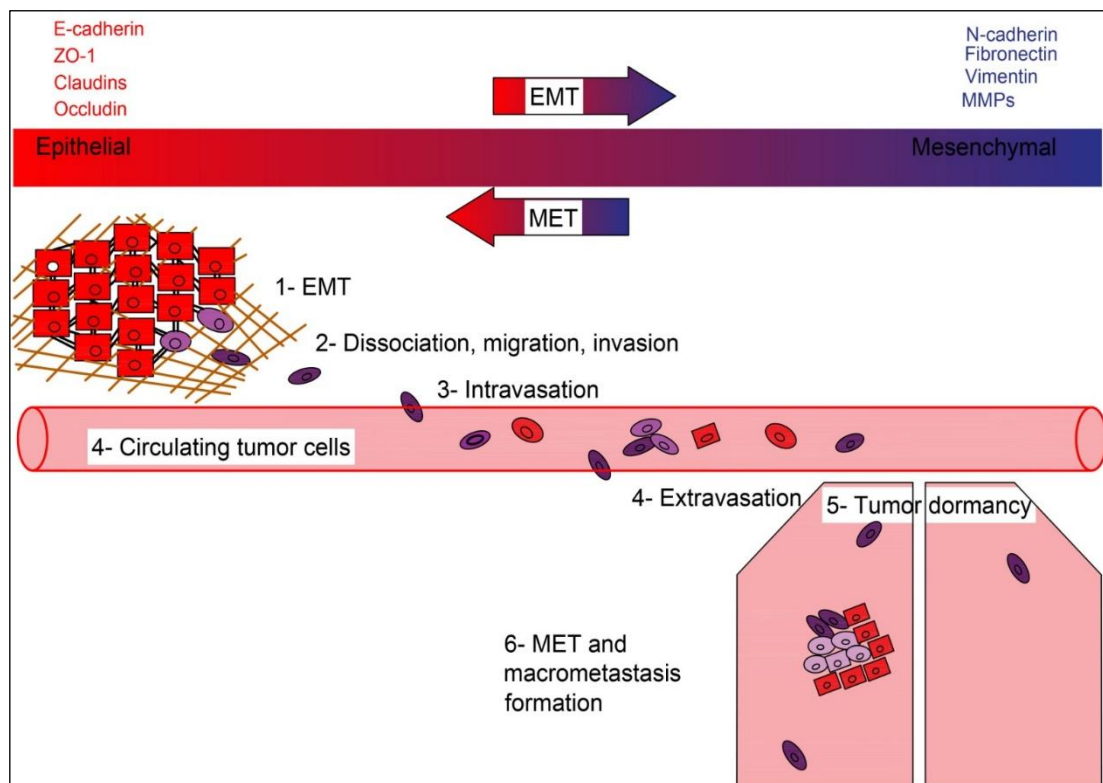


Figure 2.3: The epithelial-mesenchymal transition (EMT) of primary epithelial tumor cells. A small number of circulating tumor cells remain in the vessels and finally reach distant organs. Before tumor cells proliferate and form large-scale metastases through the mesenchymal-epithelial transition (MET) process, they can remain dormant form for a while.

EMT is represented by alterations in levels of three crucial biomarkers (E-cadherin, vimentin and N-cadherin). These alterations can cause reduced cell adhesion, polarity loss and tight junctions. During the process, epithelial cells adopt mesenchymal phenotype characteristics, particularly those such as motility and susceptibility to invasion. Many pathways such as TGF- β , WNT, NOTCH and growth factor receptor tyrosine kinases and mechanisms play role in the regulation and control of EMT. TGF- β plays a role as a major inducer of EMT. Snail, Twist, Zeb and E47

among the family of transcription factors are involved in transcriptional regulation. TGF- β increases the expression of several transcription factors such as *SNAIL1*, *SNAIL2*, *ZEB1 / 2* and *TWIST* through SMAD or non-SMAD signaling pathways. In this way, it causes down-regulation of epithelial junction proteins encoding genes. Though this changes, intercellular connections are lost and then, cells gain mesenchymal characteristics with increase of mesenchymal genes like *N-Cadherin*, *Fibronectin*, *Vimentin*. With increased expression of matrix metalloproteinases (MMPs), proteolytic extracellular matrix destruction results in cell migration and invasion. Non-coding RNAs like miRNAs and long non-coding RNAs, chromatin rearrangement and epigenetic rearrangements, alternative splicing, post-translational regulation are also involved in the EMT regulation chain [Roche, 2018], [Savagner, 2010], [Yeung and Yang, 2016], [Hao et al., 2019].

2.5. The Mechanism of ER Stress

Endoplasmic reticulum (ER) organelle has roles in translocation, folding, posttranslational modification of proteins, and lipid biosynthesis. ER provides an oxidizing environment for membrane folding and secretory proteins transported to cell plasma surface, lysosomes and Golgi vesicles. ER stress response occurs as a result of cytotoxic conditions such as pH change, excessive production of proteins, reduction of disulfide bonds and unfolded protein accumulation and aggregation. During these stress conditions, ER activates specific signaling pathways named unfolded protein response (UPR) to restore protein balance [Faitova et al., 2006], [Sano and Reed, 2013].

UPR is induced by signal transducers in the transmembrane ER. UPR contains inositol-requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF)-6. These ER stress sensors, which form the UPR mechanism, are normally connected with certain chaperones so they can remain inactive. Following endoplasmic reticulum stress, IRE1 oligomerizes in ER membrane and triggers cytosolic domain kinase and endoribonuclease functions. By IRE1 endoribonuclease activity, Xbp1 induces mRNA splicing and *sXbp1* (spliced Xbp1) acts as a transcription factor encoding proteins which increase ER protein folding

capacity and misfolded ER protein degradation, thus preserving the cell via decreasing ER stress stimulation. In another UPR signaling pathway, PERK protein dimerizes and autophosphorylates after activation and phosphorylates eukaryotic initiation factor 2 α (eIF2 α). Thus, overall protein translation decreases and the translation of some transcription factors such as ATF4 increases. ATF-6 forms the 3rd signalling pathway of the UPR mechanism and traffics from ER to Golgi apparatus during stress and is cleaved to a transcription factor that provides UPR gene expression [Almanza et al., 2019], [Lin et al., 2008], [Clarke et al., 2014].

2.6. Transcriptome Analysis

Transcriptome is termed as all of the RNA transcripts found in a particular cell for a particular developmental stage or physiological state. Analyzing the transcriptome is essential to realise the mechanisms related with basic development and disease as well as interpreting the functional elements of the genome. Transcriptome analysis is a great tool to figure out of the pathways that control cell fate, development and disease progression in a host. RNA sequencing (RNA-Seq) is an developing modern technology that utilizes next generation sequencing (NGS) or deep sequencing to perform transcriptome profiling. RNA-seq has quickly replaced technologies such as microarray due to its better resolution and higher reproducibility. RNA-Seq allows analysis of unknown genes and transcript isoforms with sequence identification, and expression levels of even low-level transcripts can be measured with this technique. It is also a fast and useful perspective for gen set research and identification of functional genes and molecular markers [Sudhagar et al., 2018], [Yang and Kim et al., 2015].

In this thesis, RNA-seq technique was used to describe the differentially-expressed-genes (DEGs) and pathways after treating *BMAL1* knockout (KO) or wild-type (WT) MDA-MB-231 cells with DNA-damaging chemotherapeutic drugs or ER stress inducing drugs as cellular stress generating reagents.

3. MATERIALS

3.1. Chemicals

Methanol (947.046, Sigma), Ethanol (920.026, Sigma), Agarose (351010, Peqlab), HEPES (P05-01100, PAN), LB Broth Medium (LBP03, Caisson Labs, 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), Cisplatin (P4394, Sigma), Doxorubicin (D1515, Sigma), Tunicamycin from Streptomyces sp. (T7765, Sigma), 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) were used in this study.

3.2. 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, Invitrogen) were used.

3.3. Kits

Total RNA was obtained using NucleoSpin RNA Purification Kit (Macherey Nagel, Germany) following manufacturer's instruction. cDNA was synthesized using ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs (NEB)-USA). For qRT-PCR assays Luminaris HiGreen qRT-PCR Master Mix (Thermo Scientific, USA) was used with gene specific primers. NEBNext® Poly (A) mRNA Magnetic Isolation Module (NEB E7490) and NEBNext® Ultra RNA Library Prep Kit for Illumina® (E7530) kits were used in the preparation of RNA-Seq samples.

Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, USA) were used for the protection of proteins in cell extracts from various proteases. Bradford Protein Assay Dye (Bio-Rad, USA) and Pierce™ BCA Protein Assay Kit (Thermo Scientific, US) were utilized for total protein quantitation. Protein Molecular Weight Marker (New England Biolabs, USA) was used to monitor the progress of electrophoretic run and analysis conditions of proteins. To define the approximate protein size and to follow the running of proteins on the gel during electrophoresis Protein Molecular Weight Marker (New England Biolabs, USA) was used. Chemiluminescent HRP Substrate (Advansta, USA) and SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Scientific, US) were used for protein level detection by western blot analysis.

3.4. Antibodies

The following primary antibodies were utilized for determining their relevant proteins: Anti BMAL1 (1:2000, Bethyl Labs Inc., TX., USA, A302-616A); Anti cleaved PARP Ab (1:2000, Cell Signaling Technology (CST), 9541s); Anti actin (1:5000, Cell Signaling Technology (CST), 4967); Anti Xbp1 (1:2000, Abcam, 37152). Secondary antibodies HRP-labeled anti-mouse and anti-rabbit antibodies (Cell Signaling Technology) were used in blocking solution at 1:5000 dilution.

3.5. Cell line, Growth Media and Solutions for Cell Culture

MDA-MB-231 cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM Dulbecco's Modified Eagle Medium (DMEM, 41965-039, Gibco, Invitrogen), supplemented with 10% FBS (Fetal Bovine Serum (FBS), 10270-076, Gibco, Invitrogen), 100 U / mL penicillin, 100 µg / mL streptomycin (100X Stock solution, 15140-122, Gibco, Invitrogen), and 1% MEM nonessential amino acids solution (100X Stock solution, 11140050, Gibco, Invitrogen). Cells were subcultured using 0.05% Trypsin-EDTA (25300-054, Gibco Invitrogen) solution after washing cells with 1XPBS obtained from 10X-Phosphate Buffered Saline (6505-OP, Calbiochem).

3.6. Oligonucleotids Used for Quantitative Real-time PCR

18 up-regulated or downregulated genes selected from RNA-seq data were analysed via Quantitative RT-real time PCR (qPCR).

Table 3.1: List of primers.

Gene name	Oligonucleotid sequence	
	Forward	Reverse
EFEMP1	5'GCTAAGCAGTGACAGGCTCA3'	5'TATCCCTGGGGGCACATACA3'
EPB41L4A	5'ATGGTTGATTCAGCGCTCA3'	5'CCCATCTGAACACTGGGACC3'
FAM47E	5'GTCCTGGTCTTCATGAAAATGGAA3'	5'TGAAGGTCTGCAAGTAACTCCTC3'
FGF13	5'TGAGGACAGCACTTACACTCTG3'	5'GCTCATGGATTTGCCTCCGT3'
MGAT4A	5'GGTGTGTAGCCAACCTGGA3'	5'AGATCCGGCGCTTGAAACAT3'
MMP1	5'AGGGGAGATCATCGGGACAA3'	5'GGCTGGACAGGATTTTGGA3'
NEO1	5'GTCTGGAGGGAAAGGAAGCC3'	5'GATGGGGTTTGGGTCTGGAG3'
SIRPA	5'GCCCACAGGGATGATGTGAA 3'	5'TGTGATATCATTTGTGTCCTGTGT
STOM	5'GGTGTGGTCTATTACCGCGT 3'	5'CTTCGGCTGCAATAACCTTGG3'
ARHGAP30	5'CCTTGAGTGCCCTGCTCTAC 3'	5'TGGCTCCACAGAGAACTCCT3'
CADM4	5'GTGTTGACGTGTGCTGTCAC3'	5'TCCCTGTTTCATCCAAGCCAC3'
FERMT3	5'GAGAAAGGAGGGCAGGAAGCv	5'CTCCTCTCCCACAAACACCC3'

Table 3.1: Continued

GALNT9	5'AACATCCCCATGTCGAACCC3'	5'GAGTCAGGCAAGAAGGCTGT3'
KCNAB2	5'AGCGTGAGAAAGTGGAGGTG 3'	5'CGGAGCTGACTCCCTCATTC3'
LCP1	5'AGCGAAGTTCTCCCTGGTTG3'	5'TGGCAGGGCATACTCTTG3'
SPANXB1	5'GCCAATGAGGCCAACAAGAC3'	5'CTCCATTTGGTCGGGGTTGA3'
TAGLN	5'AGGTCTGGCTGAAGAATGGC 3'	5'CTCTGTTGCTGCCCATCTGA3'
TIMP4	5'TGCAACTACATCGAGCCCTG3'	5'TTAGGGGCCGAGATGGTACA3'
hGAPDH	5'TGCACCACCAACTGCTTAGC3'	5'ACAGTCTTCTGGGTGGCAGTG3'

3.7. Buffers and Solutions

Table 3.2: Buffers used in nucleic acid related studies.

10X TBE: 108g Tris base, 55g Boric acid, 40 ml 0.5M EDTA (pH 8.0)

Table 3.3: Buffers used in Western Blot Analysis:

<u>-10X SDS Running Buffer</u> 250 mM Tris-Base/pH: 8.3, 1.90 M Glycine, 1% SDS
<u>-10X TBS</u> 200 mM Tris-Cl /pH: 7.6, 1.5 M NaCl
<u>-1X Transfer Buffer</u> 10% 10X SDS Running Buffer, 20% Methanol
<u>-1X TBST</u> 1X TBS, 0.1% Tween-20
<u>-5% Blocking Solution</u> 5% non-fat dry milk in 1X TBS-T
<u>-4X Protein Loading Dye (Laemmli)</u> 250 mM Tris-Cl/pH: 6.8, 8% SDS, 40% Glycerol, 20% β -mercaptoethanol, 0.008% Bromophenol Blue
<u>-Cell Lysis Buffer (RIPA Radio Immuno Precipitation Assay)</u> 750 mM NaCl, 250 mM Tris/pH: 8.0, 0.1% SDS, 1.0% NP-40

Table 3.4: List of SDS-PAGE stacking and separating gel recipe.

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

3.8. Equipments

Table 3.5: List of equipments.

2100 BioAnalyzer	Agilent/USA
Centrifuges	521-1647 refrigerated (Micro Star 17R-VWR/USA) 521-1646 (Micro Star 17-VWR/USA) 521-2844 (Mini Star-VWR/USA) NF400/Nuve (02-1827)
Chemiluminescence Imaging System	ChemiDoc™ XRS+System (Bio-Rad/USA)
Incubator (CO ₂)	Thermo-Scientific/USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad/USA
Inverted Microscope	(Nikon/Eclipse/E100)
Laminar Flow Cabinet	Hera-Safe Class II Cabinet (Thermo Scientific/USA)
Microplate Reader	Varioskan Flash (Thermo Scientific/USA)

Table 3.5: Continued

Power Supply	Bio-Rad/USA
PCR Thermal Cycler	41798 (Bio-Rad/USA)
TransBlot Transfer System	1704155 (Bio-Rad/USA)
Qubit Fluorometer	Thermo Scientific/USA
NanoDrop	206-26300-48 (Shimadzu-Biotech)
Vortex	Scientific Industries/USA
Water Bath	Wisd-Germany
Agarose Gel Electrophoresis	1704406 (Bio-Rad/USA)

4. METHODS

4.1. Cell Culture

BMAL1 knockout MDA-MB-231 cells were generated using the LentiCRISPRv2 system [Sanjana et al., 2014], and we selected the *BMAL1* KO-T1-L1 clone for this study from [Korkmaz et al., 2018]. In a similar way, a mock MDA-MB-231 cell line was generated using LentiCRISPRv2 plasmid which contained sgRNA sequence of GTATTACTGATATTGGTGGG that does not hit human genome according to a previous study [Doench et al., 2016]. Hereafter, mock-targeted control and *BMAL1* knockout MDA-MB-231 cells are called WT and KO, respectively, in respect to the status of *BMAL1* gene and for the sake of easy following. Cells were cultured in completed Dulbecco's-Modified-Eagle-Medium (DMEM) supplemented with penicillin/streptomycin in the same mixture at 100 U/ml and at 100 µg/ml concentrations, respectively, 1% non-essential amino acids, and 10% fetal bovine serum (all from Thermo Fisher Scientific, USA) in a humidified incubator at 37 °C under 5% CO₂ condition.

4.2. Drug Treatments

Cells were treated with a bulky-DNA adduct forming agent (cisplatin) or, a double-stranded break forming agent (doxorubicin) and bacterial and eukaryote N-acetylglucosamine transferase inhibitor (tunicamycin) agent (all from Sigma-Aldrich, USA). 5 mM cisplatin stock was dissolved in 0.9% (w/v) NaCl solution, 5 mM doxorubicin was prepared in double distilled sterile water, and 5 mg/ml tunicamycin was prepared in DMSO. For tunicamycin treatment group, cells were exposed to DMSO at 1:2000 volume/volume ratio. In order to expose the cells to cellular stresses, cells were seeded in 100-mm dishes at a concentration of 6×10^5 and permitted to culture overnight. In the following day, cells were treated with cisplatin and doxorubicin at 20 µM and 2.5 µM final concentrations for 16 h, respectively, while cells were treated with tunicamycin at 2.5 µg/ml final concentration for 8 h. For the

tunicamycin treatment, we selected this dose and time point because it was in the linear range of the XBP1 splicing to our experience in this cell line. A schematic description of the experimental design and treatments was given in Figure 4.1 which was taken from [Emisoglu-Kulahli et al., 2021].

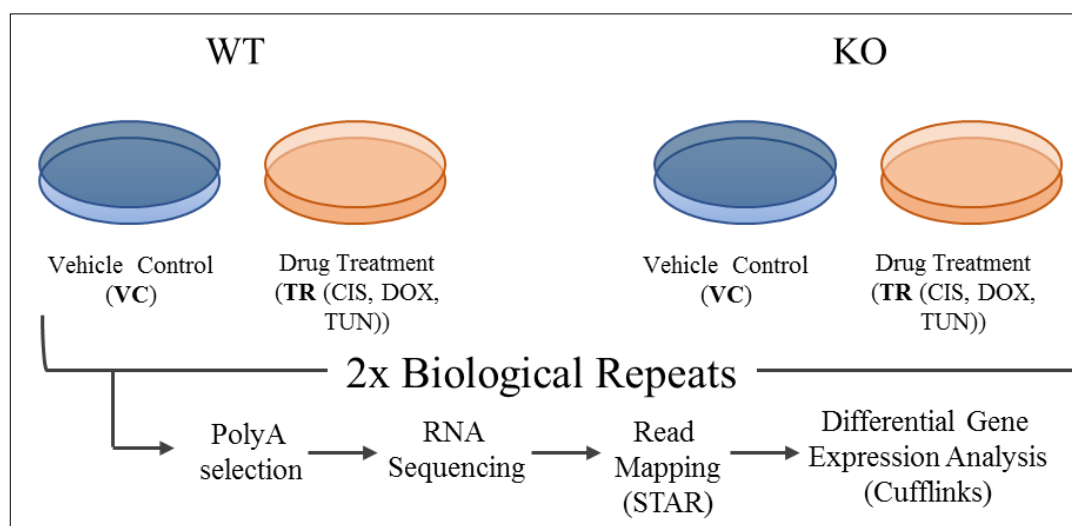


Figure 4.1: Experimental design, RNA-Seq analysis procedure.

The main aim was to identify the mechanism of the differential sensitivity of MDA-MB-231 cells to apoptosis following *BMAL1* gene deletion by CRISPR/Cas9-based genome editing. MDA-MB-231 cells were infected with lentiviruses expressing either non-targeting control sgRNA/Cas9 or sgRNA/Cas9 targeting *BMAL1* gene, and single cell-derived clones were named WT or KO, respectively. Both cell lines were treated (TR) with DNA-damaging agents cisplatin (CIS), doxorubicin (DOX), or ER stress-inducing agent tunicamycin (TUN) alongside with vehicle control (VC) solutions as mock treatments. The RNA-seq procedure was illustrated as the flowchart in Figure 4.1.

4.3. Apoptosis Assay

Apoptosis was triggered by incubating cells cisplatin (CIS) (Sigma) and doxorubicin (DOX) (Sigma). MDA-MB-231 cells were treated with 20 and 40 μ M CIS, 2.5 and 5 μ M DOX for 16 hours. Apoptosis assay was performed by probing for cleaved PARP. Actin signals were used for normalization.

4.4. ER Stress Induction

Tunicamycin was used to induce ER-stress and ER-stress response was followed by probing for spliced active for XBP-1 called sXBP-1. Activation of ER-stress response causes an unconventional splicing in XBP-1 mRNA which generates a larger and active protein product, therefore correlating with ER-stress response. After 4 hours tunicamycin treatment, sXBP-1 signal was detected with an XBP-1 antibody (Abcam, UK) and signal was normalized to actin signal.

4.5. Western Blotting

4.5.1. Sample Preparation (to extract proteins from adherent cells)

Cells were typically harvested by removing media, and adding appropriate amount of ice-cold 1X PBS to cells. Cells were collected from culture dishes via a cold scraper then the cell suspension was softly transferred into a pre-cooled microcentrifuge tubes. Tubes were centrifuged at 5000 rpm for 3 minutes. Following spin process, the supernatant was discarded. Appropriate amount of ice-cold RIPA lysis buffer, prepared shortly before using, were added to the pellet, it was lysed in RIPA lysis buffer by incubating on ice for 20 minutes. During this incubation, the pellet/lysate were homogenized at every 5 minutes by gently pipetting. After incubation, the tubes were centrifuged for 15 minutes, at 13000rpm, +4°C. The supernatant was transferred to a new tube and kept on ice (or freeze at -20 °C). With a small volume (2 μ 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 μ l) on each BSA standard and each of the 2 μ l samples in 96 well plate. Absorbance was measured at 595nm. Using 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 μ 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 briefly spinned.

4.5.2. Gel Electrophoresis

For the separation and analysing proteins based on their molecular size, 8% SDS-PAGE gels were used. It was poured into prepared gel chambers and combs were put on gel directly and the gel was allowed to polymerize. After polymerisation, combs were taken off and equal volumes of the samples were loaded into SDS gel wells, one of the wells was loaded by appropriate size and volume of desired protein marker. All empty wells were loaded with 1X Laemmli Buffer. The samples were run for 15 minutes at 80 V, the voltage was increased up to 120-150V.

4.5.3. Transfer of Proteins to the Membrane

Following separation of proteins by SDS-PAGE, proteins were transferred onto nitrocellulose membrane. For transferring of proteins to nitrocellulose membrane Trans-blot Turbo Transfer System (Biorad Transblot Turbo Transfer System, Serial No: 690BR011760) was used. 4 blotting papers and 1 nitrocellulose membrane with the same dimensions of the SDS PAGE gel were soaked into 1X transfer buffer and transfer sandwich was prepared (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 ddH₂O).

Transfer Sandwich was assembled by the components on the cassette in the order as follow:

- Top (-) Cassette electrode (cathode)
- Two Blotting papers
- Gel
- Blotting Membrane
- Two blotting papers
- Bottom (+) cassette electrode (Anode)

Air bubbles were removed from the sandwich by a roller. Transfer was performed at 12V constant voltage for 60 min for proteins which have low molecular weight and 90 min for proteins which have high molecular weight at transblot transfer system.

4.5.4. Blotting and Antibody Incubation

Following the transfer, the membrane was blotted in 5 % nonfat milk powder dissolved at 1X TBS-T, at room temperature, for 1 hour to block non-specific binding sites. The nitrocellulose membrane was incubated with appropriate primary antibody solution overnight at 4°C or for 2 hours on a rocking table 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 of primary antibody solution).

The membrane was then washed for 4 times (5-15-5-5 minutes respectively) with 1X TBS-T on an orbital shaker and incubated in horseradish peroxidase (HRP) conjugated secondary antibody (diluted in TBST as recommended by manufacturers) for 1 hour at room temperature. Then, the membrane was washed again for 4 times (5-15-5-5 minutes respectively) with 1X TBS-T on an orbital shaker.

4.5.5. Imaging and Data Analysis

Proteins were then visualised by incubating nitrocellulose membranes in chemiluminescence reagent. The chemiluminescent substrate was applied to the membrane according to the manufacturer's recommendation:

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 (In our university; BIORAD Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software, Universal Hood II, Serial No 721BR04545 is used).

4.6. Wound Healing Assay

4×10^5 cells were seeded into 6 well plate for each cell. After they reached high confluency, cells were washed with 1X PBS and the medium was replaced with a serum-free starvation medium for cell cycle arrest. After 24h incubation, cells were washed with 1X PBS and a scratch was made via a tip. Thereafter, cells were gently washed with 1X PBS again to clear cell debris, and then DMEM with serum was added into the wells. The cells were placed into the incubator to allow migration. Migration was checked by Leica inverted microscope at every 24 hours (Images were taken at 0h, 24h and 48 hour time-points). Four biological replicates were used. Results were quantified by ImageJ 1.x Software.

4.7. Total RNA isolation

Total RNA was isolated from drug- or mock-treated KO and WT cells using NucleoSpin RNA Purification Kit (Macherey Nagel, Germany) following manufacturer's instruction. Cells were typically harvested by removing media, appropriate amount of 1X PBS was added and aspirated. 0.05% Trypsin-Edta solution was added and incubated in 37 °C for 2 minutes. Detached cells were collected by adding complete DMEM. Cell suspension was softly transferred into microcentrifuge tubes, centrifuged at 5000 rpm for 3 minutes. Cells were lysed with 350µl of buffer LBP.

After mixing, the lysates were transferred to NucleoSpin gDNA Removal column (Yellow ring) and centrifuged for 30 seconds at 11000 x g. After adding 100 µl of RNA Binding solution (BS) and pipetting up and down, whole lysates were transferred to RNA Plus Column (light blue ring) and were centrifuged for 15 seconds at 11000 x g. Columns were washed by adding 200 µl of WB1; 600 µl WB2 (centrifuged at 11000 x g for 15 seconds) and finally 250 µl WB2. Columns were dried at 11000 x g centrifugation for 2 minutes, and then columns placed to new collection tubes. Total RNAs were eluted from columns via adding 30µl Rnase free water for 2 times at 11000 x g centrifugation for 1 minutes. Quality and quantity measurements of isolated total RNAs were analyzed using Qubit (Thermo Fisher Scientific, USA) and BioAnalyzer (Agilent, USA).

4.8. RNA-seq Library Preparation

Library preparation can be carried out either with the help of poly (A) selection or by the removal of rRNA technique. In this thesis, library preparation based on poly (A) selection to enrich for polyadenylated transcripts was carried out.

Basically, library preparation consists of following steps; Purification and fragmentation of mRNA, Single strand cDNA Synthesis, Double strand cDNA Synthesis, End repair, 3' Adenylation, Adapter Ligation, PCR Amplification and Quality Control of Libraries (Figure 4.2).

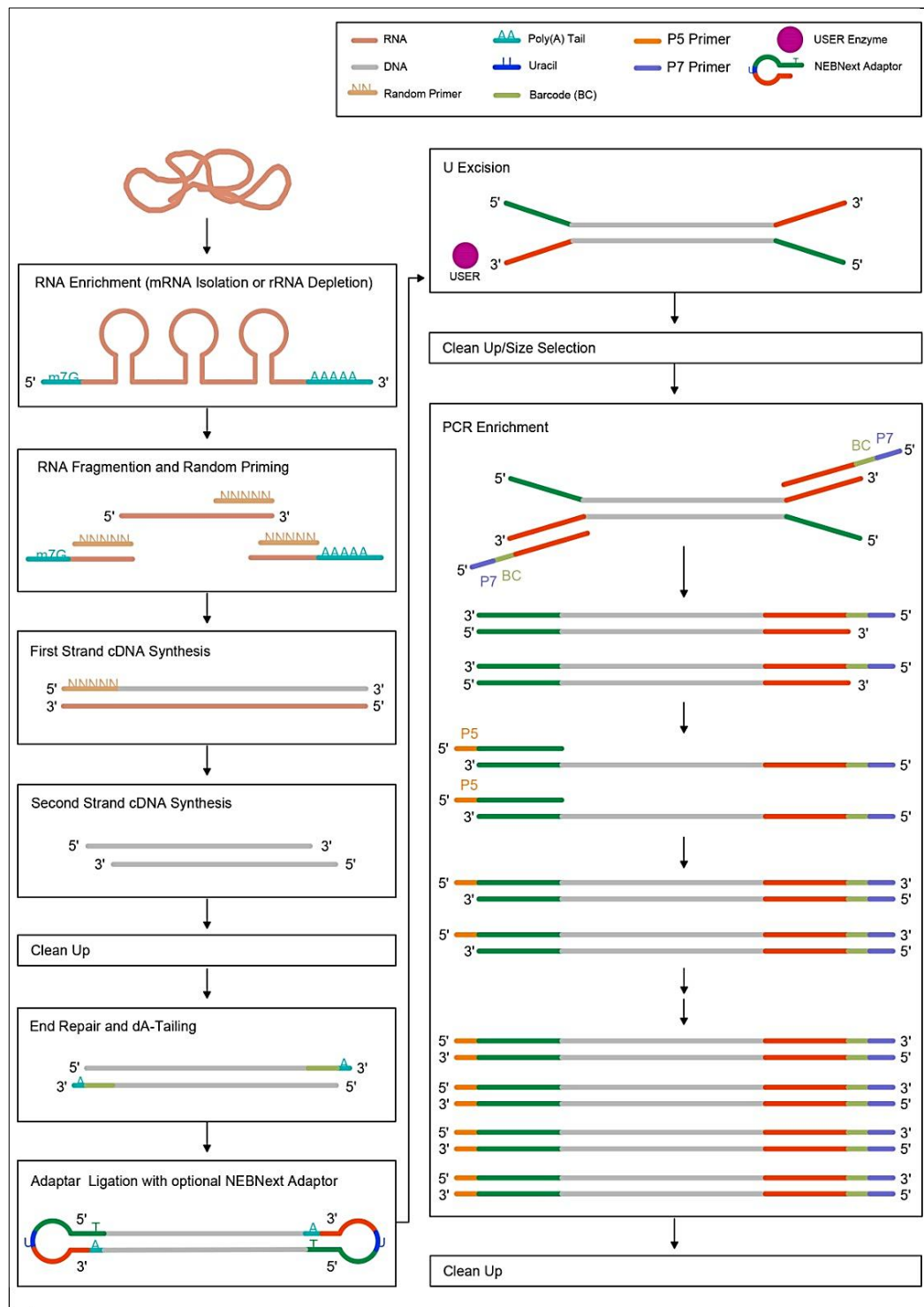


Figure 4.2: RNA-sequencing library preparation protocol. Library preparation starts with poly-A selection from 100 ng-1µg total RNA with magnetic beads. The double strand cDNA becomes ready for adaptor ligation by phosphorylation and addition of A-tail. PCR amplified library is ready for clustering and sequencing (NEBNext Ultra RNA Library preparation protocol for Illumina).

Total RNA was isolated from drug- or mock-treated KO and WT cells using NucleoSpin RNA Purification Kit (Macherey Nagel, Germany). Quality and quantity measurements of isolated total RNAs were analyzed using Qubit (Thermo Fisher Scientific, USA) and BioAnalyzer (Agilent, USA).

RNA samples which have OD_{260/280} value between 1.8 to 2.0, 28S:18S ≥ 1 ratio and RNA integrity number (RIN) ≥ 8 were used for library construction. For each sample, 2.0 μ g total RNA was incubated with Poly(A) mRNA Magnetic Isolation Module kit (New England Biolabs, USA) in order to select poly (A)-tailed RNA population and was then fragmented using the fragmentation buffer.

First strand cDNA synthesis was performed from the fragmented short RNA fragments using random hexamer primers. The second strand cDNA was synthesized with DNA polymerase I and RNase H. Double-stranded cDNAs were extracted by using AMPure XP beads (Beckman Coulter, USA). After elution with suspension buffer, the 3'-end adenine nucleotide was added. Lastly, sequencing adapters were connected to the fragments. The enrichment of cDNA fragments were performed via PCR amplification.

Before sequencing, enriched amplicons of approximately 260 bp were analyzed using the quality control 2100 BioAnalyzer (Agilent, Palo Alto, CA, USA), and electropherogram and gel images were evaluated.

The cDNA libraries for control (WT) and *BMAL1* knockout (KO) cells (which were treated with drugs) were carried out in two biological replicates using the Illumina HiSeq2500 sequencing platform (Illumina/USA) through a commercial sequencing service (Macrogen Europe B.V., Netherlands).

Illumina requires a number of steps before sequencing. In this process, which is called cluster or generation-bridge amplification, firstly the denatured library fragments were flowed into a flow cell and hybridized on the complementary Illumina adapter oligos. For complementary fragments, amplifying bridge amplification PCR was used. It was resulted in clusters of the same single-stranded library fragments. Then the prepared fragments sequenced using reversible terminator nucleotides. After laser stimulation and fluorescence detection, base pairs were identified. Finally raw data were achieved (Figure 4.3).

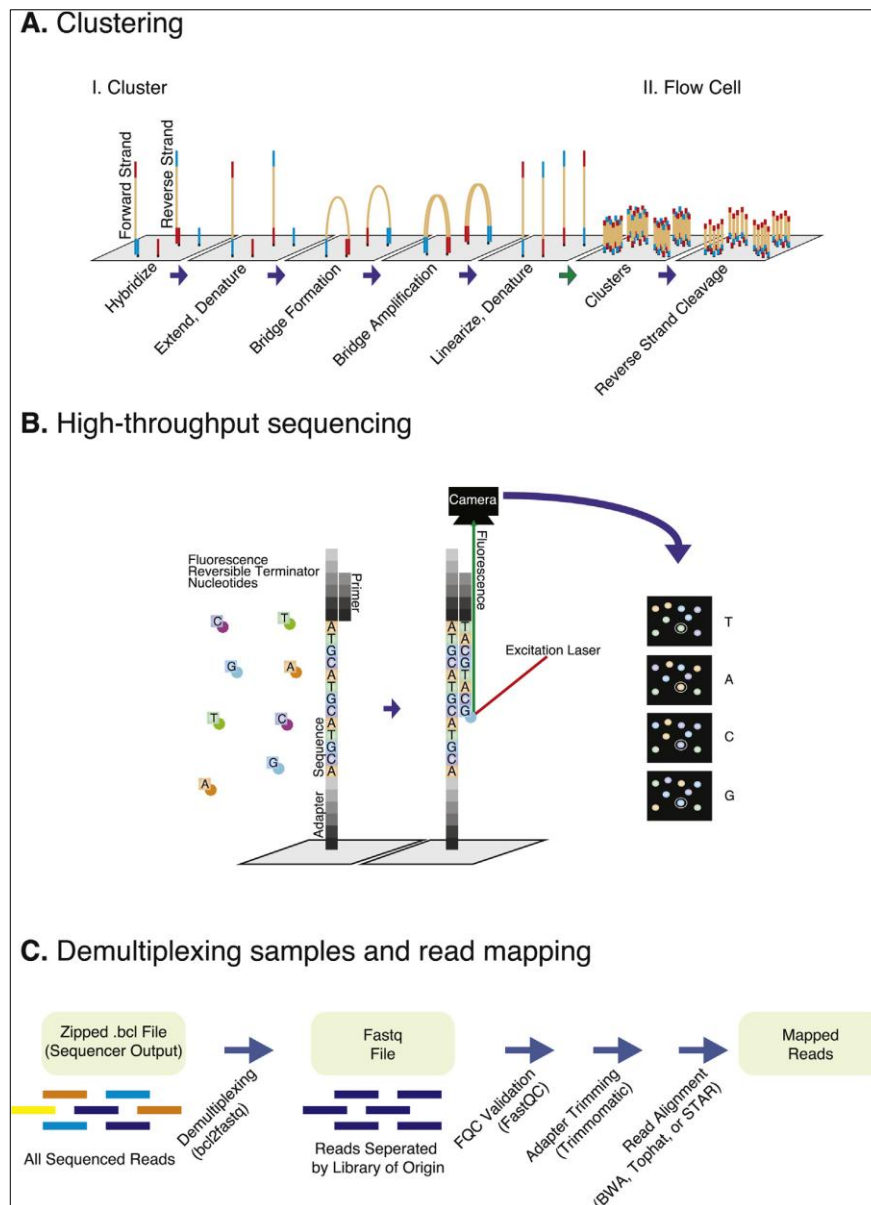


Figure 4.3: Illumina sequencing-data processing workflow [Chaitankar et al., 2016].

4.9. Transcriptome Analysis

The transcriptome analysis was performed by following pipelines that were published previously [Sarayloo et al., 2017]. Mainly, the fluorescent images were converted into sequences. Quality value calculations were performed using the FastQC program (Babraham Bioinformatics, UK). For cleaning and enhancing the quality of the reads, adaptor contaminations or low-quality bases (quality score < Q30) were removed using the program Trimmomatic (v0.35) [Bolger et al., 2014]. All cleaned sequence data (2×100 bp in length) were high quality, and these reads in fastq files were deposited in the NCBI Gene Expression Omnibus (GEO) database with the accession number GSE157069. The reads were aligned to the human reference genome GRCh38 using STAR aligner (v2.5.2) program [Dobin et al., 2013]. Transcript-level R2 values among replicates of each condition were determined by using correlation analysis of heatmap.2 function in R programming. Following the mapping procedure, Cuffdiff command in Cufflinks suite [Trapnell et al., 2012] with default parameters with false discovery rate (FDR) ≤ 0.01 was used to identify differentially expressed genes (DEGs) among different conditions. The differential expression analysis was performed by quartile library normalization, pooled dispersion estimation methods, and fragments per kilobases of exons per million mapped read (FPKM). Fold changes calculation has been made.

4.10. Identification of Differentially Expressed Genes

In this study we aimed to determine genes which were differentially expressed in response to drug treatment with *BMAL1* gene deletion in MDA-MB-231 cells (designated as “KO”). As a mock control, we used MDA-MB-231 cells infected with lentivirus particles which express a nontargeting sgRNA sequence (designated as “WT” in respect to the status of *BMAL1* gene) to eliminate any non-specific changes originating from the presence of active CRISPR/ Cas9 system in cells. As we mainly asked why the deletion of *BMAL1* gene sensitized cells to DNA-damaging drugs, each gene was assigned a DEG score as stated in the alteration they displayed upon (cisplatin, doxorubicin, or tunicamycin) drug treatment (TR) or vehicle control (VC)

treatment in comparison of the *BMAL1* knockout (KO) cells to the mocktransfected (WT) control cells, as shown in Equation (4.1):

$$DEG\ Score = \log 2 \left(\frac{\left(\frac{KO_TR}{KO_VC} \right)}{\left(\frac{WT_TR}{WT_VC} \right)} \right) \quad (4.1)$$

Tunicamycin was applied as a negative or another control treatment because it does not induce DNA damage but activates an ER stress response. Briefly, for each condition, e.g., drug treated WT cells versus vehicle or drug treated KO cells versus vehicle, DEGs were determined separately as stated above. Then, two lists of DEGs were merged, and FPKM readings of these genes were used in Equation (4.1) to determine DEGs when KO cells were treated with drugs. Significantly DEGs were identified which showed a total DEG Score more than 1, marking a change more than two fold.

4.11. Gene Set and KEGG Pathway Enrichment Analysis

To determine the enriched gene sets, we used the gene set enrichment analysis (GSEA) by providing DEG values calculated from equation as preranked input to GSEA program [Subramanian et al., 2005] with default parameters. Separate DEG lists were provided for each drug. Then, p values of significant gene sets (< 0.05) were given in legends where they are relevant.

UV_RESPONSE_DN and ESTROGEN_RESPONSE_EARLY (which are significant for cisplatin treatment) sets were made up of 144 and 200 genes in which 27 and 20 genes were regulated by cisplatin from 17 and 58 founder gene sets, respectively. INFLAMMATORY_RESPONSE (which was significant for doxorubicin treatment) set is made up of 130 genes in which 19 genes were regulated by doxorubicin from 1 founder gene set. The obtaining of the founder gene sets are performed through expression profiling by array [Subramanian et al., 2005].

ClueGo extension of Cytoscape (v.3.6.0) was used to find significantly altered KEGG pathways for each DEG lists (p value $\leq$ 0.05).

4.12. Protein-Protein Interaction Network Analysis

To construct protein-protein interaction (PPI) network for common DEGs in cisplatin- and doxorubicin-treated KO cells, a list of genes was submitted to STRING database to retrieve the experimentally determined PPI data. For a better network representation, a total of 15 ribosomal, proteasomal, or polymerase-related genes were excluded from the list due to their high degree of interactions with various proteins (Appendix A, Table A1.1). Finally, 318 genes (Appendix B, Table B1.1) were used to establish PPI network. We let STRING database to include a maximum of 50 additional interactors per protein just in the first shell. These interactors were identified from experimental data and have confidence score of 0.4 or higher. The PPI was analyzed using STRING interaction network [Jensen et al., 2009], [Szkarczyk et al., 2015]. In PPI networks, proteins were visualized as nodes, and protein-protein interactions were shown as edges. Proteins having high number of interactions are named as “hub” proteins. Hub proteins are considered to have critical regulatory roles in the network [Ekman et al., 2006]. Properties of PPI network were calculated by using Cytoscape (v. 3.6.0). Top 12 proteins having the highest betweenness centrality values were determined as the bottleneck proteins. Betweenness centrality is calculated as how frequently a node appears among all shortest paths connecting two nodes. Bottleneck proteins are located on the intersection of different paths and may regulate the communication between them [Yu et al., 2007]. Force Directed Layout algorithm in Cytoscape was used to visualize PPI network. Detached nodes were not shown.

4.13. Validation of RNA-seq Data with qPCR

18 up-regulated or down-regulated genes selected from RNA-seq data were analyzed by qPCR to validate RNA-seq analysis. cDNA was synthesized from total RNA with ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs (NEB)-USA) following manufacturer's instruction and qRT-PCR was performed via CFX Connect Real-Time PCR Detection System (BioRad, USA) using Luminaris HiGreen qRT-PCR Master Mix (Thermo Scientific, USA) with the gene specific

primers shown in Table 3.1. The *GAPDH* gene that its expression level did not vary between samples, was selected as internal reference gene. qPCR programme amplification had the following cycling protocol: 95 °C 10 seconds denaturation, 60 °C for 20 seconds annealing, and 72 °C for 30 seconds of elongation, 40 cycles. All reactions were performed with 2 biological replicates (each sample with two technical replicates), and the data were analysed according to *GAPDH* transcript level in each sample via the $2^{-\Delta\Delta CT}$ method. GraphPad Prism version 7 (GraphPad software, USA) was used for mRNA expression data analysing.

5. RESULTS

The disruption of the circadian clock by a gene deletion such as *BMAL1* gene would have wide-range effects on many different pathways. Because of this general regulatory outcome, it is expected that a connection occurs among the circadian clock and carcinogenesis, but it is too difficult to analyze this relationship by considering/analyzing pathways individually. Therefore, we decided to analyze the changes in the transcriptome in *BMAL1* knockout (KO) or wild-type (WT) MDA-MB-231 cells to reveal molecular mechanism of sensitization of knockout cells to DNA damage-induced apoptosis and further induction of invasion capacity of the cells at genomewide level by RNA sequencing (RNA seq). Moreover, we also decided to analyze another pathway not related to DNA damage response in our assays. For this aim, we analyzed the transcriptome of tunicamycin-treated *BMAL1* KO and WT cells in parallel.

5.1. Confirmation of Mutations by Western Blotting Analysis of MDA-MB-231 WT and KO cells

In this study; we were interested in the mechanism of this pleiotropic phenotype by exploring the changes at the transcriptome level. Since the introduction of CRISPR/Cas9 system into cells might have unprecedented effects on transcriptome, instead of using parental MDA-MB-231 cells as the control, we generated a more proper control by infecting MDA-MB-231 cells with lentiviral particles of LentiCRISPRv2.non-targeting sgRNA which served a mock control in our assays. *BMAL1* knockout mutant MDA-MB-231 cells was named as “KO,” while its mock control (with non-targeting sgRNA/Cas9) was named as “WT” in respect to the wild-type status of *BMAL1* gene throughout the text. We validated the *BMAL1* status of the cells by analyzing *BMAL1* protein level with immunoblotting applying specific anti-Bmal1 antibody against the C terminus of the protein at parental (+), *BMAL1* knockout (KO) and mock-infected (WT) MDA-MB-231 cells (Figure 5.1). We confirmed that single cell-derived clones represented the phenotype that was similar to our previous findings [Korkmaz et al., 2018].

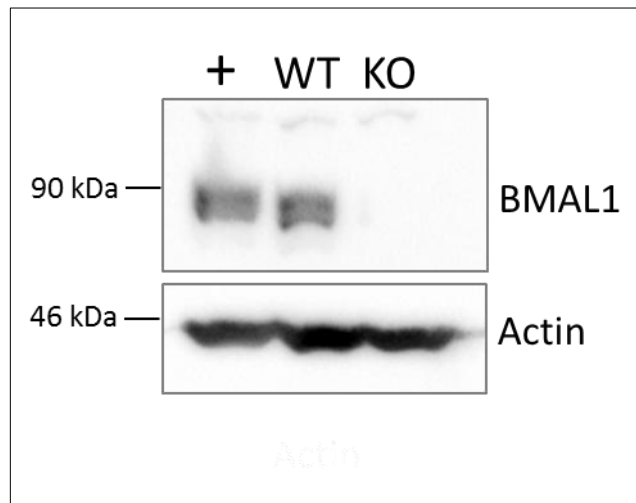


Figure 5.1: Confirmation of *BMAL1* gene knockout at MDA-MB-231 cells. The *BMAL1* protein level was detected in parental (+), *BMAL1* knockout (KO) and mock-infected (WT) MDA-MB-231 cells. Actin antibody was used as loading control for Bmal1 immunoblotting.

5.2. Effect of *BMAL1* Knockout Mutation on DNA Damage - induced Apoptosis at MDA-MB-231 WT and KO cells

We have previously found out that the deletion of *BMAL1* gene by CRISPR/Cas9 gene editing technique in MDA-MB-231 invasive breast cancer cells sensitized these cells to DNA damage induced apoptosis compared to parental cells. We wanted to confirm the sensitization of the *BMAL1* knockout MDA-MB-231 cells to DNA damage-induced apoptosis.

Apoptosis was analyzed by probing for cleaved PARP (which is a widely accepted marker for apoptosis), and was normalized to actin signals obtained by immunoblotting. KO cells had an increased apoptosis as probed for cleaved PARP after cisplatin and doxorubicin treatments (Figure 5.2).

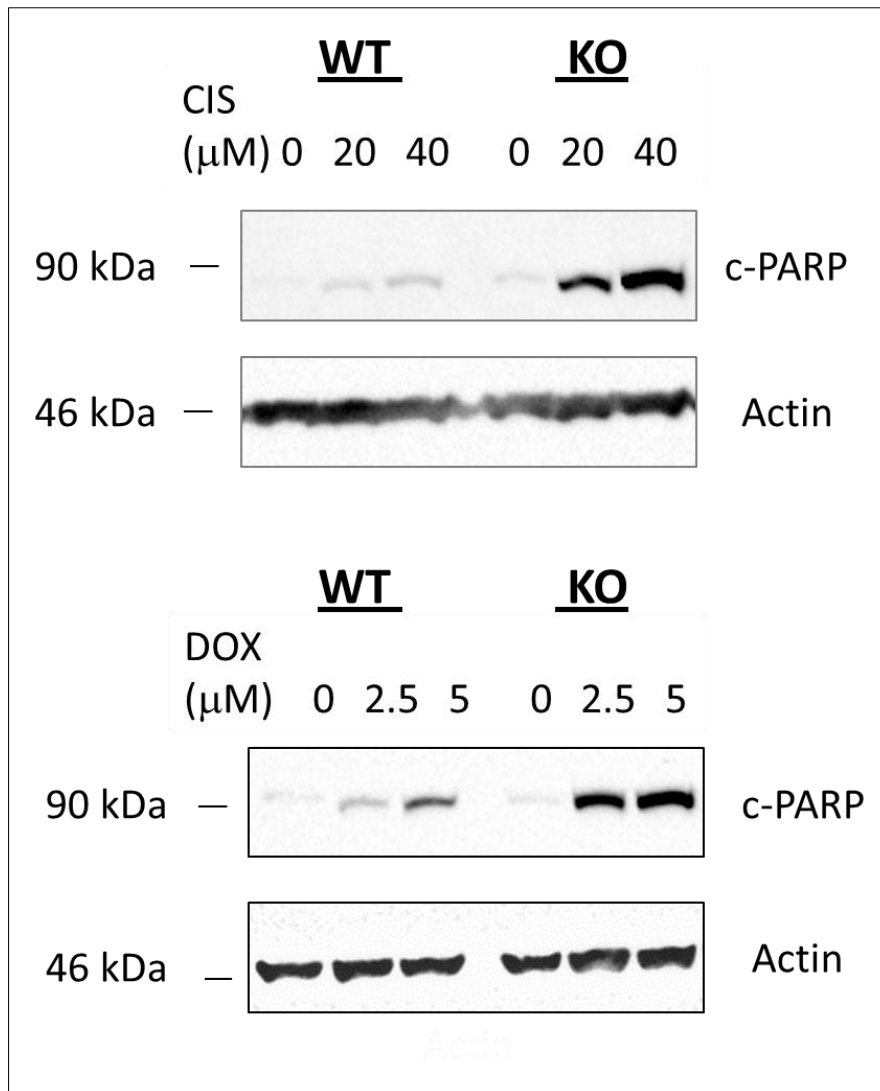


Figure 5.2: The MDA-MB-231 cells were treated with cisplatin (CIS) at doses 20-40 μ M and doxorubicin (DOX) at doses 2,5-5 μ M for 16 hours. Cleaved-PARP (c-PARP) was detected as the marker for apoptosis and actin was used as loading control. Numbers and marks on the left of each figure show the positions of the corresponding molecular size markers in kDa.

5.3. Effect of *BMAL1* Knockout Mutation on ER Stress Response at MDA-MB-231 WT and KO cells

We wanted to analyze another pathway irrelevant to the sensitization of KO cells to apoptosis after DNA damage. For this purpose, we selected ER stress response pathway and treated the cells with tunicamycin as an ER stress inducer. Both WT and KO cells had a similar formation of spliced XBP1, which is an indicator of ER stress response (Figure 5.3).

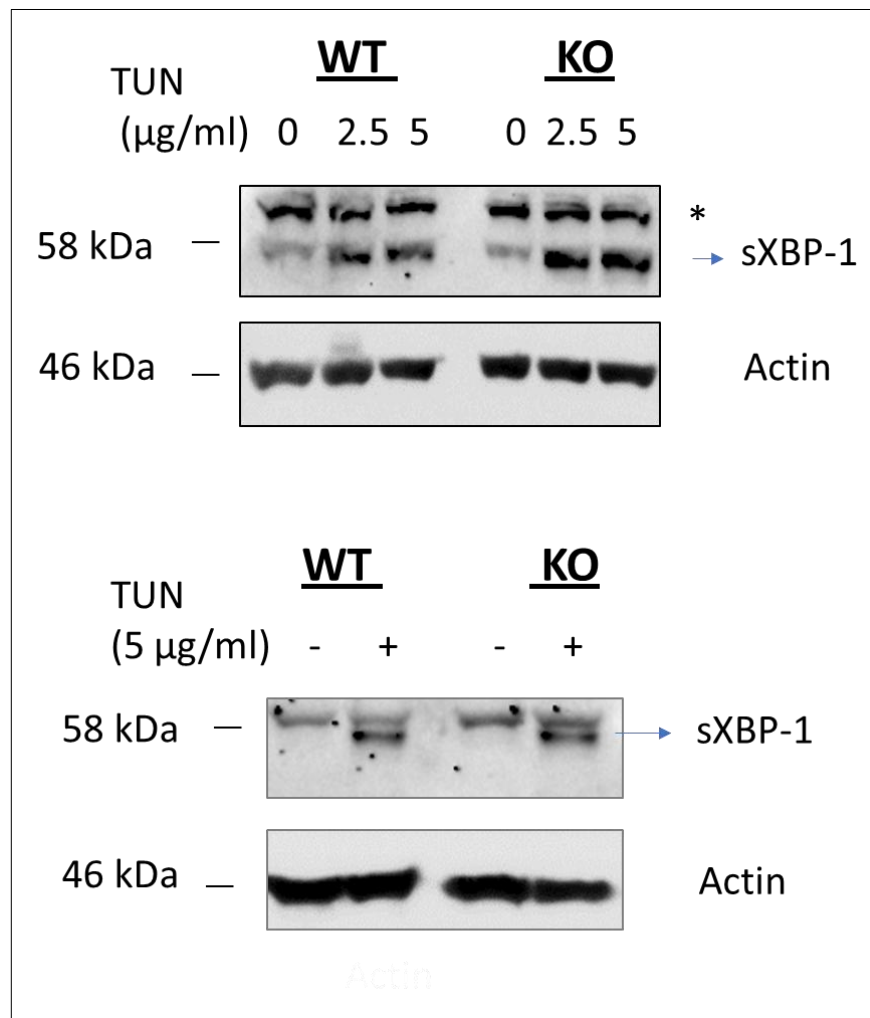


Figure 5.3: Effect of *BMAL1* mutation on ER stress response induced by tunicamycin at MDA-MB-231 cells. The cells were treated with tunicamycin (TUN) at doses from 2.5 and 5 µg/ml, in cell culture media for 4 hours. Spliced (active) form of XBP1 (sXBP1) was detected as the output marker for tunicamycin-induced ER-stress response. Since sXBP1 signal and a cross-reacting band were merged, we run the samples for a longer period to confirm that sXBP1 signal was not much different between WT and KO cells.

5.4. Effect of *BMAL1* Knockout Mutation on Migration Rates of MDA-MB-231 WT and KO Cells

In our previous study, we found while the *BMAL1* deletion made the cells sensitive to chemotherapeutic agents, it also increased invasion capacity of the cells [Korkmaz et al., 2018]. To confirm KO cells had increased motility, movements of WT and KO cell populations were analysed by wound-healing assay. An increase in the cellular motility was confirmed by wound-healing assay (Figure 5.4).

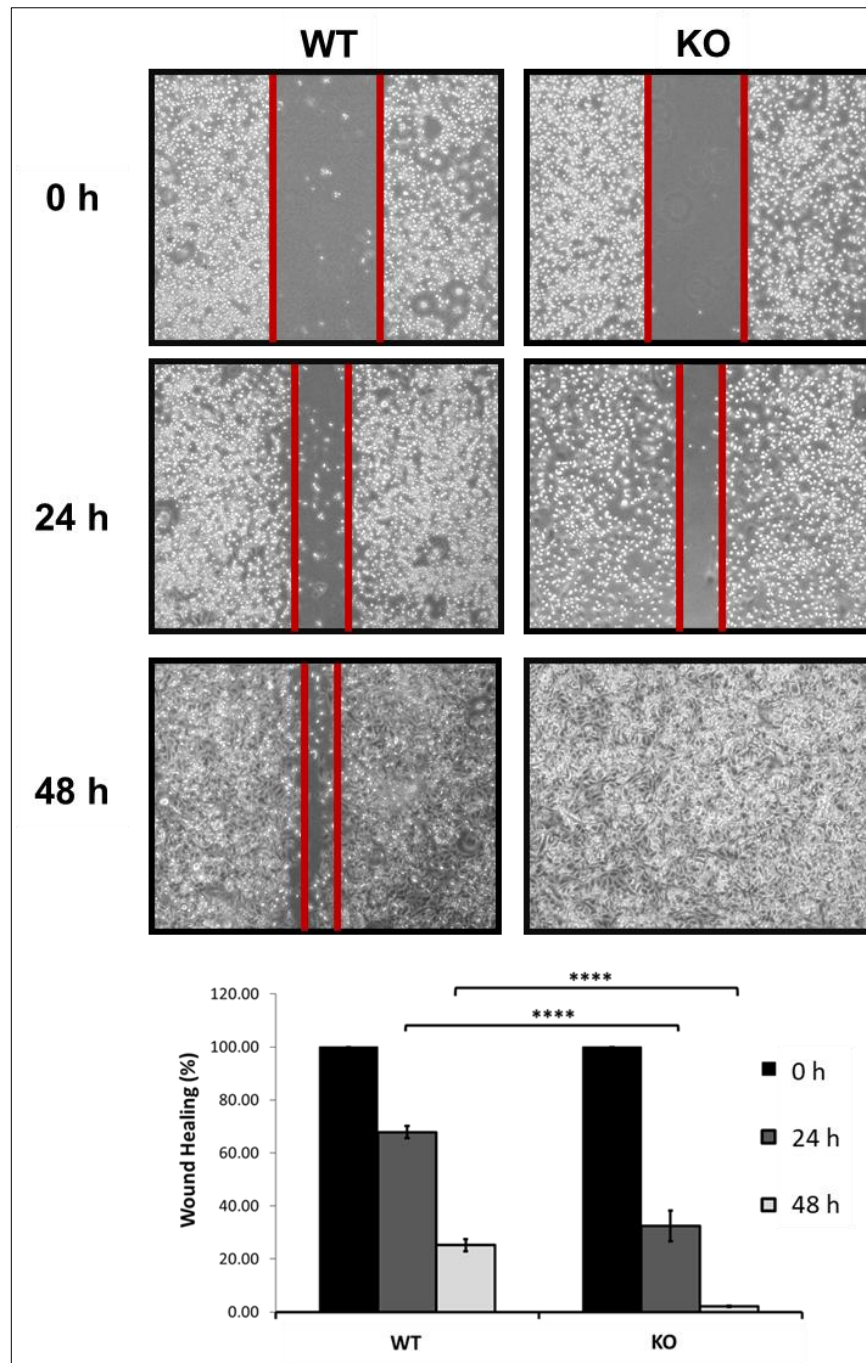


Figure 5.4: Effect of *BMAL1* mutation on Migration Rates of MDA-MB-231 WT and KO cells. KO cells were used as a positive control. Representative images show the scratch (wound) at t=0 h, t=24 h and t=48 h. Marks (red lines) was placed to locate the open area on the scratch. Images were taken by using Leica inverted microscope. Magnification 5X. The graph shows the percentage of wound healing in WT, and KO cells under starvation condition. The open area was calculated at the indicated time points by ImageJ Software (Free Accessible Image Processing Program, www.imagej.net). All results are representative of four independent experiments. Statistical Analysis was done with 24 h and 48 h data by one-way ANOVA followed by Tukey's post hoc tests for pairwise comparison. (****P< 0.0001).

5.5. RNA Extraction and RNA-seq Library Preparation

Total RNA was isolated from drug- or mock-treated KO and WT cells. In order to control any degradation and genomic DNA contamination, RNA samples were analyzed in 1% agarose gel.

The successful performance of transcriptome sequencing analysis is greatly relying on the quality and quantity of total RNA. Therefore, the quantity and quality of the RNA samples were evaluated using the Qubit® 2.0 spectrophotometer. Total RNA amount and also RIN (The RNA integrity number) values showing RNA integrity were determined (Table 5.1).

Table 5.1. RNA quantity and purity measurements of drugs treated WT and KO cells.

Sample	RIN value	260/280 ratio	Concentration (ng/μl)
WT_VC_Rep1	8,56	2,07	733
WT_VC_Rep2	8,49	2,09	565
KO_VC_Rep1	8,24	2,08	1057
KO_VC_Rep2	8,25	2,07	477
WT_CIS-20_Rep1	8,26	2,1	573,1
WT_CIS-20_Rep2	8,45	2,08	464
KO_CIS-20_Rep1	8,16	2,09	658
KO_CIS-20_Rep2	8,69	2,09	747
WT_DOX-2.5_Rep1	8,54	2,08	452
WT_DOX-2.5_Rep2	8,17	2,09	275
KO_DOX-2.5_Rep1	8,36	2,1	514
KO_DOX-2.5_Rep2	8,55	2,08	410
WT_TUN-2.5_Rep1	8,46	2,08	630
WT_TUN-2.5_Rep2	8,29	2,08	443
KO_TUN-2.5_Rep1	8,14	2,1	807
KO_TUN-2.5_Rep2	8,25	2,07	716

Quality and quantity controls of samples for sequencing after library preparation were evaluated using 2100 BioAnalyzer (Agilent, Palo Alto, CA, USA). As stated in the Tru-seq. RNA sample preparation protocol, the size of the samples was between 200-400 bp, and it was confirmed by both electropherogram (Figure 5.5) and agarose gel electrophoresis. Subsequently, the samples were sequenced using the Illumina Truseq platform.

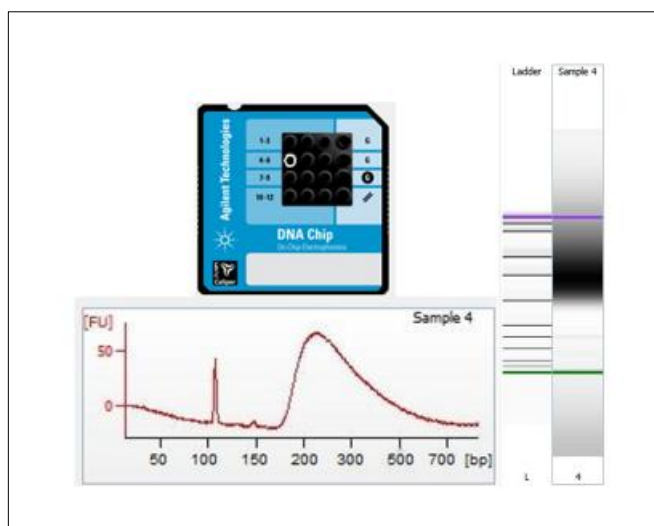


Figure 5.5: Electropherogram and gel images showing the quality and quantity controls of RNA libraries obtained with the Bioanalyzer.

5.6. Mapping Reads to Reference Genome

The cells were exposed to cisplatin, doxorubicin, and tunicamycin (all indicated as treatment, TR) or solvents as vehicle controls (designated as VC), and total RNA was isolated, sequenced and demultiplexed. STAR Aligner was used for mapping to the human reference genome GRCm38. Results showed that the reads were uniquely mapped to gene loci. Read numbers of each replicates from WT and KO cells that were treated with indicated agents or solvents are shown (Table 5.2).

Table 5.2. Read numbers of each replicates from WT and KO cells treated with indicated agents.

WT									
		VC_1	VC_2	CIS_1	CIS_2	DOX_1	DOX_2	TUN_1	TUN_2
Number	Uniquely Mapped	8.88E+06	9.16E+06	2.03E+07	2.04E+07	1.71E+07	1.78E+07	1.59E+07	1.68E+07
of Reads	Unmapped	2.92E+05	3.06E+05	6.03E+06	6.07E+06	5.07E+06	5.27E+06	4.09E+06	4.32E+06
KO									
		VC_1	VC_2	CIS_1	CIS_2	DOX_1	DOX_2	TUN_1	TUN_2
Number	Uniquely Mapped	1.29E+07	1.31E+07	1.65E+07	1.64E+07	1.56E+07	1.55E+07	1.79E+07	1.67E+07
of Reads	Unmapped	3.35E+06	3.39E+06	4.98E+06	4.94E+06	4.89E+06	4.85E+06	4.84E+06	4.53E+06

5.7. Correlation Matrix of Reads from Biological Replicates

Correlation matrix of reads from biological replicates of each sample with Pearson's correlation tests indicated that the replicates have $R^2 > 0.98$ (Figure 5.6).

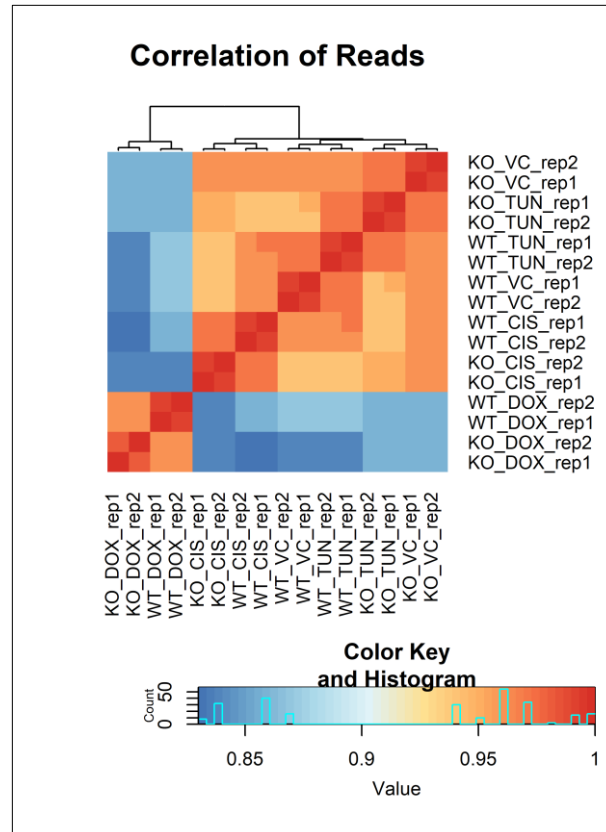


Figure 5.6: Correlation matrix of reads from biological replicates of each sample.

5.8. Identification of Differentially Expressed Genes in KO Cells Compared to WT Cells in Response to Treatments

Transcripts with an FPKM reading of 0 in one of the 8 samples were discarded as they caused splitting errors. All of these quality control analyzes gave the opportunity to further examination of the data to determine differentially expressed genes (DEGs). DEGs were determined by selecting genes with at least twofold change (Figure 5.7 (a)). Only data from readings that specifically mapped to reference genome were used for all analyzes performed. All multiple mappers were

eliminated. Differential expression analysis was performed using Cuffdiff command in the Cufflinks suit [Trapnell et al., 2012], [Tardu et al., 2016], [Tardu et al., 2017]. To calculate the fold change in the number of mapped fragments per kilobase of exon per million mapped reads (FPKM) among drug/treated and mock/treated samples Cuffdiff were used. Procedure of selecting differentially expression gene and formulation of upregulated and down-regulated genes after treatments was shown in Figure 5.7 (a). Analysis of the DEG results showed that 4295, 8704, 2832, 4734, 8959, and 2558 genes were differentially regulated in response to cisplatin (CIS), doxorubicin (DOX), and tunicamycin (TUN) in WT and KO cells, respectively (Figure 5.7 (a, b and c)). DEG analysis resulted in a total of 60,658 transcripts. To determine the genes which were differentially expressed after cisplatin or doxorubicin treatment with *BMAL1* deleted platform, affected genes of interest were determined by comparison of changes in FPKM value for each transcript in KO cells treated with drugs (KO_TR) versus mock-treated (KO_VC) to the change in WT cells treated with drugs (WT_TR) versus mock-treated (WT_VC). A gene was assessed significant for changed drug reply in KO versus WT cells only when it indicated more than twofold change. In this case, the absolute value of the DEG score in Equation (4.1) is greater than 1 (Figure 5.7 (a)). This analysis generated in a total of 1137, 1531, and 461 transcripts for cisplatin, doxorubicin, and tunicamycin, respectively (Figures 5.7 (a-c)). Genes scoring above 1 were considered.

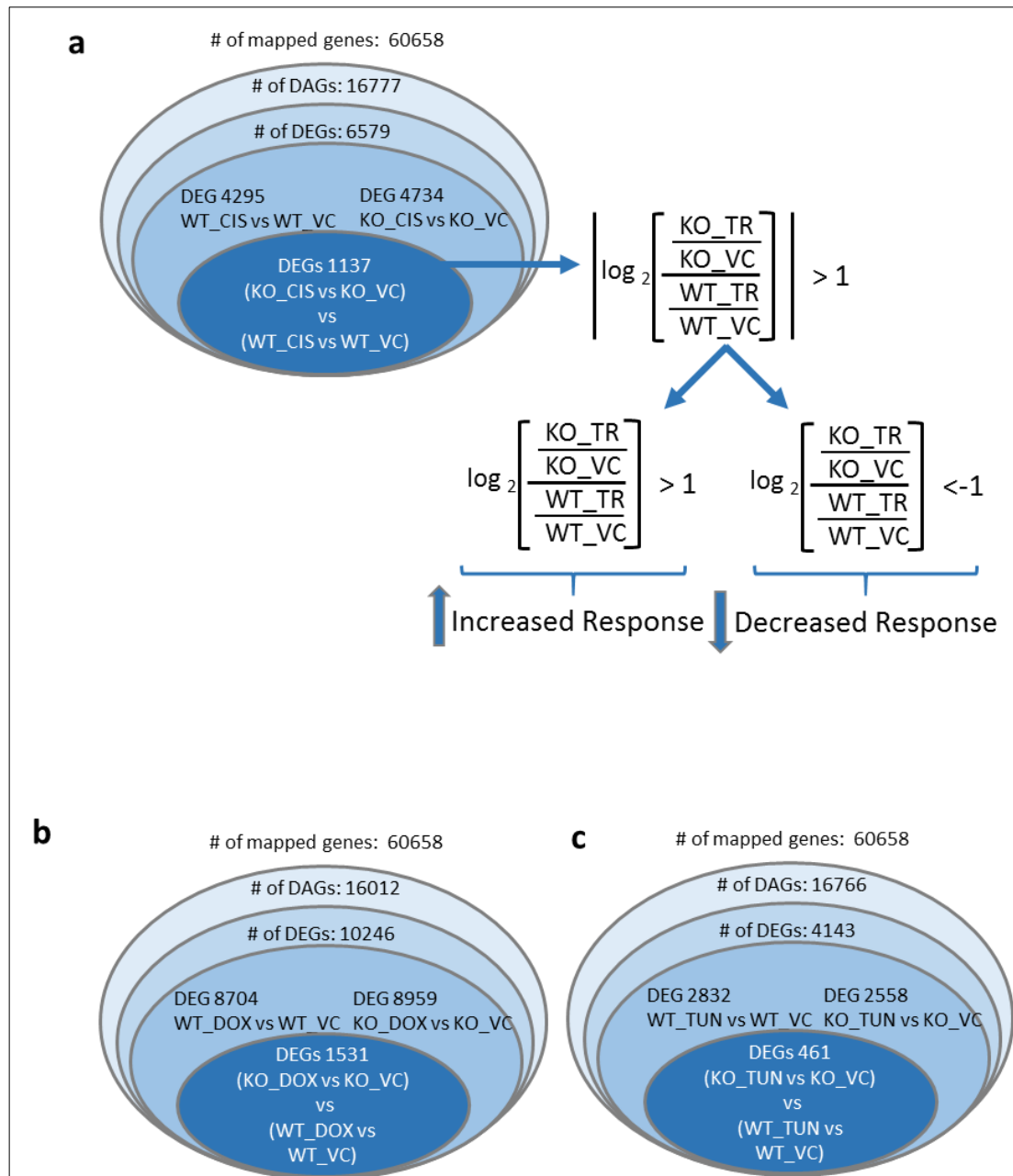


Figure 5.7: Analysis of methodology of the significantly differentially expressed genes. (a) each gene was specified a DEG score based on the change in the change they displayed after treatment in comparison of KO cells to WT cells as shown in the equation where a twofold change was set as cut off to select differentially expressed genes when KO cells treated with (a) CIS, (b) DOX, or (c) TUN. To evaluate the ratio, readings of KO_TR vs KO_VC to WT_TR vs WT_VC were calculated as shown in the formula. CIS, cisplatin; DOX, doxorubicin; TUN, tunicamycin; DAG, differentially analyzable genes; DEG, differentially expressed gene.

Among differentially regulated genes in response to cisplatin (CIS), doxorubicin (DOX), and tunicamycin (TUN) in WT and KO cells 2332, 4425, and 1565 DEGs were up-regulated, and 1963, 4279, and 1267 DEGs were down-regulated in WT cells, while 2269, 4537, and 1558 DEGs were up-regulated, and 2465, 4422, and 1000 DEGs were down-regulated in KO cells (Figure 5.8a.). Differentially analyzable genes were shown as up- or down-regulation or no change in response to treatments (Figure 5.8 (a)). Among DEGs obtained from Equation (4.1), 298, 600, and 226 genes were up-regulated, and 839, 931, and 177 genes were down-regulated as a result of cisplatin, doxorubicin, and tunicamycin treatment, respectively (Figure 5.8 (b)). The status of the up-regulated, down-regulated, or unchanged DEGs were individually plotted for each group (Figure 5.8 (c)).

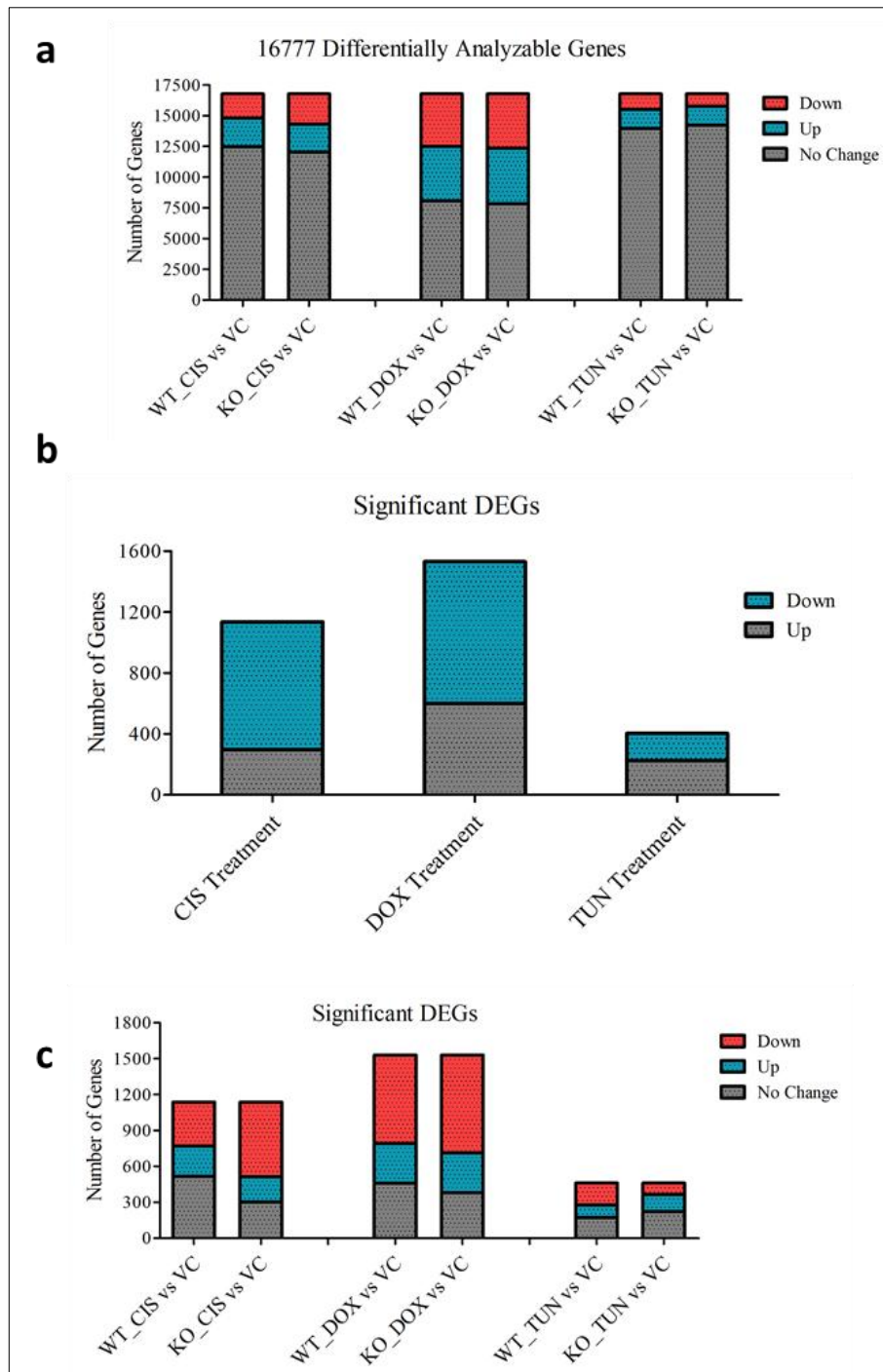


Figure 5.8: Analysis of number of differentially analyzable genes and differentially expressed genes. (a) Regulation of a total number of analyzable genes after WT or KO cells were treated with CIS, DOX, or TUN. (b) Total number of differentially expressed genes and (c) their regulation after cells were treated with CIS, DOX, or TUN. CIS, cisplatin; DOX, doxorubicin; TUN, tunicamycin.

5.9. Classification of Differentially Expressed Genes

To gain insight of the type of DEGs after treatments, all genes were classified (Figure 5.9). In all treatments, most of the DEGs were protein-encoding ones (954, 1246, and 332 genes for cisplatin, doxorubicin, and tunicamycin, respectively). In the second order, 75 long non-coding RNAs (lncRNAs), 44 processed pseudogenes, and 25 transcribed unprocessed pseudogenes populated DEGs for cisplatin treatment. On the other hand, 114 lncRNAs, 103 processed pseudogenes, and 24 transcribed unprocessed pseudogenes populated DEGs for doxorubicin. In summary, protein encoding, lncRNA, and processed or unprocessed pseudogenes commonly populated DEGs in both cisplatin and doxorubicin treatments. Tunicamycin treatment results in DEGs which are classified as 332 protein-encoding RNAs, 47 lncRNAs, 30 processed pseudogenes, and 16 unprocessed pseudogenes.

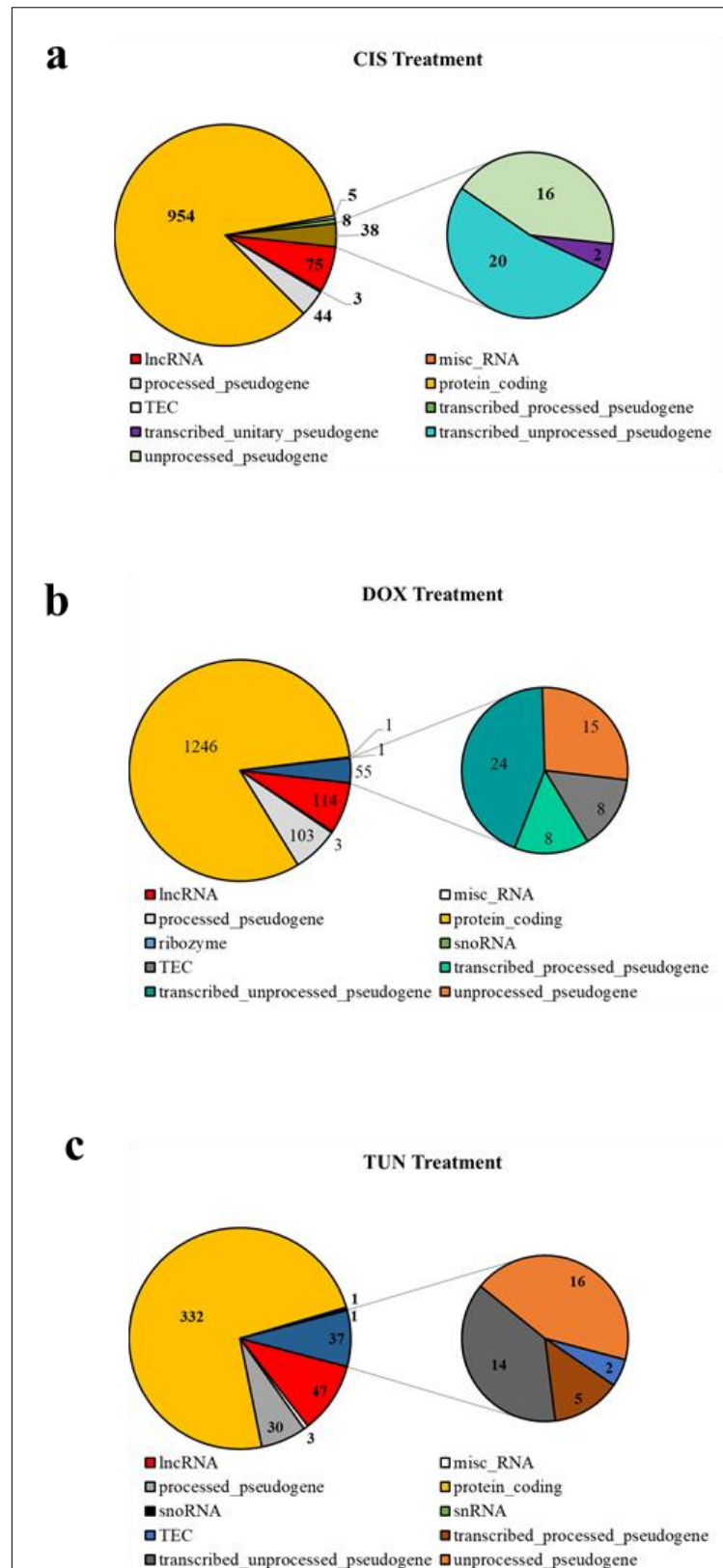


Figure 5.9: The classification of differentially expressed genes. DEGs were classified according to the nature of the genes for (a) CIS, (b) DOX, or (c) TUN treatment conditions. CIS, cisplatin; DOX, doxorubicin; TUN, tunicamycin.

5.10. Correction of Zero Division Errors Allows Gain of Information on Non-coding Genes

Genes having 0 FPKM values might be important if they are detectable in any conditions such as after drug treatments. Therefore, genes with a 0 FPKM value were included to calculation by adding 0.001 values to each FPKM values [Rapaport et al., 2013]. Importantly, the majority of these “zero cases” are processed pseudogenes and protein coding genes, and some of these changes are at quite significant levels. The list of genes having 0 FPKM values were shown in Table 5.3, 5.4, 5.5 for cisplatin, doxorubicin, and tunicamycin treatments, respectively.

Table 5.3. The list of genes having 0 FPKM values for cisplatin treatment.

Gene ID	Gene	WT_VC	WT_CIS	KO_VC	KO_CIS	DEG Score
ENSG00000022556	NLRP2	0,001	0,009	2,605	1,332	-4,071
ENSG00000080224	EPHA6	1,192	0,280	0,350	0,001	-6,362
ENSG00000101188	NTSR1	0,001	0,054	6,561	0,771	-8,857
ENSG00000101463	SYNDIG1	0,075	0,001	0,943	0,088	2,807
ENSG00000105784	RUNDC3B	1,499	0,131	0,090	0,001	-2,974
ENSG00000111452	ADGRD1	1,612	0,299	0,016	0,001	-1,584
ENSG00000117069	ST6GALNAC5	1,962	5,350	0,112	0,001	-8,250
ENSG00000129682	FGF13	15,294	2,670	0,269	0,001	-5,553
ENSG00000130600	H19	0,675	2,953	0,001	0,001	-2,128
ENSG00000134716	CYP2J2	1,426	2,308	0,001	0,082	5,654
ENSG00000137757	CASP5	0,001	0,808	0,766	2,939	-7,717
ENSG00000138639	ARHGAP24	2,088	0,300	0,505	0,001	-6,184
ENSG00000138696	BMPRI1B	0,799	0,080	0,306	0,001	-4,937
ENSG00000139173	TMEM117	1,382	0,185	0,528	0,001	-6,147
ENSG00000139364	TMEM132B	0,011	0,001	2,769	0,075	-1,736
ENSG00000139970	RTN1	2,017	0,606	0,089	0,001	-4,737
ENSG00000141404	GNAL	1,880	0,217	0,072	0,001	-3,045
ENSG00000142606	MMEL1	0,001	0,363	0,001	1,827	2,332
ENSG00000145526	CDH18	0,001	0,001	11,851	2,974	-1,995

Table 5.3: Continued

ENSG00000148219	ASTN2	2,625	0,066	1,940	0,001	-5,609
ENSG00000153930	ANKFN1	0,989	0,189	0,164	0,001	-4,967
ENSG00000155974	GRIP1	2,963	0,083	0,303	0,001	-3,086
ENSG00000163395	IGFN1	0,001	0,076	4,877	17,093	-4,432
ENSG00000164283	ESM1	0,907	4,759	0,001	0,001	-2,391
ENSG00000165072	MAMDC2	1,364	0,188	0,120	0,001	-4,054
ENSG00000167676	PLIN4	0,010	0,001	1,171	0,310	1,418
ENSG00000174827	PDZK1	0,001	0,044	1,156	0,244	-7,719
ENSG00000175445	LPL	1,065	2,808	0,001	0,017	2,685
ENSG00000176343	RPL37AP8	0,001	1,569	0,340	1,407	-8,564
ENSG00000178081	ULK4P3	0,001	0,830	0,102	0,679	-6,962
ENSG00000180287	PLD5	1,518	0,398	0,001	0,026	6,604
ENSG00000181126	HLA-V	0,001	1,690	1,951	1,599	-11,010
ENSG00000184613	NELL2	2,687	0,792	0,101	0,001	-4,889
ENSG00000184795	UNC93B5	0,001	1,965	1,982	2,864	-10,408
ENSG00000185710	SMG1P4	0,001	1,040	0,899	1,057	-9,789
ENSG00000197826	C4orf22	1,541	0,181	0,806	0,001	-6,567
ENSG00000198203	SULT1C2	0,001	0,100	2,614	0,780	-8,386
ENSG00000200795	RNU4-1	0,001	37,683	15,519	13,960	-15,354
ENSG00000204379	XAGE1A	0,001	0,812	0,522	0,770	-9,103
ENSG00000205002	AARD	2,669	4,184	0,001	0,109	6,125
ENSG00000205420	KRT6A	0,001	11,232	7,222	7,597	-13,382
ENSG00000213253	RPL12P42	0,001	1,512	0,601	1,367	-9,377
ENSG00000213343	RPL21P18	0,001	1,219	2,429	2,078	-10,477
ENSG00000213942	RP1-102E24.1	0,001	0,780	0,753	0,791	-9,535
ENSG00000214222	TUBBP2	0,001	1,447	1,141	1,144	-10,495
ENSG00000214263	RPSAP53	0,001	0,986	0,823	0,750	-10,078
ENSG00000214541	RPS4XP3	0,001	0,767	2,102	1,137	-10,469
ENSG00000214914	RPL23AP3	0,001	1,976	2,126	2,345	-10,807
ENSG00000215016	RPL24P7	0,001	0,830	0,282	1,282	-7,513

Table 5.3: Continued

ENSG00000215326	GPX1P2	0,001	0,881	0,645	1,109	-9,002
ENSG00000217026	RPL10P1	0,001	1,017	0,085	0,302	-8,162
ENSG00000221676	RNU6ATAC	0,001	31,355	4,690	37,783	-11,926
ENSG00000223638	RFPL4A	0,001	0,001	0,472	1,372	1,539
ENSG00000225082	DAP3P1	0,001	4,261	2,975	2,767	-12,162
ENSG00000225499	RPL15P4	0,001	0,937	0,491	1,384	-8,377
ENSG00000225953	SATB2-AS1	0,001	0,917	0,500	1,414	-8,341
ENSG00000226439	RP11-621K7.1	0,001	1,601	0,844	0,949	-10,474
ENSG00000226499	RP5-882O7.1	0,001	4,936	7,230	5,916	-12,559
ENSG00000227359	AC017074.2	0,001	0,856	1,534	1,448	-9,825
ENSG00000227617	CERS6-AS1	1,894	0,001	0,001	0,001	10,887
ENSG00000227850	SEPT2P1	0,001	1,152	2,002	0,001	-21,137
ENSG00000228015	AC074338.5	0,001	15,131	20,080	24,990	-13,570
ENSG00000228078	HLA-U	0,001	0,001	1,820	0,001	-10,829
ENSG00000230330	HMG2P3	0,001	1,003	0,347	0,480	-9,504
ENSG00000230929	RP11-395C3.1	1,655	1,820	0,001	2,364	11,070
ENSG00000231064	RP11-263K19.4	0,001	0,282	0,001	0,844	1,581
ENSG00000231369	RP1-40G4P.1	0,001	0,941	0,396	0,557	-9,388
ENSG00000231684	EIF1P3	0,001	0,957	0,230	0,326	-9,404
ENSG00000233868	AC009302.2	0,001	0,212	0,001	0,748	1,820
ENSG00000234513	AC073072.7	0,001	1,268	0,434	0,303	-10,829
ENSG00000234589	RP11-253E3.1	0,001	2,648	0,993	2,562	-10,003
ENSG00000235028	HMG2P3	0,001	1,256	0,001	0,499	-1,332
ENSG00000236453	AC003092.1	0,591	0,656	0,001	1,237	10,123
ENSG00000236468	RP11-104D3.2	0,001	0,507	1,048	0,475	-10,128
ENSG00000236574	RP11-340I6.4	0,001	0,472	1,653	0,001	-19,575
ENSG00000237732	AC010980.2	0,379	1,262	0,001	0,675	7,664
ENSG00000238151	MLLT10P1	0,001	0,219	0,752	0,001	-17,329
ENSG00000239719	RP4-800G7.1	0,001	1,695	6,911	0,001	-23,482
ENSG00000241431	RPL37P6	0,001	4,746	2,244	4,439	-11,228

Table 5.3: Continued

ENSG00000241746	RP11-941H19.1	0,001	1,313	1,757	1,307	-10,786
ENSG00000241877	RP11-84H6.1	0,001	1,025	0,852	0,472	-10,853
ENSG00000242692	RPS27AP1	0,001	0,864	0,659	0,280	-10,989
ENSG00000243181	RP11-734J24.1	0,001	0,851	0,782	1,194	-9,122
ENSG00000243988	RPS24P17	0,001	0,924	0,621	1,094	-9,035
ENSG00000244004	RP11-1396O13.2	0,001	1,195	0,861	1,508	-9,414
ENSG00000245112	SMARCA5-AS1	0,001	6,446	1,879	4,614	-11,359
ENSG00000249799	SNRPCP13	0,001	4,389	2,564	0,584	-14,233
ENSG00000249908	BRD9P2	0,001	10,750	8,815	9,950	-13,217
ENSG00000250081	CTD-2116N20.1	0,338	0,236	0,001	0,973	10,444
ENSG00000250268	ALG1L14P	0,001	3,017	0,895	1,367	-10,947
ENSG00000250342	SNRPCP16	0,001	3,691	2,567	0,442	-14,388
ENSG00000251087	ALG1L3P	0,001	1,103	0,341	0,724	-9,020
ENSG00000251313	RP11-747H12.5	0,001	1,193	0,926	1,646	-9,390
ENSG00000251521	IMPA1P	0,001	1,065	0,678	0,821	-9,780
ENSG00000257390	RP11-762I7.5	0,115	0,724	0,001	0,813	7,011
ENSG00000257635	RP11-536C10.10	0,001	1,094	0,558	0,895	-9,413
ENSG00000257956	NOP56P3	0,001	1,391	1,625	0,890	-11,310
ENSG00000259112	NDUFC2-KCTD14	0,001	6,114	1,393	4,364	-10,931
ENSG00000259976	RP11-553L6.5	0,774	0,001	0,325	0,040	6,559
ENSG00000260104	RP11-817O13.1	0,001	4,326	2,185	2,971	-11,635
ENSG00000264940	SNORD3C	0,001	3,689	0,689	1,715	-10,533
ENSG00000265185	SNORD3B-1	0,001	15,940	7,040	17,855	-12,618
ENSG00000270945	HSPE1P7	0,001	1,176	3,231	0,349	-13,410
ENSG00000273812	WI2-87327B8.2	0,001	0,107	7,229	2,065	-8,551
ENSG00000273821	RP5-963E22.6	0,214	0,564	0,001	0,765	8,182
ENSG00000277367	RP11-536C10.26	0,001	1,093	0,558	0,894	-9,412
ENSG00000277631	PGM5P3-AS1	4,894	1,312	0,374	0,001	-6,646
ENSG00000277672	RP11-386G11.11	0,001	7,598	5,659	10,215	-12,039
ENSG00000277782	RP11-775C24.5	1,209	0,001	0,532	0,375	9,736

Table 5.3: Continued

ENSG00000278249	SCARNA2	0,523	1,020	0,001	2,317	10,213
ENSG00000278588	HIST1H2BI	0,001	0,762	0,369	0,389	-9,497
ENSG00000279115	AC006386.1	0,001	1,279	2,477	2,217	-10,480
ENSG00000280744	LINC01173	0,001	0,140	1,408	0,001	-17,593

Table 5.4. The list of genes having 0 FPKM values for doxorubicin treatment.

Gene ID	Gene Name	WT_VC	WT_DOX	KO_VC	KO_DOX	DEG Score
ENSG00000007933	FMO3	1,330	3,475	0,001	0,001	-1,386
ENSG000000091128	LAMB4	1,580	0,525	0,001	0,001	1,591
ENSG00000101188	NTSR1	0,001	0,024	4,188	1,305	-6,275
ENSG00000101331	CCM2L	0,048	0,381	0,001	0,789	6,647
ENSG00000102287	GABRE	0,008	0,001	0,407	1,812	5,063
ENSG00000103154	NECAB2	0,001	0,207	0,251	1,218	-5,413
ENSG00000104808	DHDH	0,001	0,790	0,057	0,624	-6,161
ENSG00000104901	DKKL1	0,001	0,345	0,001	1,189	1,785
ENSG00000104903	LYL1	1,168	0,029	1,238	0,001	-4,928
ENSG00000105122	RASAL3	0,001	0,190	0,001	1,428	2,908
ENSG00000105550	FGF21	0,001	0,104	0,001	0,889	3,092
ENSG00000105708	ZNF14	1,321	0,029	0,697	0,001	-3,919
ENSG00000106689	LHX2	0,001	0,104	0,001	0,778	2,908
ENSG00000109819	PPARGC1A	0,317	0,033	0,858	0,001	-6,491
ENSG00000114349	GNAT1	0,001	0,897	0,001	0,267	-1,746
ENSG00000117069	ST6GALNAC5	1,358	0,196	0,072	0,001	-3,369
ENSG00000117115	PADI2	0,001	0,018	0,238	1,031	-2,080
ENSG00000117594	HSD11B1	0,001	0,268	0,144	0,812	-5,568
ENSG00000125144	MT1G	0,001	0,931	0,056	1,805	-4,865
ENSG00000128242	GAL3ST1	0,001	0,051	0,608	1,395	-4,471
ENSG00000131096	PYY	0,001	0,362	0,001	2,097	2,534
ENSG00000133169	BEX1	0,001	0,903	0,076	0,123	-9,126
ENSG00000134297	PLEKHA8P1	1,439	0,001	2,824	0,372	7,565

Table 5.4: Continued

ENSG00000134716	CYP2J2	0,988	2,217	0,001	0,001	-1,166
ENSG00000135373	EHF	0,974	0,252	0,001	0,019	6,226
ENSG00000136286	MYO1G	0,009	0,001	0,573	1,288	4,288
ENSG00000137757	CASP5	0,001	0,471	0,489	1,836	-6,970
ENSG00000139970	RTN1	1,396	2,878	0,057	0,001	-6,875
ENSG00000141040	ZNF287	0,946	0,215	0,225	0,001	-5,680
ENSG00000143416	SELENBP1	0,001	0,001	0,660	4,732	2,843
ENSG00000145526	CDH18	0,001	0,001	7,563	0,867	-3,124
ENSG00000145824	CXCL14	0,001	0,001	0,132	0,754	2,515
ENSG00000151715	TMEM45B	1,447	4,387	0,001	0,001	-1,601
ENSG00000152092	ASTN1	0,216	0,913	0,001	0,001	-2,078
ENSG00000156966	B3GNT7	0,892	0,054	0,078	0,001	-2,243
ENSG00000159905	ZNF221	0,969	0,103	0,235	0,001	-4,635
ENSG00000164283	ESM1	0,627	2,536	0,001	0,001	-2,015
ENSG00000168348	INSM2	0,001	0,520	0,001	1,906	1,873
ENSG00000168754	FAM178B	0,001	0,001	0,188	0,765	2,026
ENSG00000169253	RP11-220D10.1	0,001	1,351	0,324	0,519	-9,722
ENSG00000171084	FAM86JP	1,144	0,128	1,119	0,001	-6,969
ENSG00000175315	CST6	0,144	0,001	4,463	21,419	9,431
ENSG00000175445	LPL	0,738	3,430	0,001	0,001	-2,216
ENSG00000176343	RPL37AP8	0,001	3,884	0,217	3,762	-7,805
ENSG00000178917	ZNF852	0,841	0,001	0,305	0,001	1,463
ENSG00000180543	TSPYL5	1,719	0,044	0,861	0,001	-4,476
ENSG00000181126	HLA-V	0,001	2,473	1,245	5,820	-9,047
ENSG00000181315	ZNF322	0,854	0,001	1,286	0,035	4,555
ENSG00000181359	HSP90AA6P	0,001	0,906	0,366	0,820	-8,662
ENSG00000182676	PPP1R27	0,001	0,420	0,001	1,073	1,352
ENSG00000183161	FANCF	0,476	0,001	0,825	0,049	4,812
ENSG00000184795	UNC93B5	0,001	2,775	1,264	4,597	-9,576
ENSG00000186081	KRT5	0,001	0,001	0,267	1,132	2,085

Table 5.4: Continued

ENSG00000186446	ZNF501	1,432	0,001	0,466	0,001	1,621
ENSG00000186812	ZNF397	1,161	0,001	0,909	0,347	8,789
ENSG00000189409	MMP23B	0,001	0,342	0,145	1,344	-5,208
ENSG00000196406	SPANXD	0,001	0,508	6,668	22,583	-7,229
ENSG00000196466	ZNF799	0,964	0,001	0,617	0,037	5,836
ENSG00000196550	FAM72A	5,532	0,789	3,565	0,001	-8,989
ENSG00000196793	ZNF239	0,882	0,147	1,191	0,001	-7,637
ENSG00000197566	ZNF624	0,625	0,011	0,686	0,001	-3,587
ENSG00000197575	RPS17P2	0,001	1,022	0,001	0,001	-9,997
ENSG00000197933	ZNF823	2,197	0,260	1,598	0,001	-7,561
ENSG00000198610	AKR1C4	0,001	0,399	0,204	1,208	-6,072
ENSG00000198796	ALPK2	0,063	0,001	1,172	0,223	3,579
ENSG00000200693	U3	0,001	3,062	0,442	1,400	-9,916
ENSG00000200795	RNU4-1	0,001	71,422	9,892	58,800	-13,553
ENSG00000203711	C6orf99	0,610	0,886	0,824	0,001	-10,224
ENSG00000204379	XAGE1A	0,001	2,871	0,333	2,962	-8,335
ENSG00000205362	MT1A	0,001	0,001	1,766	6,405	1,859
ENSG00000205420	KRT6A	0,001	12,862	4,610	15,374	-11,913
ENSG00000206652	RNU1-1	0,001	2,397	0,001	0,001	-11,227
ENSG00000207205	RNVU1-15	0,001	2,345	0,001	0,001	-11,195
ENSG00000207513	RNU1-3	0,001	2,397	0,001	0,001	-11,227
ENSG00000213082	PPP1R14BP2	0,001	0,920	0,368	0,742	-8,833
ENSG00000213253	RPL12P42	0,001	3,373	0,384	3,629	-8,481
ENSG00000213343	RPL21P18	0,001	1,613	1,550	4,632	-9,076
ENSG00000213467	HMGB1P37	0,001	0,913	0,524	1,898	-7,977
ENSG00000213539	YBX1P6	0,001	1,020	0,157	1,224	-7,029
ENSG00000213942	RP1-102E24.1	0,001	1,496	0,480	1,350	-9,055
ENSG00000214047	RPS11P7	0,001	0,837	0,171	0,417	-8,423
ENSG00000214222	TUBBP2	0,001	1,890	0,728	2,686	-9,001
ENSG00000214263	RPSAP53	0,001	1,696	0,525	2,382	-8,545

Table 5.4: Continued

ENSG00000214541	RPS4XP3	0,001	2,017	1,341	3,493	-9,596
ENSG00000214654	RP11-27I1.4	5,671	0,229	2,414	0,001	-6,607
ENSG00000214784	AC010468.1	0,001	1,247	0,241	1,042	-8,170
ENSG00000214914	RPL23AP3	0,001	2,975	1,357	4,670	-9,755
ENSG00000215016	RPL24P7	0,001	0,893	0,180	0,866	-7,535
ENSG00000215093	EEF1A1P29	0,001	0,402	0,336	1,148	-6,878
ENSG00000215096	IFITM8P	0,001	0,153	0,001	0,899	2,552
ENSG00000215326	GPX1P2	0,001	1,089	0,412	1,529	-8,196
ENSG00000217026	RPL10P1	0,001	1,077	0,055	1,236	-5,574
ENSG00000217027	TPT1P4	0,001	0,934	0,435	0,949	-8,744
ENSG00000219932	RPL12P8	0,001	0,790	0,251	1,826	-6,762
ENSG00000220842	RP11-572P18.1	0,001	0,792	0,649	0,658	-9,611
ENSG00000221676	RNU6ATAC	0,001	32,142	2,988	27,658	-11,762
ENSG00000223668	EEF1A1P24	0,001	0,371	0,282	1,061	-6,625
ENSG00000224023	FLJ37035	0,987	0,001	0,370	0,248	9,372
ENSG00000224677	PDIA3P2	0,001	77,329	32,484	133,012	-14,205
ENSG00000225082	DAP3P1	0,001	2,634	1,899	5,061	-9,949
ENSG00000225416	AC104843.3	0,001	2,276	0,482	1,808	-9,246
ENSG00000225499	RPL15P4	0,001	0,794	0,313	2,129	-6,869
ENSG00000226439	RP11-621K7.1	0,001	2,574	0,538	1,737	-9,640
ENSG00000226499	RP5-882O7.1	0,001	23,725	4,616	20,207	-12,404
ENSG00000227359	AC017074.2	0,001	0,917	0,979	1,275	-9,460
ENSG00000227617	CERS6-AS1	1,311	0,126	0,001	0,001	3,374
ENSG00000228015	AC074338.5	0,001	10,206	12,809	8,311	-13,941
ENSG00000228071	RPL7P47	0,001	0,849	0,001	0,323	-1,395
ENSG00000228169	PPIAP19	0,001	0,904	0,079	0,767	-6,542
ENSG00000228236	TXNP5	0,001	1,589	0,001	0,001	-10,634
ENSG00000228399	RP4-575N6.2	0,001	1,965	0,843	2,491	-9,378
ENSG00000229367	HMG2P19	0,001	0,317	0,001	1,118	1,821
ENSG00000230216	HSPB1P2	0,001	1,071	0,408	0,992	-8,783

Table 5.4: Continued

ENSG00000230228	RP11-4M23.4	0,001	0,883	0,125	0,202	-9,086
ENSG00000230330	HMGN2P3	0,001	1,522	0,222	0,702	-8,910
ENSG00000230916	RP11-10B2.1	0,001	0,895	0,098	0,555	-7,305
ENSG00000231304	SGOL1-AS1	0,001	0,074	0,001	0,869	3,544
ENSG00000231327	AC016700.5	0,001	1,039	0,365	1,445	-8,037
ENSG00000231369	RP1-40G4P.1	0,001	1,326	0,253	0,715	-8,873
ENSG00000231747	AC079922.2	0,001	0,921	0,001	0,210	-2,135
ENSG00000232065	LINC01063	0,001	1,533	0,686	0,886	-10,213
ENSG00000232177	MTND4P24	0,001	1,546	0,001	0,178	-3,122
ENSG00000232568	RPL23AP35	0,001	0,001	0,001	0,873	9,769
ENSG00000233380	RPS2P48	0,001	0,987	0,183	1,308	-7,109
ENSG00000233594	BT3P5	0,001	0,837	0,087	0,965	-6,230
ENSG00000233654	AC093388.3	0,001	0,300	0,001	0,861	1,520
ENSG00000233929	MT1XP1	0,001	1,580	0,001	0,001	-10,625
ENSG00000234147	RP3-460G2.2	0,622	2,621	0,001	0,146	5,112
ENSG00000234513	AC073072.7	0,001	1,329	0,277	1,328	-8,116
ENSG00000234589	RP11-253E3.1	0,001	3,681	0,634	3,477	-9,391
ENSG00000234648	AL162151.3	0,001	8,368	1,021	14,222	-9,231
ENSG00000235028	HMGN1P30	0,001	1,582	0,001	0,727	-1,122
ENSG00000235175	RPL26P37	0,001	1,045	0,284	1,064	-8,123
ENSG00000236060	HSPB1P1	0,001	2,100	2,022	3,611	-10,200
ENSG00000236773	RP11-365O16.1	0,001	0,880	1,287	0,001	-20,112
ENSG00000236972	FABP5P1	0,001	1,147	0,001	0,503	-1,188
ENSG00000236976	RP11-165H4.2	0,001	1,432	0,446	1,095	-9,188
ENSG00000237094	RP4-669L17.10	0,001	0,128	0,274	0,912	-5,269
ENSG00000237676	RPL30P4	0,001	1,216	0,150	1,431	-6,998
ENSG00000237943	PRKCQ-AS1	1,848	0,605	0,580	0,001	-7,570
ENSG00000238117	AP004372.1	3,179	0,951	0,001	0,365	10,254
ENSG00000238165	AC007560.1	0,001	1,673	0,301	0,481	-10,029
ENSG00000239719	RP4-800G7.1	0,001	6,066	4,414	7,937	-11,720

Table 5.4: Continued

ENSG00000240409	MTATP8P1	0,001	18,596	3,830	16,805	-12,049
ENSG00000240914	RPL15P2	0,001	1,300	0,288	0,563	-9,377
ENSG00000241431	RPL37P6	0,001	5,193	1,431	6,943	-10,064
ENSG00000241489	AF011889.5	0,001	22,849	3,217	15,766	-12,187
ENSG00000241746	RP11-941H19.1	0,001	2,853	1,121	2,734	-10,192
ENSG00000242156	AC000041.8	0,001	91,335	22,570	132,637	-13,924
ENSG00000242692	RPS27AP1	0,001	0,943	0,421	1,770	-7,808
ENSG00000243007	RPL12P35	0,001	0,865	0,078	0,632	-6,742
ENSG00000243181	RP11-734J24.1	0,001	1,523	0,499	2,422	-8,294
ENSG00000243396	RP11-454H13.1	0,001	0,597	0,001	1,216	1,025
ENSG00000243494	CTC-550B14.1	0,001	1,432	0,347	0,558	-9,799
ENSG00000243554	AC004967.7	0,001	3,197	0,637	4,854	-8,713
ENSG00000243904	RPSAP5	0,001	1,912	0,219	2,606	-7,328
ENSG00000243988	RPS24P17	0,001	1,677	0,397	1,281	-9,021
ENSG00000244004	RP11-1396O13.2	0,001	1,920	0,550	2,139	-8,946
ENSG00000244703	CD46P1	0,001	3,943	1,215	5,244	-9,835
ENSG00000247317	RP11-273G15.2	0,001	0,409	0,001	0,911	1,156
ENSG00000247595	SPTY2D1-AS1	0,001	0,260	0,047	0,726	-4,063
ENSG00000249149	RP11-79P5.3	0,001	1,370	0,623	1,813	-8,881
ENSG00000249799	SNRPCP13	0,001	5,199	1,636	5,988	-10,472
ENSG00000249908	BRD9P2	0,001	5,232	5,622	2,821	-13,348
ENSG00000250268	ALG1L14P	0,001	2,017	0,571	1,848	-9,284
ENSG00000250342	SNRPCP16	0,001	2,785	1,639	4,532	-9,976
ENSG00000250794	ALG1L12P	0,001	0,929	0,285	0,001	-18,016
ENSG00000251087	ALG1L3P	0,001	0,993	0,218	0,566	-8,578
ENSG00000251313	RP11-747H12.5	0,001	1,978	0,591	2,204	-9,051
ENSG00000251521	IMPA1P	0,001	2,415	0,433	2,141	-8,932
ENSG00000254004	ZNF260	1,861	0,044	0,841	0,001	-4,330
ENSG00000254270	ERHP1	0,356	0,463	0,851	0,001	-10,112
ENSG00000254402	LRRC24	0,278	0,946	0,001	0,447	7,034

Table 5.4: Continued

ENSG00000254605	RP11-626H12.2	0,235	0,348	0,744	0,001	-10,102
ENSG00000254854	CTD-2523D13.2	0,001	0,820	0,001	0,118	-2,802
ENSG00000255262	TCEB2P2	0,001	0,914	0,001	0,215	-2,088
ENSG00000255339	RP11-411B6.6	0,001	0,963	0,030	0,084	-8,453
ENSG00000255730	CTC-435M10.3	0,001	0,684	0,231	3,506	-5,498
ENSG00000255823	MTRNR2L8	0,001	6,587	0,162	3,799	-8,131
ENSG00000256045	MTRNR2L10	0,001	2,802	0,039	2,138	-5,676
ENSG00000257790	EIF4A1P4	0,001	0,608	0,275	1,022	-7,354
ENSG00000257956	NOP56P3	0,001	0,799	1,038	0,747	-10,115
ENSG00000258011	HMGA1P3	0,001	3,073	1,095	7,024	-8,904
ENSG00000258613	RPL21P7	0,001	2,719	0,968	5,117	-9,007
ENSG00000258704	SRP54-AS1	0,001	1,672	0,371	1,710	-8,502
ENSG00000259112	NDUFC2-KCTD14	0,001	0,658	0,902	0,001	-19,178
ENSG00000259330	INAFM2	1,994	0,001	2,049	0,099	6,588
ENSG00000259863	SH3RF3-AS1	1,009	0,103	0,837	0,001	-6,415
ENSG00000260077	RP11-254F7.2	0,947	0,054	0,038	0,001	-1,129
ENSG00000260490	MYL12BP1	0,001	0,843	0,227	0,735	-8,025
ENSG00000261105	LMO7-AS1	0,372	1,467	0,001	0,248	5,976
ENSG00000261431	RP4-616B8.4	0,001	3,900	1,716	3,605	-10,858
ENSG00000261857	MIA	0,398	2,473	0,001	0,456	6,198
ENSG00000262902	MTCO1P40	0,001	1,127	0,058	2,202	-4,882
ENSG00000264940	SNORD3C	0,001	3,123	0,439	2,298	-9,220
ENSG00000265185	SNORD3B-1	0,001	26,546	4,495	42,139	-11,467
ENSG00000267308	AC004510.3	1,063	4,722	0,001	0,001	-2,151
ENSG00000267505	CTC-296K1.3	2,057	0,001	2,864	0,107	6,258
ENSG00000267601	RP11-323N12.5	0,001	26,872	7,750	32,940	-12,626
ENSG00000269910	RP11-73M18.10	4,576	1,953	3,622	0,001	-10,594
ENSG00000270722	U1	0,001	8,362	2,655	5,548	-11,966
ENSG00000270906	MTND4P35	0,001	0,804	0,066	0,674	-6,290
ENSG00000270945	HSPE1P7	0,001	1,996	2,063	3,829	-10,071

Table 5.4: Continued

ENSG00000271029	RP11-849F2.10	0,001	2,289	0,328	1,413	-9,051
ENSG00000272602	ZNF595	1,608	0,161	1,073	0,001	-6,743
ENSG00000273175	RP11-11N7.4	0,001	0,097	0,001	0,680	2,801
ENSG00000273812	WI2-87327B8.2	0,001	0,083	4,613	0,276	-10,431
ENSG00000277232	GTSE1-AS1	0,271	0,266	0,993	0,001	-9,929
ENSG00000277672	RP11-386G11.11	0,001	17,068	3,612	23,803	-11,339
ENSG00000277782	RP11-775C24.5	0,836	0,001	0,340	0,185	8,826
ENSG00000278249	SCARNA2	0,360	5,782	0,001	5,663	8,462
ENSG00000278878	CH507-145C22.3	0,001	1,009	0,174	0,663	-8,046
ENSG00000279115	AC006386.1	0,001	3,569	1,582	4,954	-10,155
ENSG00000279225	RP11-2K6.1	0,001	0,804	0,001	0,001	-9,652
ENSG00000280744	LINC01173	0,001	0,215	0,900	0,001	-17,559
ENSG00000282879	RP11-10F11.4	0,001	8,964	1,515	7,138	-10,894

Table 5.5. The list of genes having 0 FPKM values for tunicamycin treatment.

Gene ID	Gene	WT_VC	WT_TUN	KO_VC	KO_TUN	DEG Score
ENSG00000019186	CYP24A1	6,288	3,948	0,158	0,001	-6,630
ENSG00000101188	NTSR1	0,001	0,035	5,985	4,041	-5,709
ENSG00000120337	TNFSF18	0,001	1,449	0,164	2,727	-6,442
ENSG00000124194	GDAP1L1	0,027	0,132	0,001	0,980	7,650
ENSG00000127533	F2RL3	0,001	0,225	0,246	3,137	-4,138
ENSG00000132965	ALOX5AP	0,001	0,518	0,555	8,422	-5,094
ENSG00000133169	BEX1	0,001	0,906	0,108	0,354	-8,115
ENSG00000134548	SPX	0,001	0,545	0,037	1,849	-3,431
ENSG00000135373	EHF	1,352	3,675	0,001	0,095	5,121
ENSG00000137757	CASP5	0,001	1,028	0,698	2,319	-8,274
ENSG00000145934	TENM2	0,001	0,012	0,408	0,962	-2,353
ENSG00000151025	GPR158	0,001	0,008	0,913	2,009	-1,889
ENSG00000152463	OLAH	0,186	0,364	0,001	1,331	9,405
ENSG00000159337	PLA2G4D	0,001	0,098	0,707	0,218	-8,307

Table 5.5: Continued

ENSG00000163564	PYHIN1	0,001	0,675	0,351	1,000	-7,891
ENSG00000163915	IGF2BP2-AS1	0,001	0,019	0,001	2,175	6,857
ENSG00000176046	NUPR1	0,001	1,360	0,384	1,748	-8,222
ENSG00000176343	RPL37AP8	0,001	1,618	0,309	1,272	-8,621
ENSG00000181126	HLA-V	0,001	1,243	1,779	1,774	-10,284
ENSG00000184613	NELL2	2,579	1,040	0,092	0,001	-5,209
ENSG00000184795	UNC93B5	0,001	2,202	1,807	2,587	-10,587
ENSG00000185710	SMG1P4	0,001	1,511	0,820	1,465	-9,724
ENSG00000200795	RNU4-1	0,001	168,761	14,145	120,183	-14,278
ENSG00000205420	KRT6A	0,001	6,370	6,588	5,364	-12,934
ENSG00000213253	RPL12P42	0,001	0,952	0,549	0,730	-9,483
ENSG00000213343	RPL21P18	0,001	1,700	2,216	2,050	-10,843
ENSG00000214263	RPSAP53	0,001	1,100	0,750	0,914	-9,818
ENSG00000214541	RPS4XP3	0,001	1,507	1,916	1,481	-10,929
ENSG00000214914	RPL23AP3	0,001	1,251	1,939	1,709	-10,471
ENSG00000221676	RNU6ATAC	0,001	19,227	4,273	21,073	-11,929
ENSG00000222009	BTBD19	0,001	0,052	0,209	0,933	-3,541
ENSG00000224165	DNAJC27-AS1	0,881	0,131	0,001	1,341	13,143
ENSG00000224677	PDIA3P2	0,001	85,911	46,432	138,332	-14,816
ENSG00000225082	DAP3P1	0,001	2,390	2,714	3,723	-10,767
ENSG00000225416	AC104843.3	0,001	1,210	0,689	1,534	-9,086
ENSG00000226439	RP11-621K7.1	0,001	1,330	0,769	0,906	-10,141
ENSG00000226499	RP5-882O7.1	0,001	7,017	6,596	8,920	-12,341
ENSG00000227617	CERS6-AS1	1,818	0,106	0,001	0,001	4,101
ENSG00000228015	AC074338.5	0,001	8,120	18,312	18,119	-13,002
ENSG00000228399	RP4-575N6.2	0,001	1,417	1,205	0,671	-11,312
ENSG00000231684	EIF1P3	0,001	1,082	0,210	0,511	-8,800
ENSG00000233929	MT1XP1	0,001	0,001	0,001	3,450	11,753
ENSG00000234589	RP11-253E3.1	0,001	1,436	0,906	2,123	-9,259
ENSG00000236060	HSPB1P1	0,001	0,955	2,890	0,001	-21,395

Table 5.5: Continued

ENSG00000236453	AC003092.1	0,567	0,546	0,001	0,737	9,579
ENSG00000236574	RP11-340I6.4	0,001	0,001	1,508	0,001	-10,558
ENSG00000236581	STARD13-AS	1,076	0,115	0,001	0,001	3,224
ENSG00000236773	RP11-365O16.1	0,001	2,000	1,840	0,372	-13,274
ENSG00000238151	MLLT10P1	0,001	0,001	0,686	0,001	-9,422
ENSG00000241431	RPL37P6	0,001	3,702	2,046	4,581	-10,691
ENSG00000241746	RP11-941H19.1	0,001	1,590	1,602	1,672	-10,573
ENSG00000243137	PSG4	1,354	0,537	0,019	0,001	-2,891
ENSG00000244004	RP11-1396O13.2	0,001	1,088	0,785	1,289	-9,373
ENSG00000245112	SMARCA5-AS1	0,001	3,953	1,715	5,367	-10,303
ENSG00000249572	CTD-2203K17.1	0,001	1,110	0,497	0,650	-9,728
ENSG00000249908	BRD9P2	0,001	5,976	8,038	7,018	-12,741
ENSG00000250268	ALG1L14P	0,001	1,615	0,816	1,027	-10,326
ENSG00000250342	SNRPCP16	0,001	2,536	2,342	0,948	-12,613
ENSG00000250746	RP11-39C10.1	0,001	1,323	0,120	1,066	-7,221
ENSG00000251313	RP11-747H12.5	0,001	1,137	0,845	1,386	-9,437
ENSG00000255073	ZFP91-CNTF	0,001	0,202	0,381	1,341	-5,841
ENSG00000257635	RP11-536C10.10	0,001	1,444	0,509	0,829	-9,793
ENSG00000259570	RP11-671M22.4	0,001	1,412	0,646	1,087	-9,713
ENSG00000264940	SNORD3C	0,001	4,650	0,628	4,835	-9,238
ENSG00000265185	SNORD3B-1	0,001	67,021	6,424	62,336	-12,754
ENSG00000270722	U1	0,001	13,689	3,796	15,894	-11,675
ENSG00000270945	HSPE1P7	0,001	1,773	2,948	1,920	-11,411
ENSG00000273703	HIST1H2BM	0,001	0,873	0,334	1,827	-7,317
ENSG00000273812	WI2-87327B8.2	0,001	0,072	6,594	12,380	-5,264
ENSG00000277367	RP11-536C10.26	0,001	1,443	0,509	0,828	-9,792
ENSG00000277447	RP11-193E15.4	0,001	1,016	0,232	0,199	-10,212
ENSG00000277672	RP11-386G11.11	0,001	5,793	5,162	5,588	-12,386
ENSG00000277918	U1	0,001	12,591	2,541	6,275	-12,316
ENSG00000278249	SCARNA2	0,501	3,489	0,001	3,558	8,997

Table 5.5: Continued

ENSG00000279115	AC006386.1	0,001	1,966	2,260	1,255	-11,790
ENSG00000281383	CH507- 513H4.5	0,001	0,966	0,099	0,685	-7,128
ENSG00000282879	RP11-10F11.4	0,001	3,197	2,166	0,584	-13,534

The types of these genes were shown in Figure 5.10 (a and b). Whereas, the third majority of these “zero cases” are long non-coding RNA (lncRNA) (14, 28, and 9 genes for cisplatin, doxorubicin, and tunicamycin treatments, respectively) (Figure 5.10 (a-c)).

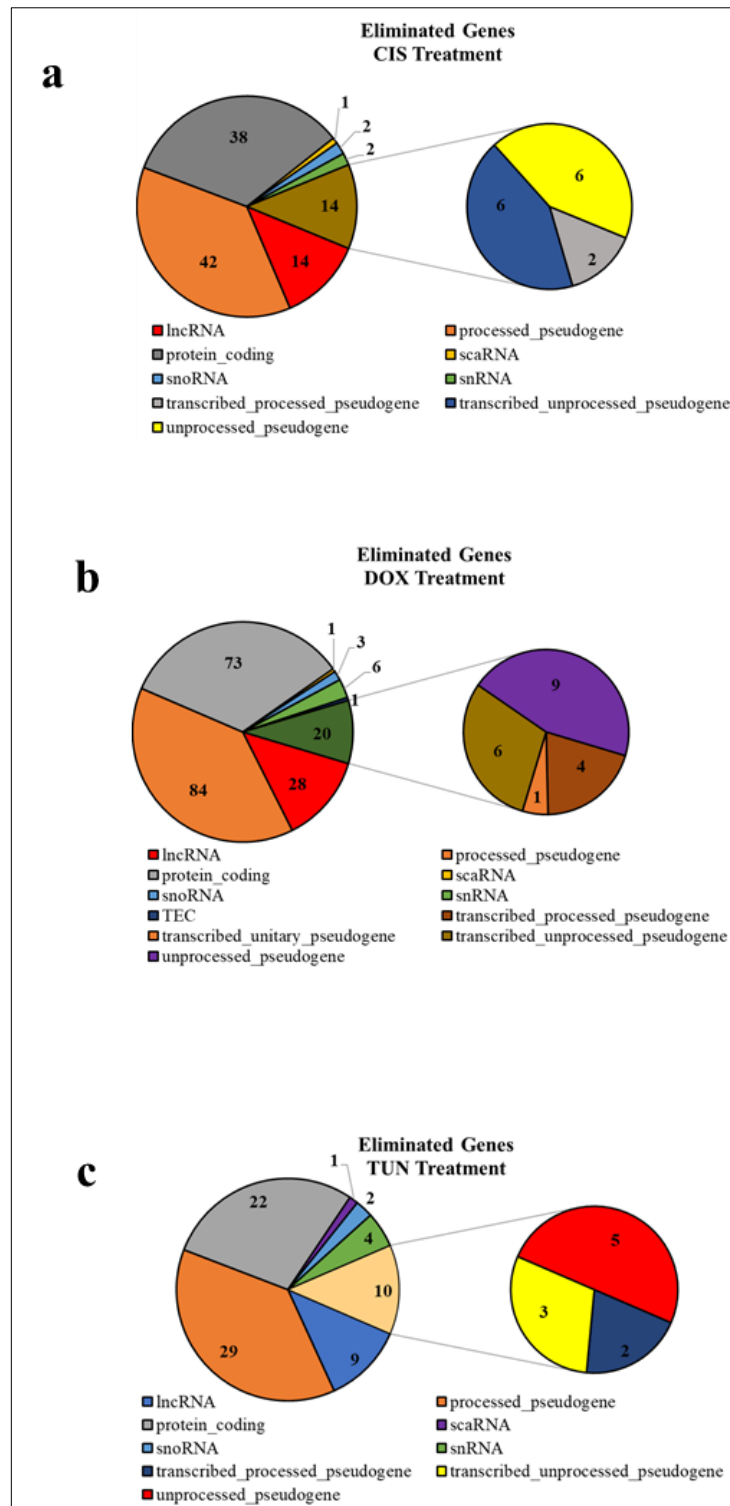


Figure 5.10: The classification of eliminated transcripts with zero FPKM values. The elimination of transcripts with zero FPKM causes information loss, particularly on processed pseudogenes and protein coding genes. Therefore, genes having zero FPKM values were included to calculation by adding 0.001 values to each FPKM values. Feature types of (a) 113 genes in CIS treatment, (b) 216 genes in DOX treatment, and (c) 77 genes in TUN treatment were eliminated because of 0 reading. CIS, cisplatin; DOX, doxorubicin; TUN, tunicamycin.

5.11. Identified Pathways in Gene Set and KEGG Enrichment Analyses May Increase Apoptosis in KO Cells Upon Exposure to Cisplatin and Doxorubicin

To perform the gene set enrichment analysis (GSEA) the differentially expressed gene score (DEG score, Equation (4.1)) for each gene between 1137 and 1531 DEGs as a preranked input was used. Enriched gene sets were sought from the MSigDB hallmark data collection utilizing the default “weighted” enrichment statistic parameter and “meandiv” normalization that includes gene sets with more than 15 and less than 5000 members. Enriched gene sets with a nominal p value < 0.05 were considered significant. The genes that make up the MSigDB hallmark gene sets display consistent expression patterns in specific biological processes in the many constituent MSigDB gene sets based on publications. Genes that display consistent expression patterns in particular biological processes in a lot of the founder MSigDB gene sets established on publications form MSigDB distinctive gene sets. These gene clusters are attentively curated to comprise only genes that are constantly observed to be required in a particular pathway, so they have high reliability. The hallmark genes `UV_RESPONSE_DN` and `ESTROGEN_RESPONSE_EARLY` after cisplatin treatment and `INFLAMMATORY_RESPONSE` after doxorubicin treatment are significantly down-regulated. In these gene sets, 27, 20, and 19 genes identified in DEGs were detected respectively (Figure 5.11 (a and b), left panels).

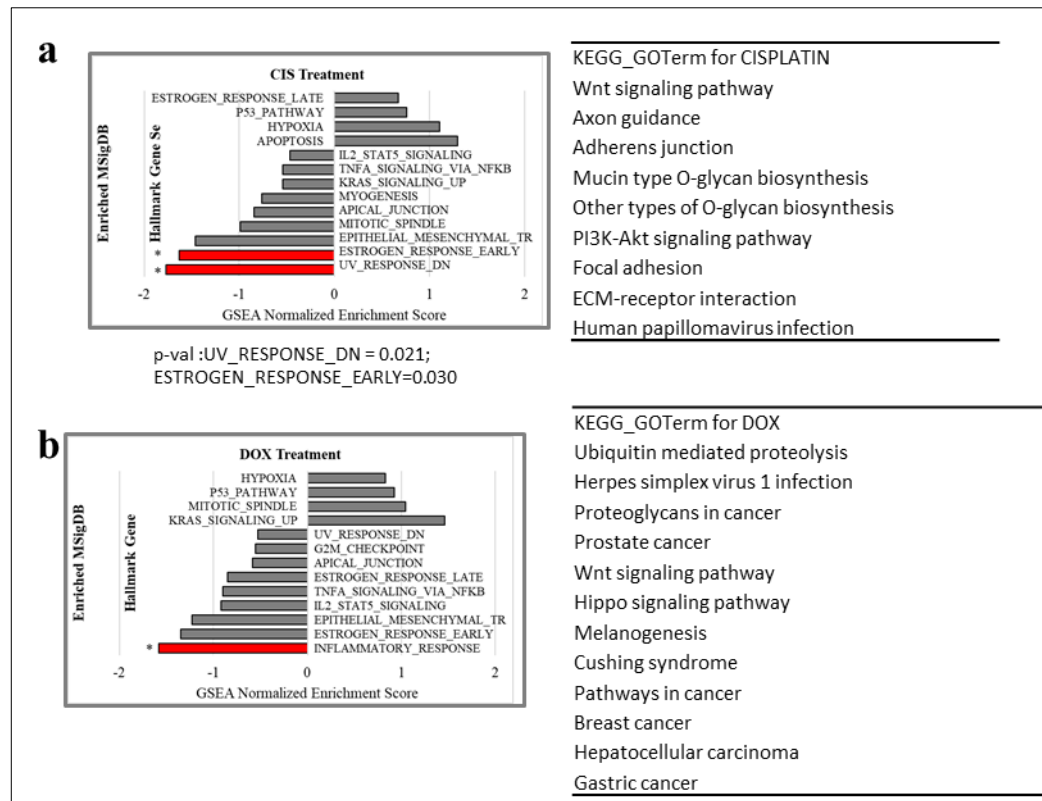


Figure 5.11: Gene Set Enrichment (GSEA) and KEGG pathway analysis. DEGs determined at the end of (a) CIS treatment and (b) DOX treatment were further evaluated in GSEA and KEGG pathway analysis. Estrogen response early (p value: 0.030) and UV response DN (p value: 0.021) gene sets were significantly down-regulated in CIS treatment. Inflammatory response (p value 0.05) gene set was significantly downregulated in DOX treatment. KEGG pathways of CIS- and DOX treated cells were presented on the right panel of (a) and (b), respectively. CIS, cisplatin; DOX, doxorubicin.

The GSEA analysis for tunicamycin did not reveal any significant hallmark genes (Figure 5.12).

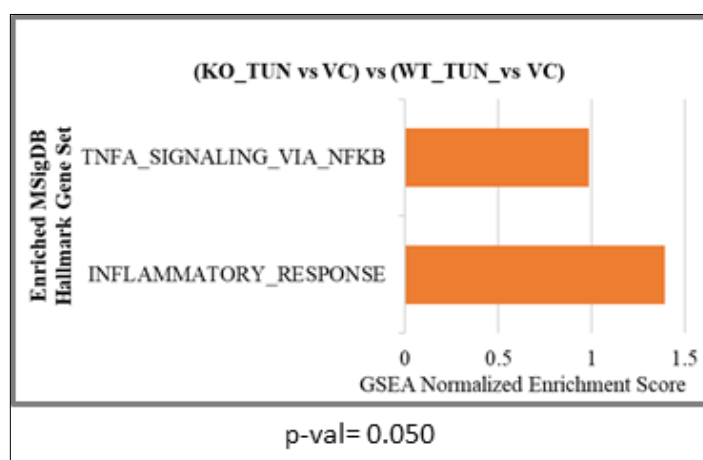


Figure 5.12: Gene Set Enrichment Analysis after TUN treatment. In agreement with the sXBP1 formation in WT and KO cells, No significant GSEA was detected after TUN treatment.

KEGG pathway enrichment was assayed by using ClueGO extension tool of Cytoscape [Bindea et al., 2009]. The 1137 and 1531 significant DEG lists were loaded as a marker list, and Homo sapiens was preferred. In the ClueGO setting sections, the pathway was chosen as KEGG, and a threshold of significance was set as “Show only pathways with p value ≤ 0.05 .” The remaining settings were left as default. The resulting gene lists in the “Functional Groups with Genes” output were given in the right panel of Figure 5.11 (a and b). We analyzed the DEGs (DEG score, Equation (4.1) between KO and WT cells (using VC-treated controls) and performed the gene set enrichment analysis (GSEA) [Subramanian et al., 2005]. HALLMARK_EPITHELIAL_MEZENCHYMAL_TRANSITION was the most significant one (p value *** < 0.001) which was in agreement with the observed phenotype (Figure 5.13 (a and b)).

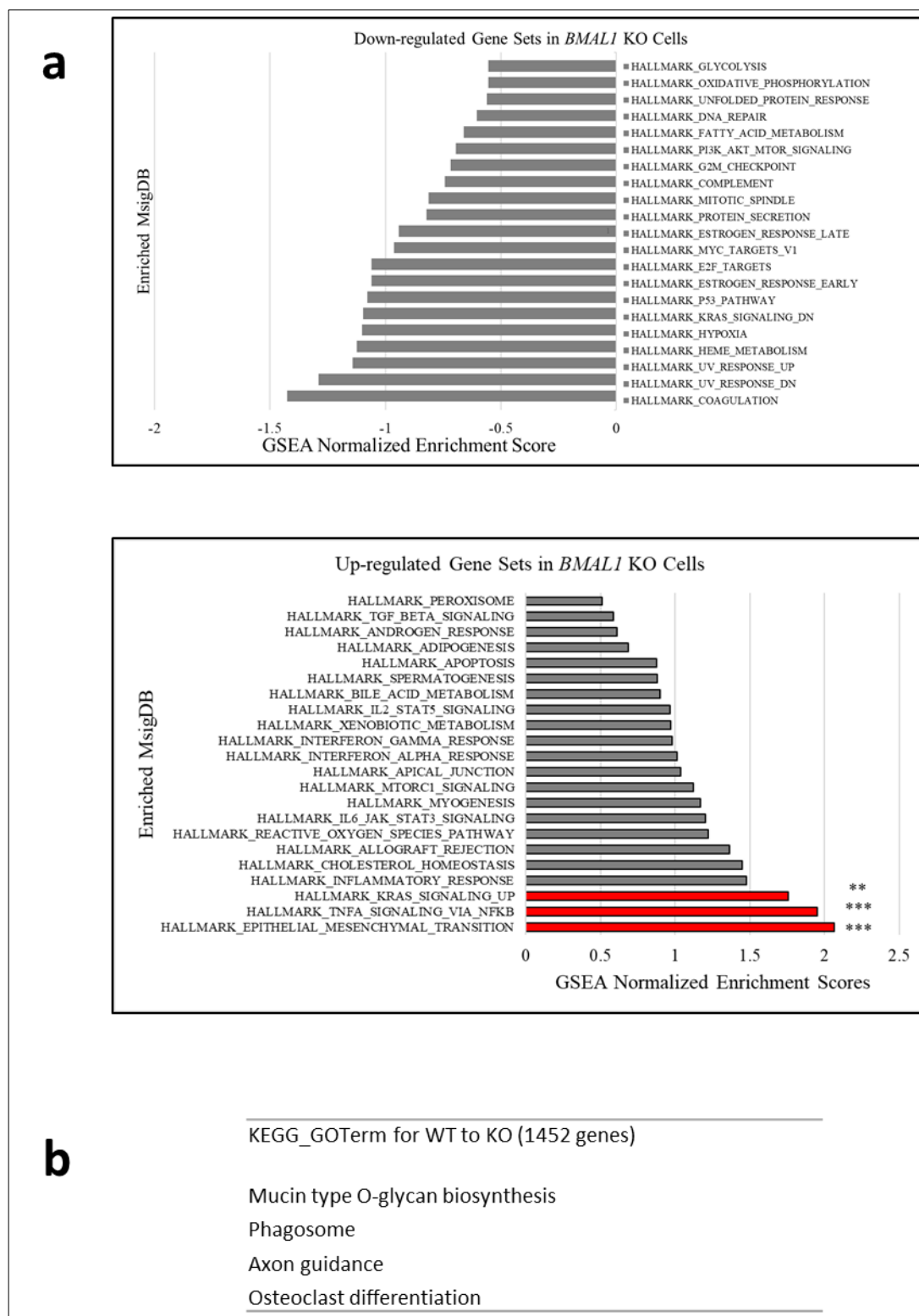


Figure 5.13: Gene Set Enrichment Analysis and KEGG GO term of DEGS without treatment. In order to reveal the Genes enriched in KO cells, DEGs were used for GSEA (KO vs WT). a) In agreement with the observed phenotype, HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION was the most significant one. b) *BMAL1* gene deletion affected many genes as KEGG GO term of DEGs was shown.

5.12. Analysis of Transcriptome Reveals EMT Regulated Genes are Significantly Up-regulated in KO Cells

Since KO cells have higher mobility and invasion capacity, we analyzed the gene expression between KO and WT cells. GSEA analysis revealed that hallmark genes for EPITHELIAL_MESENCHYMAL_TRANSITION gene set is consisted of 60 genes up-regulated with respect to *BMAL1* deletion across 105 founder gene sets. The up-regulation agrees with the observed phenotype because *BMAL1* deletion increased both cell motility (Figure 5.4) and invasion capacity [Korkmaz et al., 2018]. A role of EMT in cancer drug resistance has long been suggested because signaling pathways that support EMT were certainly known to participate to drug resistance. For example, TGF β induces EMT, and this in turn leads to the drug resistance [Shibue and Weinberg 2017]. In this study, the EMT genes were up-regulated in KO cells which is well-correlated with the increased invasive capacity of KO cells. However, the downregulation of EMT genes in cisplatin- or doxorubicin treated cells might explain why these cells get sensitized these drugs.

5.13. Protein-protein Interaction Network Analysis Helps Identify Key Protein Interactions Responsible for Sensitization of KO Cells to Cisplatin- and Doxorubicin - Induced Apoptosis

Besides the KEGG and the GSEA, protein-protein interaction network analysis of the DEGs common to cisplatin and doxorubicin treatments was also done for determination of protein interactions which may play important roles in cancer upon *BMAL1* gene deletion. For this analysis, 336 common DEGs from cisplatin and doxorubicin treatments were used. Their relevant functions were given in Figure 5.14.

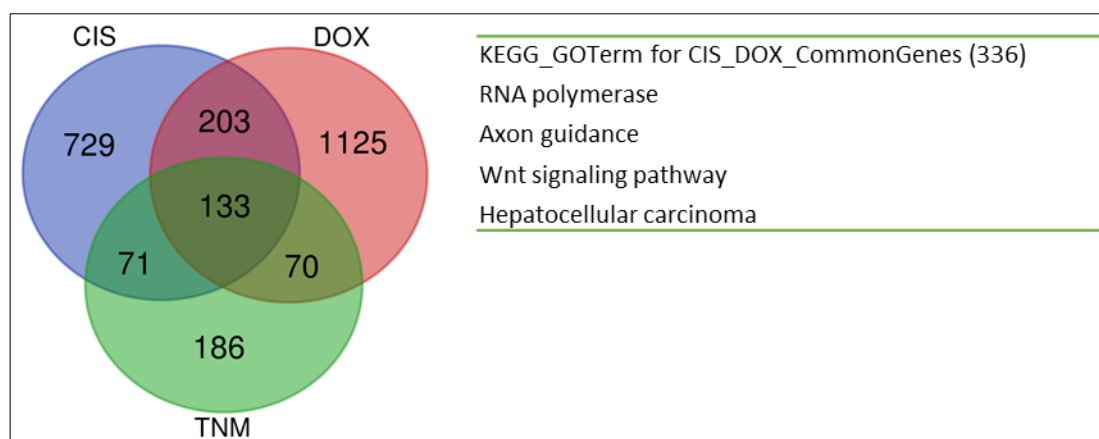


Figure 5.14: Number of common DEGs in CIS, DOX, and TUN treatments were shown in Venn schema. KEGG pathway analysis of 336 common DEGs in CIS and DOX treatments.

By using interaction data only obtained from experiments with confidence score 0.4 or higher and allowing maximum 50 additional interactor proteins, we constructed the PPIN (Figure 5.15). Proteins were shown in ellipses (nodes), and interacting proteins were connected by lines (edges). Degree and betweenness centrality of nodes were used to determine the width and height of the nodes, respectively. Cyan nodes represent down-regulated, and red nodes represent up-regulated genes in both CIS and DOX treatments. Orange nodes represent differentially regulated genes in cisplatin and doxorubicin treatments. White nodes are additional ones added by STRING database. Top bottleneck proteins were determined based on the highest betweenness values of nodes. Protein-protein interaction network obtained from common DEGs in cisplatin and doxorubicin treatments revealed that genes, e.g., *GSK3 β* , *NACC1*, and *EGFR*, in involve apoptotic pathways and their interaction partners are the principal genes regulating the response of the KO cells. In addition, *HDAC4* and *INO80* genes which remodel the chromatin structure are also key components in the PPIN. To note, *GSK3 β* was differentially regulated in cisplatin and doxorubicin treatments, while it was down-regulated in cisplatin treatment and up-regulated in doxorubicin treatment.

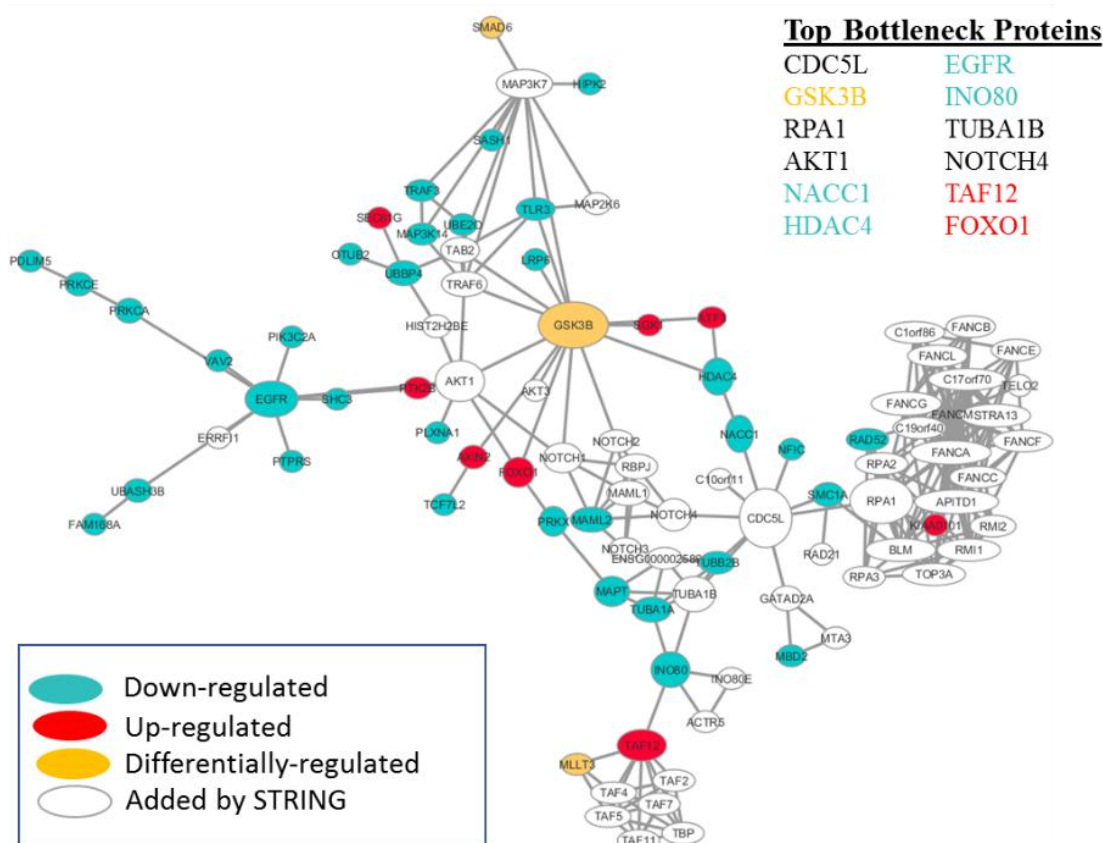


Figure 5.15: STRING network analysis of common DEGs in CIS and DOX treatments. Protein-protein interactions (PPIs) were obtained from STRING database. Analysis and visualization of PPIs were performed using Cytoscape. Interaction data only obtained from experiments were utilized with confidence score 0.4 or higher. Maximum 50 additional interactor proteins were allowed to construct the network. Proteins were shown in ellipses (nodes), and interacting proteins were connected by lines (edges). Degree and betweenness centrality of nodes were used to determine the width and height of the nodes, respectively. Cyan nodes represent down-regulated, and red nodes represent upregulated genes in both CIS and DOX treatments. Orange nodes represent differentially regulated genes in CIS and DOX treatments. White nodes are additional ones added by STRING database. Top bottleneck proteins were determined based on the highest betweenness values of nodes.

5.14. Validation of RNA-seq Data with qPCR

To validate RNA-seq analysis, we selected 18 randomly genes either up-regulated or downregulated listed in Table 3.1 at control WT and KO cells, and their expressions levels was analyzed by qPCR. Actin whose expression level did not vary between samples was used as internal reference gene. After the steps of isolating total RNA from cells and synthesizing of cDNA from total RNA, qPCR was carried out with primers. The fold change in gene expressions (folding changes calculated according to Actin gene, $2^{-\Delta\Delta CT}$ method) were in good agreement between RNA-seq and qPCR analysis (Figure 5.16). These results indicated that the RNA-sequencing results were reliable. The results obtained using both methods were comparable.

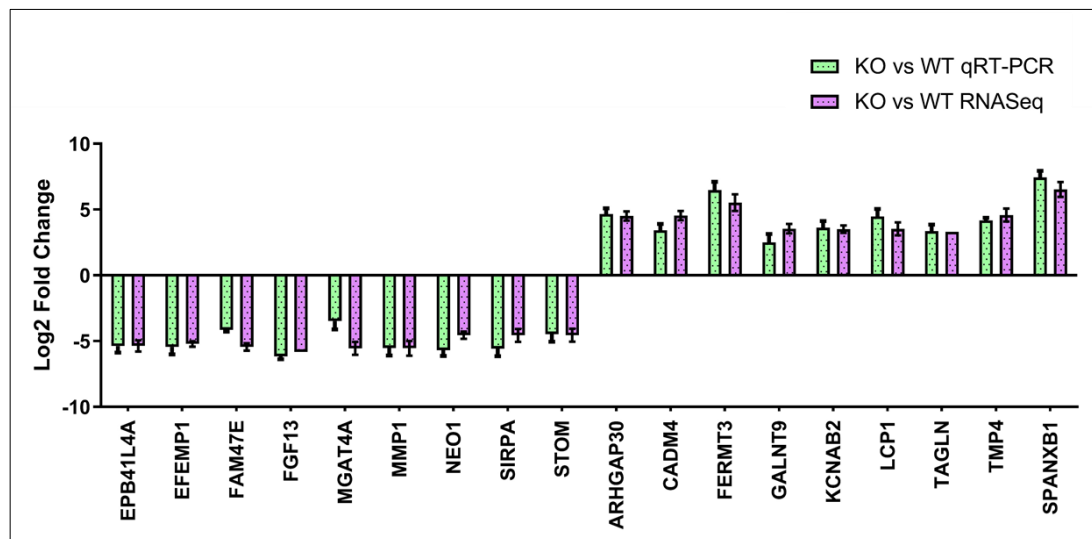


Figure 5.16: Validation of RNA-seq data with qPCR. 18 genes were selected from RNA-seq results of control WT and KO cells, and they were compared with qPCR results. Purple bars represent relative transcript levels analysed by qPCR. Green bars represent transcript levels analysed using RNA-seq. The results are averages of two technical repeats of two biological replicates.

6. DISCUSSION

The GSEA and PPI network analyses of the differentially expressed transcripts identified by RNA sequencing showed that *BMAL1* gene deletion increased anti-apoptotic and anti-tumorigenic pathways which agreed with our previous findings [Korkmaz et al., 2018]. The results obtained from the study provided the formation of the pathway model shown in Figure 5.15. This model illustrates how novel identified genes may be required for increased apoptosis after exposure to DNA damage (Figure 5.15 (a and b)). Moreover, the analysis of the pathways in tunicamycin treated samples reveals how the ER stress response is minimally affected by *BMAL1* deletion. The transcriptome analysis of *BMAL1*-deleted cells suggests that it affects multiple pathways which are in agreement with the observed pleiotropic phenotype as published previously [Korkmaz et al., 2018]. First, the increased cell motility and invasion profile are reflected by epithelial mesenchymal transition (EMT)-related gene expression profile (Figure 5.13 (a)). Another clue from KO vs WT comparison (Figure 5.13 (b)) indicates that *BMAL1* deletion affect the cell differentiation. It was suggested that *BMAL1* or *CLOCK* disruption in glioblastoma stem cells decreased the expression of core glioblastoma stem cell maintenance transcription factors, including SOX2, OLIG2, and MYC [Dong et al., 2019]. From bottleneck proteins, NACC1 is a transcriptional repressor involved in stem cell self-renewal and pluripotency conservation [Wang et al., 2006], [Ma et al., 2009]. Differentiation status of cell might affect the many event in cells such as nucleotide excision repair where the differentiation is accompanied by the increase expressions of xeroderma pigmentosum (XP) complementation group A (XPA), XPC, XPG, and XPF-ERCC1 [Li et al., 2019]. Conversely, the increased and decreased gene expression in bottleneck proteins agree with increased sensitivity to apoptosis. For example, anti-miR-135b sensitizes colorectal cancer cells to oxaliplatin-triggered cytotoxicity through the increase of *FOXO1* [Qin et al., 2018]. HDAC4 is an crucial part of the DNA damage response pathway that serves with 53BP1, and silencing of HDAC4 by RNA interference unexpectedly as well as reduced levels of 53BP1 protein, abrogated the DNA damage-induced G2 delay, and radiosensitized HeLa cells [Kao et al., 2003]. Therefore, it is possible that down-regulation of *HDAC4* may contribute to the sensitization of *BMAL1*

knockout cells to doxorubicin induced apoptosis. The significance of EGFR in regulating cellular response to cisplatin and IR treatment was previously established by demonstrating that EGFR enhances NHEJ by association with DNA-PKcs [Liccardi et al., 2011]. Ino80 is involved for efficient double-stranded break repair, and *Ino80* knockdown cells exhibit DNA repair defects [Gospodinov et al., 2011]. Down-regulation of this in doxorubicin-treated cells may contribute to the sensitization of doxorubicin-induced apoptosis. *GSK3β* is differentially regulated by cisplatin- and doxorubicin-treated KO cells. Deletion of *Cry* in *p53*-null mutant mice required inactivation of GSK3p. This does not cause the anti-apoptotic activity of NF-κB to be activated, that makes cells more sensitive to apoptosis induction by TNFα [Lee and Sancar 2011]. Even though we activated intrinsic apoptotic pathway in our model, the analysis suggests that GSK3β might also contribute to the differential sensitization of *BMAL1* knockout cells. STRING analysis put CDC5L, RPA1, NOTHC4, and AKT1 pathways among the interactions. *AKT1* knockout mice display impaired DNA damage dependent induction of p21 and increased tissue apoptosis [Bozulic et al., 2008]. For example, treating cells with doxorubicin increased the Ser-473 phosphorylation of AKT in mouse embryonic fibroblasts (MEFs) [Surucu et al., 2008]. Inhibition of the AKT pathway has been displayed to impair DNA repair after IR, resulting in radio-sensitization various cancers [Li et al., 2009]. Cell division cycle 5-like (CDC5L) protein is a cell cycle regulator of the G2/M transition and participate in DNA damage repair [Qiu et al., 2016]. Here, it is in the top of bottleneck protein, and its interaction with ATR directly indicates [Zhang et al., 2009] that it might be important for the sensitization of KO cell to apoptosis. RPA is required in all four major DNA repair pathways: nucleotide excision repair (NER), base excision repair (BER), DNA mismatch repair (MMR), and DNA double-stranded break (DSB) repair [Wold, 1997]. RPA is known to play role in DNA damage detection for NER pathway [Vasquez et al., 2002]; it also takes part in gap-filling reaction, along with PCNA, RFC, and DNA polymerase δ or ε in the late stages of NER [Aboussekhra et al., 1995]. RNAi-mediated ablations of *Notch4* in MDA-MB-231 cells displayed increased vulnerability to apoptosis, after treatment with DNA-damaging agents etoposide or 5-fluorouracil (5-FU) for 24 h [Saini and Sarin 2020]. In addition to expected data such as pathways related to DNA damage repair, apoptosis, and EMT, some pathways were unexpectedly affected. For instance, down-regulation of estrogen receptor signaling

early genes was significant after cisplatin treatment. As one member of this group, *IGF1R* (insulin-like growth factor 1 receptor) is extremely overexpressed in most malignant tissues where it serves as an antiapoptotic agent by increasing cell survival [Warshamana-Greene et al., 2005]. Therefore, down-regulation of *IGF1R* correlates with the sensitization to cisplatin-induced apoptosis.

However, inflammatory response genes were down-regulated after doxorubicin treatment. IL7 inhibits apoptosis in certain context, and its receptor is down-regulated after doxorubicin treatment [Liu et al., 2014]. Moreover, the genes from this group such as *IFNGR2*, *P2RY2*, *IL1R1*, *IL6*, and *CSF3* are known to be involved in apoptosis. This indicates that multiple genes from inflammatory response and EMT may co-operate in resistance to apoptosis and their down-regulation may sensitize KO cells to doxorubicin induced apoptosis.

Overall, the transcriptome analysis of MDA-MB-231 cells with a wild-type or deleted *BMAL1* gene reveals that *BMAL1* deletion affects multiple pathways which is not surprising considering that the circadian clock regulates 10% percent of the transcriptome. One significant change is at the differentiation status of the cells which can be also seen by the increased EMT capacity of *BMAL1* deleted cells. The differentiation status by itself may affect the stress responses such as DNA damage response. Moreover, the alterations in the DNA damage response pathways (as exemplified in the top bottleneck proteins) explain why these cells become sensitized to cisplatin- or doxorubicin-induced apoptosis.

The GSEA and PPI network analyses of the differentially expressed transcripts determined by RNA sequencing illustrated that *BMAL1* gene deletion in MDA-MB-231 cells increase apoptotic response to cisplatin and doxorubicin through multiple pathways. The DEG analysis was in agreement with the pleiotropic phenotype observed in this cell line such as sensitization to apoptosis or increased invasion/motility potential. The aim of this thesis was to describe the sensitization of this cell to DNA damage by constructing the pathway model shown in Figure 7. Overall, when we inspect the model, DEGs concentrate in pathways related to DNA damage, and differentiation status of cells is affected by the status of *BMAL1* gene. A minimal or no sensitization to tunicamycin by *BMAL1* deletion indicated that the sensitization of *BMAL1*-deleted cells to apoptosis is a specific event but not an intrinsic increased apoptotic nature of the cells.

This thesis and the presented model open new avenues for further investigation in explaining the sensitization of transformed MDA- MB-231 cells by *BMAL1* deletion upon chemotherapeutic anti-cancer drugs, cisplatin, and doxorubicin.

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BIOGRAPHY

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APPENDICES

Appendix A: 15 ribosomal, proteasomal, or polymerase-related genes list. They were excluded from the list due to their high degree of interactions with various proteins

TableA1.1: List of eliminated genes from PPI network analysis.

Eliminated Genes
AC090498.1
AC116533.1
AC135068.6
AC243732.1
AL139100.2
AL161909.1
POLR1A
POLR2J3
POLR2K
POLRMTP1
RPL23AP24
RPL7AP30
RPS27AP5
RPS2P55
RPS8P10

Appendix B: The list of genes used to construct PPI network

Table B1.1: The list of 318 genes used to construct PPI network.

Gene stable ID	Gene name
ENSG00000170161	AL512625.1
ENSG00000180066	LINC02870
ENSG00000197536	IRF1-AS1
ENSG00000223891	OSER1-DT
ENSG00000223960	CHROMR
ENSG00000225756	DBH-AS1
ENSG00000226007	BX005266.2
ENSG00000229956	ZRANB2-AS2
ENSG00000247708	STX18-AS1
ENSG00000254929	AL591684.2
ENSG00000257453	AC011611.3
ENSG00000258017	AC011603.3
ENSG00000259623	AC125257.1

Table B1.1: Continued

ENSG00000260917	AL158212.3
ENSG00000261534	AL596244.1
ENSG00000265415	AC099850.4
ENSG00000272084	AL137127.1
ENSG00000275964	AL355001.2
ENSG00000276077	CU633904.2
ENSG00000277011	AC148477.4
ENSG00000277067	CU634019.2
ENSG00000274012	RN7SL2
ENSG00000276168	RN7SL1
ENSG00000213757	RPS3AP20
ENSG00000214199	EEF1A1P12
ENSG00000224543	SNRPGP15
ENSG00000228502	EEF1A1P11
ENSG00000233270	SNRPEP4
ENSG00000233426	EIF3FP3
ENSG00000237818	RPS3AP29
ENSG00000240668	KRT8P36
ENSG00000242607	RPS3AP34
ENSG00000244289	RPS3AP35
ENSG00000247627	MTND4P12
ENSG00000248641	HMGA1P2
ENSG00000249264	EEF1A1P9
ENSG00000249855	EEF1A1P19
ENSG00000250182	EEF1A1P13
ENSG00000259612	EEF1A1P22
ENSG00000259781	HMGB1P6
ENSG00000264350	SNRPGP2
ENSG00000002016	RAD52
ENSG00000002746	HECW1
ENSG00000006062	MAP3K14
ENSG00000008083	JARID2
ENSG00000008128	CDK11A
ENSG00000011405	PIK3C2A
ENSG00000013619	MAMLD1
ENSG00000026652	AGPAT4
ENSG00000031081	ARHGAP31
ENSG00000037042	TUBG2
ENSG00000040933	INPP4A
ENSG00000049618	ARID1B
ENSG00000053254	FOXN3
ENSG00000054965	FAM168A
ENSG00000064042	LIMCH1
ENSG00000064393	HIPK2
ENSG00000066084	DIP2B
ENSG00000068024	HDAC4

Table B1.1: Continued

ENSG00000068354	TBC1D25
ENSG00000068383	INPP5A
ENSG00000068650	ATP11A
ENSG00000069493	CLEC2D
ENSG00000069702	TGFBR3
ENSG00000070018	LRP6
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ENSG00000072786	STK10
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ENSG00000073464	CLCN4
ENSG00000074964	ARHGEF10L
ENSG00000075340	ADD2
ENSG00000075391	RASAL2
ENSG00000079263	SP140
ENSG00000079308	TNS1
ENSG00000079691	CARMIL1
ENSG00000079841	RIMS1
ENSG00000080603	SRCAP
ENSG00000082458	DLG3
ENSG00000082701	GSK3B
ENSG00000088387	DOCK9
ENSG00000089723	OTUB2
ENSG00000101871	MID1
ENSG00000102024	PLS3
ENSG00000102878	HSF4
ENSG00000103227	LMF1
ENSG00000103319	EEF2K
ENSG00000104267	CA2
ENSG00000105426	PTPRS
ENSG00000105711	SCN1B
ENSG00000105875	WDR91
ENSG00000106069	CHN2
ENSG00000106683	LIMK1
ENSG00000106714	CNTNAP3
ENSG00000107957	SH3PXD2A
ENSG00000109066	TMEM104
ENSG00000109881	CCDC34
ENSG00000110841	PPFIBP1
ENSG00000111252	SH2B3
ENSG00000111961	SASH1
ENSG00000112319	EYA4
ENSG00000112379	ARFGEF3
ENSG00000112699	GMDS
ENSG00000113196	HAND1
ENSG00000113389	NPR3

Table B1.1: Continued

ENSG00000113811	SELENOK
ENSG00000114554	PLXNA1
ENSG00000115107	STEAP3
ENSG00000115183	TANC1
ENSG00000115738	ID2
ENSG00000116580	GON4L
ENSG00000118507	AKAP7
ENSG00000118515	SGK1
ENSG00000119681	LTBP2
ENSG00000119714	GPR68
ENSG00000119878	CRIP1
ENSG00000120656	TAF12
ENSG00000120784	ZFP30
ENSG00000120899	PTK2B
ENSG00000121966	CXCR4
ENSG00000123908	AGO2
ENSG00000124225	PMEPA1
ENSG00000125386	FAM193A
ENSG00000126001	CEP250
ENSG00000126778	SIX1
ENSG00000128656	CHN1
ENSG00000128833	MYO5C
ENSG00000128908	INO80
ENSG00000129667	RHBDF2
ENSG00000131323	TRAF3
ENSG00000131389	SLC6A6
ENSG00000132432	SEC61G
ENSG00000132541	RIDA
ENSG00000132846	ZBED3
ENSG00000133794	ARNTL
ENSG00000134046	MBD2
ENSG00000134531	EMP1
ENSG00000134780	DAGLA
ENSG00000134909	ARHGAP32
ENSG00000135443	KRT85
ENSG00000135905	DOCK10
ENSG00000136943	CTSV
ENSG00000136944	LMX1B
ENSG00000137225	CAPN11
ENSG00000137285	TUBB2B
ENSG00000137834	SMAD6
ENSG00000137944	KYAT3
ENSG00000138623	SEMA7A
ENSG00000138759	FRAS1
ENSG00000139292	LGR5
ENSG00000140262	TCF12

Table B1.1: Continued

ENSG00000140470	ADAMTS17
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ENSG00000142185	TRPM2
ENSG00000143776	CDC42BPA
ENSG00000144711	IQSEC1
ENSG00000145012	LPP
ENSG00000145555	MYO10
ENSG00000146648	EGFR
ENSG00000148082	SHC3
ENSG00000148655	LRMDA
ENSG00000148737	TCF7L2
ENSG00000148950	IMMP1L
ENSG00000150456	EEF1AKMT1
ENSG00000150907	FOXO1
ENSG00000151611	MMAA
ENSG00000152104	PTPN14
ENSG00000152818	UTRN
ENSG00000153993	SEMA3D
ENSG00000154127	UBASH3B
ENSG00000154229	PRKCA
ENSG00000155846	PPARGC1B
ENSG00000155918	RAET1L
ENSG00000156011	PSD3
ENSG00000156030	MIDEAS
ENSG00000156050	FAM161B
ENSG00000157193	LRP8
ENSG00000157350	ST3GAL2
ENSG00000157869	RAB28
ENSG00000157890	MEGF11
ENSG00000158234	FAIM
ENSG00000159267	HLCS
ENSG00000160190	SLC37A1
ENSG00000160293	VAV2
ENSG00000160877	NACC1
ENSG00000160991	ORAI2
ENSG00000161249	DMKN
ENSG00000161544	CYGB
ENSG00000162772	ATF3
ENSG00000162825	NBPF20
ENSG00000163040	CCDC74A
ENSG00000163110	PDLIM5
ENSG00000163362	INAVA
ENSG00000163637	PRICKLE2
ENSG00000164221	CCDC112
ENSG00000164342	TLR3

Table B1.1: Continued

ENSG00000164542	KIAA0895
ENSG00000164920	OSR2
ENSG00000165895	ARHGAP42
ENSG00000166233	ARIH1
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ENSG00000166451	CENPN
ENSG00000166455	C16orf46
ENSG00000166762	CATSPER2
ENSG00000166803	PCLAF
ENSG00000167552	TUBA1A
ENSG00000168646	AXIN2
ENSG00000168758	SEMA4C
ENSG00000168874	ATOH8
ENSG00000169239	CA5B
ENSG00000169246	NPIP3
ENSG00000169851	PCDH7
ENSG00000170190	SLC16A5
ENSG00000170412	GPRC5C
ENSG00000170523	KRT83
ENSG00000171105	INSR
ENSG00000171132	PRKCE
ENSG00000171310	CHST11
ENSG00000171408	PDE7B
ENSG00000171806	METT18
ENSG00000171843	MLLT3
ENSG00000172059	KLF11
ENSG00000172164	SNTB1
ENSG00000172197	MBOAT1
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ENSG00000172687	ZNF738
ENSG00000172731	LRRC20
ENSG00000173889	PHC3
ENSG00000173915	ATP5MD
ENSG00000174010	KLHL15
ENSG00000174695	TMEM167A
ENSG00000174705	SH3PXD2B
ENSG00000175183	CSRP2
ENSG00000175931	UBE2O
ENSG00000176845	METRNL
ENSG00000179172	HNRNPCL1
ENSG00000180530	NRIP1
ENSG00000182263	FIGN
ENSG00000182389	CACNB4
ENSG00000182704	TSKU
ENSG00000183049	CAMK1D
ENSG00000183060	LYSMD4

Table B1.1: Continued

ENSG00000183150	GPR19
ENSG00000183397	C19orf71
ENSG00000183508	TENT5C
ENSG00000183826	BTBD9
ENSG00000183853	KIRREL1
ENSG00000183943	PRKX
ENSG00000184371	CSF1
ENSG00000184384	MAML2
ENSG00000184678	H2BC21
ENSG00000184985	SORCS2
ENSG00000185164	NOMO2
ENSG00000185338	SOCS1
ENSG00000185829	ARL17A
ENSG00000186174	BCL9L
ENSG00000186523	FAM86B1
ENSG00000186868	MAPT
ENSG00000187790	FANCM
ENSG00000188064	WNT7B
ENSG00000188219	POTEE
ENSG00000188910	GJB3
ENSG00000196604	POTEF
ENSG00000196834	POTEI
ENSG00000197321	SVIL
ENSG00000197535	MYO5A
ENSG00000197555	SIPA1L1
ENSG00000197646	PDCD1LG2
ENSG00000197852	INKA2
ENSG00000203326	ZNF525
ENSG00000204160	ZDHHC18
ENSG00000204272	NBDY
ENSG00000204366	ZBTB12
ENSG00000204632	HLA-G
ENSG00000205423	CNEP1R1
ENSG00000211448	DIO2
ENSG00000212724	KRTAP2-3
ENSG00000215790	SLC35E2A
ENSG00000215915	ATAD3C
ENSG00000222038	POTEJ
ENSG00000223953	C1QTNF5
ENSG00000228696	ARL17B
ENSG00000242265	PEG10
ENSG00000251322	SHANK3
ENSG00000253626	EIF5AL1
ENSG00000261371	PECAM1
ENSG00000267645	AC105052.3
ENSG00000269028	MTRNR2L12

Table B1.1: Continued

ENSG00000270136	MICOS10-NBL1
ENSG00000270757	HSPE1-MOB4
ENSG00000273136	NBPF26
ENSG00000274791	F8A2
ENSG00000275023	MLLT6
ENSG00000275740	AC091959.3
ENSG00000275774	HNRNPCL2
ENSG00000275832	ARHGAP23
ENSG00000214110	LDHAP4
ENSG00000224287	MSL3P1
ENSG00000233369	GTF2IP4
ENSG00000106133	NSUN5P2
ENSG00000135477	KRT87P
ENSG00000185684	EP400P1
ENSG00000186704	DTX2P1
ENSG00000204622	HLA-J
ENSG00000214425	LRRC37A4P
ENSG00000215154	AC141586.1
ENSG00000234231	ANAPC1P4
ENSG00000263563	UBBP4
ENSG00000203761	MSTO2P
ENSG00000204745	AC083899.1
ENSG00000205578	POM121B
ENSG00000206341	HLA-H
ENSG00000226102	SEPTIN7P3
ENSG00000227232	WASH7P
ENSG00000227879	PSPC1P1
ENSG00000260075	NSFP1
ENSG00000279457	WASH9P

APPENDIX C: Protein Marker

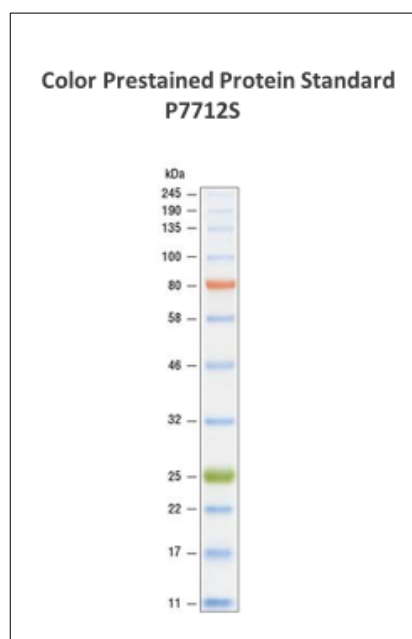


Figure C1.1: Protein Marker.

APPENDIX D: Permissions for Copyrighted Materials

Table D.1.1: Permissions for copyrighted materials.

FIGURE NO	LICENCE DATE	LICENCE NO	COPYRIGHT OWNER
Figure 4.3 [Chaitankar et al., 2016].	-	Free to: Share — copy and redistribute the material in any medium or format The licensor cannot revoke these freedoms as long as you follow the license terms.	-
Figure 4.1 [Emisoglu-Kulahlı et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.1 [Emisoglu-Kulahlı et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.2 [Emisoglu-Kulahlı et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.3 [Emisoglu-Kulahlı et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature

Table D1.1: Continued

Figure 5.4 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.6 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.7 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.8 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.9 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.10 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.11 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.12 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.13 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.14 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.15 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Figure 5.16 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature
Table 5.2 [Emisoglu-Kulahli et al., 2021]	Jan 23, 2021	4994910490121	Springer Nature

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

Emisoglu-Kulahli H., Gul S., Morgil H., Ozcan O., Aygenli F., Selvi S., Kavakli I. H., Ozturk N., (2021), “Transcriptome analysis of the circadian clock gene *BMAL1* deletion with opposite carcinogenic effects”, Functional Integrative Genomics, 21, 1-16.