

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**INVESTIGATING PROTEIN VARIATIONS OF BAG-1 IN WILD TYPE AND
BAG-1 KNOCKOUT MCF-7 BREAST CANCER CELLS**



Ph.D. THESIS

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Molecular Biology Genetics and Biotechnology Department

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**DOĞAL TİP VE BAG-1 SİLİNİMİŞ MCF-7 MEME KANSERİ HÜCRELERİNDE
BAG-1'İN PROTEİN DEĞİŞİKLİKLERİNİN İNCELENMESİ**

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To my lovely daughter,



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ABBREVIATIONS

AAV	: Adeno-associated virus
AGO	: Argonaute protein
AJCC	: American Joint Committee on Cancer
AMT	: Amoeboid mesenchymal transition
ATM	: Ataxia telangiectasia mutated
ATR	: Ataxia telangiectasia and Rad3 related
Bag-1	: Bcl-2-associated athanogene 1
Bag-1 KO	: Bag-1 knockout plasmid vector-transfected cell model
Bag-1 OE	: Bag-1 overexpression plasmid vector-transfected cell model
BLAST	: Basic Local Alignment Search Tool
Cas	: CRISPR-associated proteins
CBh	: Chicken β -actin hybrid
Control KO	: Control knockout plasmid vector-transfected cell model
CRISPR	: Clustered regularly interspaced short palindromic repeats
crRNA	: Crispr RNA
DAVID	: Database for Annotation, Visualization, and Integrated Discovery
DCIS	: Ductal Carcinoma in situ
DGCR	: Double-stranded RNA binding protein DiGeorge syndrome critical region 8
DiOC₆	: 3,3'-dihexyloxacarbocyanine iodide
DNA	: Deoxyribonucleic acid
DNA-PK	: DNA dependent protein kinase
dNTP	: Deoxynucleoside triphosphate
DSB	: Double-strand break
ECM	: Extracellular matrix
EF1a	: Elongation Factor 1a
EMT	: Epithelial-mesenchymal transition
ER	: Estrogen Receptor
ERBB2	: Epidermal Growth Factor 2
FAK	: Focal adhesion kinase
FDA	: Food and Drug Administration
FDR	: False Discovery Rates
Gab1	: Grb2-associated binding protein 1
GFP	: Green Fluorescent Protein
Grb2	: Growth factor receptor-bound protein 2
HDR	: Homology-directed repair
HER2	: Human epidermal growth factor receptor 2
HGF	: Hepatocyte growth factor
Hsp90	: Heat shock protein 90
IHC	: Immunohistochemistry
IRES	: Internal ribosome entry site
JAK	: Janus kinase
LoxP	: Locus of X-over P1

MA	: Mean average
MAPK	: Mitogen-activated protein kinase
MAT	: Mesenchymal amoeboid transition
MET	: Mesenchymal epithelial transition
miRNA	: Micro RNA
miRISC	: miRNA induced silencing complex
MMP	: Matrix metalloproteinases
mTOR	: Mammalian target of rapamycin
mTORC1	: Mammalian target of rapamycin complex 1
mTORC2	: Mammalian target of rapamycin complex 2
NGS	: Next-generation sequencing
NHEJ	: Non-homologous end joining
NLS	: Nuclear localization signal
Pax2	: Paired box 2
P-bodies	: processing bodies
PCR	: Polymerase chain reaction
PDK1	: Phosphoinositide-dependent kinase 1
PI3K	: Phosphoinositide 3-kinase
PIP2	: Phosphatidylinositol-4,5-bisphosphate
PIP3	: Phosphatidylinositol-3,4,5-trisphosphate
Pol II	: RNA polymerase II
pre-miRNAs	: precursor miRNAs
pri-miRNAs	: primary miRNAs
PPI	: Protein-protein interaction
PTEN	: Phosphatase and tensin homolog deleted on chromosome 10
PTK	: Protein tyrosine kinase
Rb	: The retinoblastoma protein
RFP	: Red Fluorescent Protein
ROCK	: RHO-associated protein kinase
ROS	: Reactive Oxygen Species
RTK	: Receptor Tyrosine Kinase
SBS	: Sequencing by synthesis
SEER	: Surveillance, Epidemiology, and End Results
sgRNA	: Single-guide RNA
STAT	: Signal transducer and activator of transcription
STRING	: Search Tool for the Retrieval of Interacting Genes/Proteins
TALEs	: Transcription activator-like effectors
TALENs	: Transcription activator-like effector nucleases
TPM	: Transcripts per million
TSC1/2	: Tuberous sclerosis complex 1 and 2
UTR	: Untranslated region
XPO5	: Exportin 5
ZEB1	: Zinc finger E-box binding homeobox 1
ZFNs	: Zinc Finger Nucleases

SYMBOLS

C	: Celcius
L	: Liter
ml	: milliliter
M	: molar
mM	: millimolar
μl	: microliter
μm	: micrometer





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INVESTIGATING PROTEIN VARIATIONS OF BAG-1 IN WILD TYPE AND BAG-1 KNOCKOUT MCF-7 BREAST CANCER CELLS

SUMMARY

The multifunctional Bag-1 protein which is known for its anti-apoptotic role has many critical direct or indirect interaction partners in the cell. According to these interactions, the Bag-1 protein plays an important role in the decision mechanism between survival or death of cancer cells. In previous studies determining the chromosomal location of the *Bag-1* gene, it has been stated that this gene is associated with neurodegenerative diseases such as Alzheimer's and Parkinson's, and cancer. It has been shown that the change in the expression level of Bag-1 protein in the cell is associated with many different cancer types. In addition, it has been demonstrated that Bag-1 may provide clinical benefit as a prognostic marker in the determination of breast cancer, which is the first among women with its incidence. Elevated levels of Bag-1 are generally associated with breast cancer growth, development, and aggressiveness. In particular, interactions with the Hsc70/Hsp70 chaperone family are effective in the long-term survival of breast cancer cells under stress conditions. Our previous studies showed that silencing the Bag-1 protein increases drug-induced apoptosis in breast cancer. Although there are several studies on the association of changes in the expression level of the Bag-1 protein with cancer, the biological aspect of the complete deletion of the *Bag-1* gene in breast cancer cells is not detailed explained. Therefore, this thesis study consists of two parts covering the molecular function of the deletion of the *Bag-1* gene in MCF-7 breast cancer cells. The first part of the thesis consists of the generation of Bag-1 knockout cells with the CRISPR/Cas9 system, the validation and characterization, and the investigation of the effect of Bag-1 deficiency on the viability and cellular response of MCF-7 cells. The second part of the thesis includes the profiling of differentially expressed microRNAs and the determination of the molecular targets of these significantly differentially expressed miRNAs in wild-type and Bag-1 knockout MCF-7 breast cancer cells.

In the first part of the thesis, the Bag-1 deficient MCF-7 cells were generated by CRISPR/Cas9, one of the current gene-editing techniques, and the Bag-1 knockout cell models were validated and characterized. In this study, a plasmid containing the leader RNA sequences was specifically designed for 3 different exon regions of the Bag-1 gene co-transfected with another plasmid that performs the repair mechanism by homologous recombination. Bag-1 knockout cells were grown from a single cell colony, and the deletion of Bag-1 was controlled by the lack of Bag-1 expression in selected cell colonies. Bidirectional Sanger sequencing was performed in the selected colony that showed the loss of Bag-1 expression, and the deletions were determined in both chains of *Bag-1* gene regions. Studies showed that the viability, proliferation, and colony formation potentials of MCF-7 cells were decreased in Bag-1 knockout conditions compared to wt cells. To examine the relationship between breast cancer development and Bag-1 deficiency, the phosphorylation profiles of receptor tyrosine kinases were determined which have important roles in breast cancer. For this purpose,

phosphorylation of 39 different kinase proteins functioning in 11 different signaling pathways was determined by receptor tyrosine kinase array. The expression level of phospho-Akt Ser 473 was observed markedly increased in Bag-1 deleted MCF-7 cells based on the normalized densitometry and immunoblotting analysis. Therefore, to examine the relationship between Akt phosphorylation and Bag-1 deprivation, MK-2206, a potential Akt inhibitor, was used to examine the upstream and downstream signaling pathways of the Akt signaling pathway in wild-type, Bag-1 deleted and Bag-1 overexpressed MCF-7 breast cancer cells. Considering the Akt-mediated regulation of actin filaments and the epithelial-mesenchymal transition in cancer, our study showed the expression profiles of β -actin and α -actinin were markedly downregulated in Bag-1 knockout cells, whereas Akt inhibition upregulated the expression of these proteins. The epithelial-mesenchymal transition properties of Bag-1 knockout MCF-7 cells were determined by downregulation of epithelial E-cadherin, and upregulation of N-cadherin expression levels. To confirm the potential role of Akt phosphorylation-mediated actin dysregulation in Bag-1 knockout cells, Bag-1 isoforms (S, M, and L) specifically were transiently transfected into Bag-1 deleted MCF-7 cells, respectively. Specific transfection of Bag-1 isoforms into Bag-1 knockout cells caused downregulation of Akt phosphorylation and mesenchymal markers, while upregulated expression levels of actin cytoskeletal proteins β -actin, α -actinin, and the focal adhesion proteins. In the first part of the thesis, it was concluded that the deletion of Bag-1 increased stress-induced Akt activation in MCF-7 cells, caused more mesenchymal and stemness properties through cytoskeletal remodeling.

In the second part of the thesis, non-coding miRNAs, which have important roles in gene expression regulation in wt and Bag-1 knockout cells were determined by high throughput sequencing. The target genes and molecular signaling pathways associated with significantly differentially expressed miRNAs were determined. For this purpose, total RNA was isolated in wt and Bag-1 KO cells, and small RNA sequencing was performed by next-generation sequencing after the generation of the cDNA library. The results obtained after the sequencing were analyzed using Galaxy open, a web-based platform. Initial quality control of raw reads of sequencing was performed by FASTQC, then adapter sequences were trimmed with the Cutadapt program, and sequences between 18-35 base pairs in length were selected. The obtained reads were aligned to the GRCh37-hg19 human genome with the Bowtie2 program and annotated with the human mature miRNA data library with the featureCounts program. Differential expression analysis was performed with DESeq2 and the expression levels of the miRNAs were determined as the fold change between wt and Bag-1 deleted cells. Within the sequenced 25 miRNAs, 11 miRNAs were found significantly upregulated and 14 miRNAs were downregulated, and 14 of 25 miRNAs were experimentally validated. hsa-miR-21-3p/-5p, hsa-miR-429, hsa-miR-200a-5p, hsa-miR-27a-3p, hsa-miR-342-5p, hsa-miR-345-5p, hsa-miR151a-3p, has-miR-146b-5p were found significantly downregulated compared to wt samples, and hsa-miR-130b-3p, hsa-miR-4521, hsa-miR-887-3p, hsa-miR-767-5p, hsa-miR-31-5p ve hsa-miR-10a-5p upregulated. The biological and functional enrichment of significantly expressed differentially expressed miRNAs in Bag-1 knockout cells compared to wt cells was performed using GO and KEGG enrichment analysis. hsa-miR-429 was selected as a potential miRNA in Bag-1 KO MCF-7 cells because of the downregulation both in bioinformatics and validation qRT-PCR assay. It was observed from our results that the predicted gene targets of hsa-miR-429 were phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha (PIK3CA), kinase insert domain receptor (KDR), GRB2 associated binding protein 1 (GAB1),

Rac family small GTPase1 (RAC1), vascular endothelial growth factor A (VEGFA), Cbl proto-oncogene (CBL), syndecan 2 (SDC2), phospholipase C gamma 1 (PLCG1), E1A binding protein p300 (EP300), and CRK like proto-oncogene, adaptor protein (CRKL) which are significantly enriched in PI3K/Akt signaling, cell migration, cell adhesion, focal adhesion, integrin binding, and actin cytoskeleton regulation.

The thesis study, which consists of two interconnected parts, is a novel understanding of the elucidation of the molecular mechanism of Bag-1 deletion in breast cancer cells.





DOĞAL TIP VE BAG-1 SİLİNİMİŞ MCF-7 MEME KANSERİ HÜCRELERİNDE BAG-1'İN PROTEİN DEĞİŞİKLİKLERİNİN İNCELENMESİ

ÖZET

Anti-apoptotik rolü ile bilinen Bag-1 proteinin hücre içerisine direk ya da indirek olarak etkileşimde bulunduğu bir çok kritik etkileşim partneri bulunmaktadır. Bu etkileşimleri sayesinde Bag-1 proteini özellikle kanser hücrelerinin canlılık ya da ölüm arasındaki karar mekanizmasında önemli bir rol üstlenmektedir. Bag-1 geninin kromozomal lokasyonunun belirlenmesi amacıyla yapılan çalışmalarda bu genin başta kanser olmak üzere Alzheimer, Parkinson gibi nörodejeneratif hastalıklarla ilişkisi olduğu belirtilmiştir. Bag-1 proteinin hücre içerisindeki ifade seviyesi değişiminin bir çok farklı kanser tipi ile ilişkisi belirlenmiş ve kadınlar arasında görülme sıklığı ile birinci sırada yer alan meme kanserinin belirlenmesinde prognostik bir belirteç olarak klinik fayda sağlayabileceği ortaya konmuştur. Bunun yanında, artan Bag-1 seviyesi genellikle meme kanseri büyümesi, gelişmesi ve agresifliği ile ilişkilendirilmiştir. Özellikle Hsc70/Hsp70 şaperon ailesi ile yapmış olduğu etkileşimler meme kanseri hücrelerinin stres koşulları altında uzun süreli hayatta kalmalarında etkili olduğu görülmüştür. Bunun yanında, Bag-1 proteinin susturulmasının meme kanserinde ilaçla tetiklenen apoptozu arttırdığı yaptığımız çalışmalar ile belirlenmiştir. Bag-1 proteinin ifade seviyesindeki değişikliğin kanser ile ilişkisine ait bir dizi çalışma olmasına rağmen, meme kanseri hücrelerinde Bag-1 geninin tamamen silinmesinin biyolojik yönü hakkında çok sınırlı bilgi bulunmaktadır. Bu nedenle bu tez çalışması, MCF-7 meme kanseri hücrelerinde Bag-1 geninin silinmesinin moleküler fonksiyonunun araştırılmasını kapsayan iki bölümden oluşmaktadır. Tezin ilk bölümü Bag-1 geninin CRISPR/Cas9 sistemi ile silinmesi, karakterizasyonu, MCF-7 hücrelerinin canlılığına olan etkisinin araştırılması ve hücre canlılığını tetikleyici sinyal yollarındaki proteomik değişimlerin incelenmesini içermektedir. Tezin ikinci bölümü ise Bag-1 geninin silinmesine özgü olarak MCF-7 meme kanseri hücrelerindeki miRNA (mikroRNA) profilinin çıkarılması ve miRNA'lardan önemli derecede değişiklik gösterenlerin moleküler hedeflerinin belirlenerek hücre içerisindeki fonksiyonlarının araştırılmasını içermektedir.

Tezin ilk bölümünde güncel gen düzenleme tekniklerinden CRISPR/Cas9 ile Bag-1 geninin silindiği MCF-7 meme kanseri hücre modeli oluşturulmuş ve karakterizasyon çalışması yapılmıştır. Bu yöntemde 7 eksondan oluşan Bag-1 geninin 3 farklı ekson bölgesine spesifik olarak tasarlanan lider RNA dizilerini içeren bir plazmit ile homolog rekombinasyon ile tamir mekanizması gerçekleştiren ikinci bir plazmit eş zamanlı olarak MCF-7 meme kanseri hücrelerine transfekte edilmiştir. Tek bir hücre kolonisinden büyütülerek elde edilen bu hücre modelinde Bag-1 geninin silinmesi farklı kolonilerdeki Bag-1 ifadesinin yoksunluğu ile kontrol edilmiştir. Seçilen spesifik koloni büyütülerek iki yönlü olarak Sanger dizileme analizi sonrasında her iki zincirde de Bag-1 geninde delesyonlar olduğu tespit edilmiştir. Karakterizasyonu yapılan Bag-1 geni silinmiş hücrelerin doğal tip hücrelere kıyasla MCF-7 meme

kanseri hücrelerinin canlılık, proliferasyon ve koloni oluşturma potansiyellerini azalttığı belirlenmiştir. Meme kanseri gelişimi ile Bag-1 yoksunluğu arasındaki ilişkinin incelenmesi için meme kanseri gelişiminde önemli rolleri olan reseptör tirozin kinaz proteinlerinin fosforilasyon profilleri belirlenmiştir. Bu amaçla 11 farklı sinyal yolunda rolü olan toplam 39 farklı kinaz proteinin fosforilasyonu reseptör tirozin kinaz yolak tarama testi ile tespit edilmiştir. Normalize densitometrik analizlere ve immunoblot analizlerine göre p-Akt Ser 473 ifade seviyesi Bag-1 silinmiş hücrelerde önemli derecede artış gösterdiği belirlenmiştir. Bu sebeple Akt fosforilasyonu ve Bag-1 yoksunluğu arasındaki ilişkinin incelenmesi için potansiyel bir Akt inhibitörü olan MK-2206 kullanılarak doğal tip, Bag-1 silinmiş ve Bag-1 aşırı ifadesine sahip MCF-7 meme kanseri hücrelerinde Akt sinyal yolunun üst ve alt sinyal yollarına ait hedeflerin total protein profilleri belirlenmiştir. Akt aracılı aktin filamentlerinin regülasyonu ve epitel-mezenkimal dönüşüm profili incelendiğinde β -actin ve α -actinin proteinlerinin Bag-1 silinmiş hücrelerde önemli derecede azaldığı ancak Akt inhibisyonunun bu proteinlerin ifadesinde artışa sebep olduğu görülmüştür. Bag-1 silinmiş hücrelerdeki mezenkimal dönüşümün belirteci olarak da N-cadherin ifadesinin artışı gözlemlenmiş ancak Akt inhibisyonu ile N-cadherin ifadesinin azaldığı immunoblotting analizi belirlenmiştir. Bag-1 silinmesi ile Akt fosforilasyonu aracılı aktin düzensizliğinin potansiyel rolünü doğrulamak için Bag-1 silinmiş MCF-7 hücrelerine spesifik olarak Bag-1 izoformları (S, M, L) geçici olarak transfekte edilmiş ve Bag-1 izoformları sırası ile hücrelere geri kazandırılmıştır. Bag-1 izoformlarının spesifik olarak Bag-1 silinmiş hücrelere geri kazandırılması Akt fosforilasyonunda ve mezenkimal belirteçlerin ifadelerinde azalmaya sebep olurken aktin iskeleti proteinlerinden β -actin, α -actinin ve fokal adhezyon proteinlerinin ifade seviyelerindeki artış hem immunoblot analizi hem de immüno boyama ile gösterilmiştir. Tezin ilk kısmında Bag-1 geninin silinmesinin MCF-7 hücrelerinde strese-bağlı Akt aktivasyonunu arttırdığı, adhezyon ve hücre iskelet yapılarının bozularak farklılaşma sürecine girdiği ve strese bağlı-uzun süreli büyüme durumu gösterdikleri kanısına varılmıştır.

Tezin ikinci bölümünde, MCF-7 doğal tip ve Bag-1 silinmiş hücrelerde gen anlatımı düzenlenmesinde önemli rolleri olan kodlama yapmayan miRNA'lar yeni nesil dizileme yöntemi ile saptanmıştır. Belirlenen bu miRNA'ların regüle ettiği hedef genlerin ve bu genlerin rol aldıkları moleküler sinyal yolları belirlenmiştir. Bu amaçla, MCF-7 doğal tip ve Bag-1 silinmiş hücrelerden total RNA izole edilmiş, cDNA kütüphanesinin oluşturulmasının ardından yüksek verimli yeni nesil dizileme yöntemi ile küçükRNA dizilemesi yapılmıştır. Yapılan dizileme çalışmasının ardından elde edilen sonuçlar çevrimiçi usegalaxy.org. internet platformu kullanılarak analiz edilmiştir. Dizilemenin ilk kalite kontrolü FASTQC tarafından yapılmış, ardından adaptör diziler Cutadapt programı ile kırılmış ve uzunluğu 18-35 baz çifti arasında olan diziler seçilmiştir. Elde edilen dizilerin Bowtie2 programı ile GRCh37-hg19 insan genomu ile sıralanmış ve feauteCounts programı ile insan olgun miRNA data kütüphanesi ile anotasyonu gerçekleştirilmiştir. Differansiyel ekspresyon analizi DESeq2 ile yapılmış ve belirlenen miRNA'ların ifade seviyeleri wt ile Bag-1 silinmiş hücreler arasındaki kat değişimi olarak ifade edilmiştir. GO ve KEGG zenginleştirilmesi sonucu diferansiyel olarak eksprese edilen miRNA'ların biyolojik fonksiyonları elde edilmiştir. Sekanslama sonucunda 2576 miRNA elde edilmiş ve bunlardan 25 tanesinin diferansiyel olarak eksprese olduğu belirlenmiştir. Bag-1 silinmiş hücrelerde, 25 tane miRNA'dan 11 tanesinin ifadesinin önemli oranda arttığı ve 14 tanesinin de önemli oranda ifadesinin azaldığı belirlenmiştir. Önemli oranda ifade seviyesi değişikliği gösteren miRNA'ların toplamda 14 tanesinin kantitatif PCR ile ifade seviyeleri doğrulanmıştır. Bag-1 silinmiş hücrelerde, hsa-miR-21-3p/-5p, hsa-

miR-429, hsa-miR-200a-5p, hsa-miR-27a-3p, hsa-miR-342-5p, hsa-miR-345-5p, hsa-miR151a-3p, has-miR-146b-5p'nin ifadesinin doğal tip hücrelere göre azaldığı, hsa-miR-130b-3p, hsa-miR-4521, hsa-miR-887-3p, hsa-miR-767-5p, hsa-miR-31-5p ve hsa-miR-10a-5p'nin ifadesinin arttığı belirlenmiştir.

Birbirine bağlı iki bölümden oluşan tez çalışması, meme kanseri hücrelerinde stabil olarak Bag-1 geninin silinmesi, bu genin silinmesinin hücre içerisinde meydana getirdiği genomik, transkriptomik ve proteomik değişikliklerin belirlenmesi ve bu değişikliklerin birbirleri ile olan ilişkisinin anlaşılması için ilk çalışma olmaktadır.





1. INTRODUCTION

Breast cancer is the most common cause of cancer-related death with the highest incidence rates among women worldwide. The fact that breast cancer is a heterogeneous disease like many other cancer types due to factors in gene-environment interactions. Considering the genetic factors, the high penetrance of BRCA1, BRCA2, ATM, and CHEK2 mutations contributes to breast cancer incidence [1]. Besides, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and Ki-67 are important diagnostic factors for this disease [2]. Despite such known genetic risk factors, breast cancer does not progress in the same way in all patients. Therefore, novel molecular biomarkers have been required to find specific therapies for different patients.

As one of the potential additional molecular biomarkers, Bag-1 (Bcl-2-associated athanogene 1) has been known for its anti-apoptotic function and survival targeting role in the development of breast cancer. Clinical studies have demonstrated that Bag-1 has been used as a prognostic biomarker predicting postoperative prognosis and understanding the potential benefits of chemotherapy [3], [4]. Considering the results of a recent systematic review with Bag-1 meta-analyses in 2017, high levels of Bag-1 were associated with increased pro-survival data in breast cancer patients. Of the twenty clinicopathological studies using cohorts of approximately 100 patients between 1999-2016, fourteen showed that elevated levels of Bag-1 expression were a prognostic marker in improved breast cancer outcomes. Besides, immunohistochemical staining of Bag-1 in the clinic also contributed to the therapies in the different risk groups of patients' better outcomes [5].

1.1 Purpose of Thesis

High levels of Bag-1 mRNA and protein expression have been shown to provide clinical benefit as a prognostic marker for the detection of breast cancer and were associated with growth, development, and aggressiveness of breast cancer. Although many studies are showing that increased Bag-1 expression and the development of

breast cancer are correlated, there is very limited research on the biological function of complete deletion of Bag-1 gene in breast cancer. Therefore, the main purpose of the thesis was to understand the molecular function of the Bag-1 deficiency in the development of breast cancer. The first part of the thesis involves the comparison of survival and proliferation of MCF-7 breast cancer cells under Bag-1 knockout and Bag-1 overexpressed conditions and investigation of cellular pathways associated with survival, differentiation, and metastasis. The second part of the thesis consists of understanding the expression, regulation, or dysregulation of miRNA mechanism associated with the survival of MCF-7 cells in Bag-1 deficiency. We performed miRNA screening since the change in many signaling pathways in Bag-1 deficiency can be post-transcriptionally regulated of genes in these signaling pathways by miRNAs. The target genes and associated molecular pathways have been identified by high-throughput screening to lead the development of future Bag-1-based therapy approaches.

1.2 Hypothesis

Considering the studies that elevated Bag-1 expression enhances breast cancer development and the silencing of Bag-1 increases the chemotherapeutical effects, we hypothesized that if the Bag-1 gene is completely deleted the cell viability and breast cancer progression would be completely affected. In addition, we also hypothesized that the alternation of breast cancer cellular response is due to both Bag-1 deficiency and the regulation of other mechanisms in the cell because of the heterogenic characteristics of breast cancer.

2. CHAPTER II- INVESTIGATION OF THE MOLECULAR FUNCTION OF BAG-1 DELETION BY COMPARING DIFFERENT BAG-1 EXPRESSION LEVELS IN MCF-7 CELLS

2.1 Purpose

Considering the positive association between high Bag-1 expression and cell survival mechanism in breast cancer cells is regarded as a potential predictive marker of the clinical outcome of breast cancer [6]. However, downregulation of Bag-1 expression sensitized and enhanced the apoptotic potential of cisplatin and paclitaxel in MCF-7 cells [7]. Despite the evidence about survival-related functional roles of Bag-1 in breast cancer, there has been limited data about its other cellular functions. In the first part of the thesis, we aimed to generate a Bag-1 knockout (KO) MCF-7 breast cancer cell model using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR-associated 9 (Cas9) technique comparing the effect of different Bag-1 expression levels (basal, knockout, and overexpressed) on the EMT signaling and actin remodeling.

2.1.1 Breast cancer

Breast cancer is the most commonly diagnosed cancer type with an estimated 2.3 million new cases, and the highest morbidity among women worldwide but the mortality rates are decreasing due to early diagnosis and combinatorial chemotherapeutical approaches [8]. According to the GLOBOCAN 2020 data, breast cancer constitutes 11.7% of all cancer cases and ranks fifth in the cause of cancer mortality with 685,000 deaths. Breast cancer showed the highest incidence (24,5%) and mortality rates (15,5%) among women of all ages in the world. The prevalence and mortality rates of breast cancer increase depending on age, approximately 95% of the new breast cancer cases have been seen in women 40 years of age and older and mostly in developed countries [9]. Figure 2.1 showed the region-specific incidence and mortality rates of breast cancer worldwide.

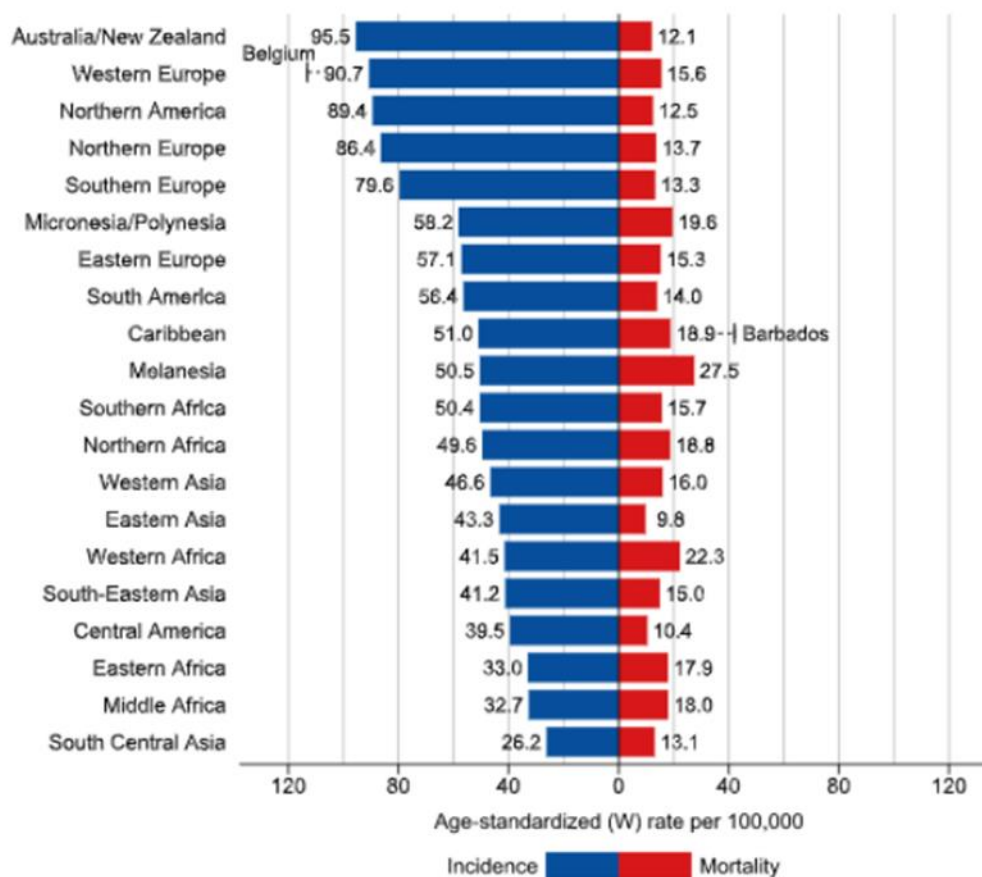


Figure 2.1 : Breast cancer estimated age-standardized incidence and mortality rates in 2020 among women worldwide (GLOBOCAN 2020, Global Cancer Observatory).

2.1.1.1 Risk factors

There are important epidemiological studies that identify several risk factors for the current knowledge of the genetic and environmental risk factors of breast cancer. Considering that women have higher breast cancer developing rates than men, being a woman alone is a risk factor. 266,120 invasive and 63,960 non-invasive new cases of breast cancer were diagnosed in American women in 2019. However, 2,550 men were diagnosed with breast cancer, corresponding to 1 percent of the total population. The main reason for this difference is that hormonal stimulation in women is higher than in men [10]. Diet, insufficient physical activity, obesity, hormone levels, higher alcohol consumption are considered as well-established risk factors [11]. GLOBOCAN 2018 data were shown that increased body mass index was the most common factor associated with breast cancer risk (Figure 2.2).

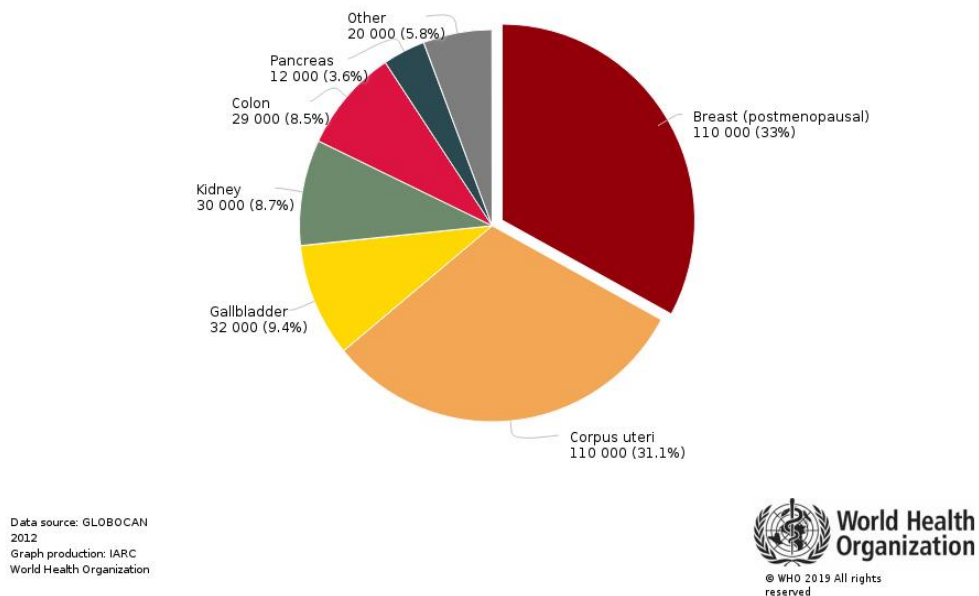


Figure 2.2 : Cancer cases among females worldwide are attributable to excess body mass index, shown by anatomical site as percentages (GLOBOCAN 2018, Global Cancer Observatory).

Considering the hormonal levels, experimental and epidemiological studies have shown that elevated levels of endogenous sex steroids such as androgens and estrogens increase breast cancer development [12]. Long-term exposure to increased levels of estrogen, which is mostly used in postmenopausal hormone replacement therapy, is also associated with an increased risk of breast cancer [13].

Menarchy at a younger age, older-age pregnancy and, nulliparity are also well-established risk factors for breast cancer due to long-time exposure of hormone levels [14]. On the contrary, giving birth at a younger age and breastfeeding have a protective role on breast cancer risks [15].

Based on the population- or family-based epidemiologic studies BRCA1 and BRCA2 are identified as high-penetrance genetic mutations which cause the heterogeneity of the disease [16]. These two genes are highly expressed in breast tissue and have roles in the double-strand break repair of DNA and mutations of these genes are account for 15-20% of familial breast cancer cases [17].

Considering the family history as a risk factor, about 20% of breast cancer patients have this disease in their first-degree relative. Genetic polymorphisms are also considered risk factors for breast cancer. For example, single nucleotide polymorphisms of XRCC2 and XRCC3 which have a role in the homologous recombination of DNA double-strand break influence the breast cancer risk [18].

Although there are many unchanged risk factors such as genetic factors, several breast cancer risk factors can be reduced with simple lifestyle changes. Avoiding taking extra hormones, being at a healthy weight, limiting alcohol, and tobacco use, and eating nutritious foods helps to reduce the risk of developing breast cancer.

2.1.1.2 Breast cancer biomarkers and subtypes

The Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), and Ki67 are important diagnostic and predictive biomarkers for the prognosis of breast cancer. ER exists in two forms ER α and ER β but since ER α is the form where ER function is most frequently validated, ER α is also referred to as ER. Estrogen receptor alpha (ER α) as the main steroid hormone receptor in breast cancer is associated with the oncogenic growth of cancer due to the expression in 70% of invasive breast cancers. In the endocrine therapy of invasive breast cancer, patients are exposed to selective drugs such as; tamoxifen; selective estrogen receptor modulator, anastrozole, letrozole, or exemestane; aromatase inhibitors, fulvestrant; estrogen receptor downregulators which are targeting ER α and PR in the clinical applications. Therefore, ER and PR are targeted in adjuvant or neoadjuvant treatments as predictive endocrine therapy markers [19], [20]. In many previous studies, it has been shown that ER or PR-positive patients account for 60% of all breast cancer patients and receptor-positive tumors showed better outcomes than receptor-negative tumors in endocrine therapy [21]. Epidermal growth factor 2 (ERBB2, HER2) as a transmembrane receptor is overexpressed 20% of breast cancer. Triple-negative breast cancer is consist of 15% of breast cancers and is known as ER, PR, or HER2 negative and is known as the most invasive breast cancer type [22]. Ki67 levels are also used in the meta-analyses and high Ki67 levels are associated with poor outcomes of breast cancer patients [23].

The level of ER, PR, HER2 as classical immunohistochemistry (IHC) markers and Ki67 are the main causes of the formation of breast cancer subtypes. The breast cancer classification and subtyping were identified by using 456 breast cancer cDNA clones and the tumors were classified into five intrinsic subtypes according to the gene expression patterns. There are three different subtypes of breast cancer which are Luminal A, Luminal B, HER2-overexpression, basal and normal-like, and triple-negative [24], [25]. The basic breast cancer sub-types, IHC status, tumor grade, and patient outcome was shown in Table 2.1.

Table 2.1 : The five intrinsic subtypes mapped according to their IHC status. The table was modified from [24].

Subtype	IHC status	Outcome
Luminal A	ER+, PR+, HER2-, Ki67-	Good
Luminal B	ER+, PR+, HER2 -, Ki67+	Intermediate
Luminal B	ER+, PR+, HER2 +, Ki67+	Poor
HER-2 overexpression	ER-, PR-, HER2+	Poor
Basal	ER-, PR-, HER2-, basal marker +	Poor
Normal-like	ER+, PR+, HER2-, Ki67-	Intermediate

Following these classifications, several other studies also categorized the breast cancer subtypes such as the identification of 6 groups consisting of 3 luminal-like, 1 HER2-like, and 2 basal subtypes [26].

In addition to gene expression profiling analyses, studies exploring the protein expression profiles also revealed five groups. The first two subgroups showed luminal epithelial cell profile, hormone receptor positivity, lack of basal epithelial characteristics, HER2 overexpression. The other two subgroups exhibited HER2 overexpression, hormone receptor negativity, different MUC1, and E-cadherin expression. The last group was classified as their higher basal epithelial marker and p53 expression, lack of hormone receptor expression, and weak cytokeratin and luminal epithelial expression [27].

Luminal-like tumor subtypes

The luminal-like tumors consist of Luminal A and Luminal B subtypes and they are steroid hormone-receptor-positive (ER+, PR+) but differ in the HER2 expression profile. Moreover, considering the ER-related gene expressions, Luminal A tumors higher expression profile of ER-associated genes than Luminal B, but Luminal B tumors have a higher expression profile of proliferative genes than Luminal A. Therefore, Luminal B tumors showed have high grade than Luminal A subtype [28]. According to the Caroline Breast Cancer Study, 64.3% of all patients have luminal breast tumors, and among them, 54.3% have Luminal A subtype [29].

HER2 overexpression tumors

HER2 overexpressed tumors have a poor prognosis and grade 3 character. These types of tumors are more sensitive to taxane-derived chemotherapeutic drugs than luminal breast tumors [30]. Moreover, anti-HER2 monoclonal antibody trastuzumab is the frequently treated drug in this type of tumor.

Basal tumors

Basal tumors are referred triple-negative (ER-, PR-, HER2-) due to the loss of hormone receptor and HER2 expression, and high expression basal and proliferation markers with low BRCA1 expression but p53 mutation. Basal tumors have grade 3 tumor characteristics [31]. These tumor subtypes are the most aggressive form of breast cancer with the non-response character to standard conventional applications which increase the metastasis feature towards bone and lymph nodes [32].

2.1.1.3 Stages, diagnosis, and prognosis of breast cancer subtypes

The mammogram and biopsies are the basic methods for breast cancer diagnosis. Mostly benign tissues were diagnosed through mammograms and biopsies are used for the microscopic analysis of suspected tissues [33].

Following diagnosis, the extent and stage of cancer are defined for further treatment and therapy options. American Joint Committee on Cancer (AJCC) and Surveillance, Epidemiology, and End Results (SEER) staging systems are used for the determination and the statistical analysis of breast cancer tumors. AJCC defines the stages based on the clinically anatomic information of tumor extension, lymph nodes, and metastases depending on the cancer types. For breast cancer staging the tumor size has an important impact on prognosis.

Considering the intrinsic breast cancer subtypes, Luminal A type of tumors show a better prognosis with ER+, PR+, HER2- feature. Luminal B type showed ER+, PR+, HER2+, triple positive feature. The prognosis showed worse characteristics with HER2 overexpressed subtypes ER-, PR-, HER2+. The poor prognosis is observed in basal tumors with triple-negative condition ER-, PR-, HER2-. Therefore, it is determined that ER, PR positivity are important factors for a better prognosis (Figure 2.3).

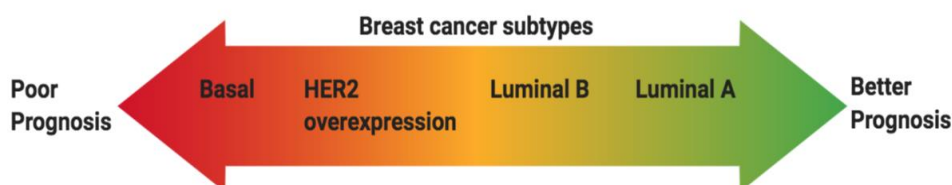


Figure 2.3 : The intrinsic breast cancer subtype-related patient prognosis.

2.1.1.4 Therapy

Breast cancer is a heterogeneous disease with a combination of biological, pathological, and clinical implications, and different features require distinct therapy regimes. There are two main therapy methods in the treatment of breast cancer including local and systemic. Local therapy is based on the surgical removal of non-metastatic tumors and regional lymph nodes from the breast. Besides, radiation is also known as another local therapy option.

Systemic therapy can be neoadjuvant (preoperative) or adjuvant (postoperative) or a combination of both applications before surgical resection. Although neoadjuvant therapy is frequently common in solid tumors such as breast cancer, the difference in breast cancer subtypes is an important criterion for determining the application of therapy. Hormonal therapy is based on the use of endocrine agents like antiestrogens known as the main systemic therapy for ER/PR+ and HER2- Luminal A and B breast cancer [34]. One of the most known antiestrogens is tamoxifen which is based on the competitive inhibition of estrogen's binding to ER. A meta-analysis of 20 ER-positive patients showed that 5 years of adjuvant treatment of tamoxifen as a standard form of endocrine treatment decreased the recurrence of the disease by 39% and mortality by 30% [35]. However, tamoxifen treatment did not show a significant effect on the mortality rates of ER-negative patients [36]. In addition, aromatase inhibitors such as exemestane or letrozole can be used in endocrine therapy. Adjuvant treatment of aromatase inhibitors in postmenopausal hormone-receptor-positive patients showed a higher survival ratio compared to the 5-year tamoxifen treatment. Therefore, starting the usage of aromatase inhibitors was found more appropriate after 2-3 years of tamoxifen treatment [37].

Antibody-based therapy is used in the treatment of HER2+ breast cancers individually or in combination with endocrine therapy. Trastuzumab (Herceptin) is a monoclonal antibody that interacts and suppressed the extracellular region of HER2. Although trastuzumab has been used as a standard first-line anti-HER2 therapy, the development of resistance resulted in the use of trastuzumab with other therapies such as small kinase inhibitors lapatinib, gefitinib, and neratinib [38]. It was also suggested that following trastuzumab treatment, taxane, trastuzumab emtansine, or other monoclonal antibody pertuzumab can be administered in neoadjuvant therapies [39].

2.1.1.5 Personalized medicine

The traditional “one-dose-fits-all” standard approach to pharmaceutical applications has become ineffective in clinical treatment due to individual drug toxicities and treatment failures. According to the studies, 38% to 75% of patients do not respond to treatment due to the large variabilities in diseases and patients [39]. Genetic variations in individuals require different clinical treatment strategies for personalized outcomes and drug responses. Therefore, personalized medicine has improved as an innovative approach that aims to change drug application through increasing its effectiveness and avoiding adverse drug reactions based on personal genetic information [40]. Personalized medicine has four major features which are specified as being personalized, preventative, predictive, and participatory. The first characteristic of personalized medicine means that it is personalized that the knowledge of genetic variations of individuals or specific sub-groups allows effective personalized treatment options. The preventative feature is based on identifying treatment options that may be effective on the disease by early prediction. According to the clarification of breast cancer, susceptibility genes showed that genetic mutations in the BRCA1 and BRCA2 genes have a higher potential to develop breast cancer [41]. Personalized medicine uses molecular diagnostic tools and predictive biomarkers to provide information for improved treatment options. Through these features, healthcare costs and adverse treatment reactions are minimized, so patients can be more participatory in the treatment process. Cancer drugs approved by the Food and Drug Administration (FDA) for different types of breast cancer cases are shown in Table 2.2.

To further develop basic science, investigators need to determine how genotype influences phenotype and more specific personalized medicine approaches. Therefore, targeted gene studies, whole-genome sequencing, and site-specific nuclease technologies gained importance in the last decade.

Table 2.2 : Drugs approved for breast cancer (Data from National Cancer Institute).

Drugs Approved to Prevent Breast Cancer			
Brand Name	Compound	Aim of the use	FDA
Evista	Raloxifene Hydrochloride	It is used to decrease invasive breast cancer who have a high risk of developing the disease or who have osteoporosis.	Yes
Nolvadex	Tamoxifen Citrate	It is used to decrease the chance of invasive breast cancer in patients who have had surgery and radiation therapy for Ductal carcinoma in situ (DCIS).	Yes
Drugs Approved to Treat Breast Cancer			
Brand Name	Compound	Aim of the use	FDA
Verzenio	Abemaciclib	It is used for hormone receptor-positive (HR+) and HER2 negative (HER2-) and is advanced or has metastasized breast cancer types	Yes
Abraxane	Paclitaxel Albumin-stabilized Nanoparticle Formulation	It is used to treat breast cancer that has recurred or metastasized.	Yes
Kadcyla	Ado-Trastuzumab Emtansine	It is used as adjuvant therapy in patients with early-stage breast cancer.	Yes
Taxotere	Docetaxel	It is used to treat locally advanced or has metastasized and has not gotten better with other chemotherapy. It is also used with doxorubicin hydrochloride and cyclophosphamide.	Yes
Efudex	Fluorouracil Injection	It is used to treat breast, colorectal, gastric, and pancreatic cancer.	Yes
Faslodex	Fulvestrant	It is used in postmenopausal women with hormone receptor-positive (HR+) and HER2-negative advanced cancer that has not been treated with hormone therapy.	Yes
Herceptin Herzuma Ogivri	Trastuzumab	It is used to treat hormone receptor-negative or high-risk cancer. As combination therapy with Doxorubicin hydrochloride, cyclophosphamide, and either paclitaxel or docetaxel; or Docetaxel and carboplatin.	Yes
Rheumatrex	Methotrexate	It is used to treat breast cancer and acute lymphoblastic leukemia that has spread to the central nervous system or to prevent it from spreading there.	Yes

2.1.2 Genome editing techniques

Since recombinant DNA technologies gained importance in biology, targeted genome engineering applications have become the most widely used technique. With this development, researchers are effectively able to delete, insert or modify the DNA sequences in any cells or organisms to investigate the exact functional organization and genetic or epigenetic variations. Direct modulation of DNA sequences provided a broad range of applications in medicine, biology, and biotechnology including examples of gene therapy, targeted drug design, and biofuel productions. The

manipulation of DNA bases in targeted gene editing allows the engineered nucleases to induce site-specific double-strand breaks (DSBs) and these breaks are modified in the form of gene deletion, insertion, or correction by non-homologous end joining (NHEJ) of breaks or to homology-directed repair (HDR) through [42].

HDR is based on homologous recombination with integrating exogenous repair sequences into the donor site that contain sequence homology. The error-prone NHEJ pathway also allows insertions or deletions in the absence of an exogenous HDR template [43]. In NHEJ, DSBs are repaired by protein factors that ligate the two broken ends of DNA without a homologous DNA template. The ligation occurs directly or causes insertions or deletions (indels) [44]. The other repair mechanism HDR requires a homologous DNA template which can be endogenous or donor as a guide for repair mechanism [45].

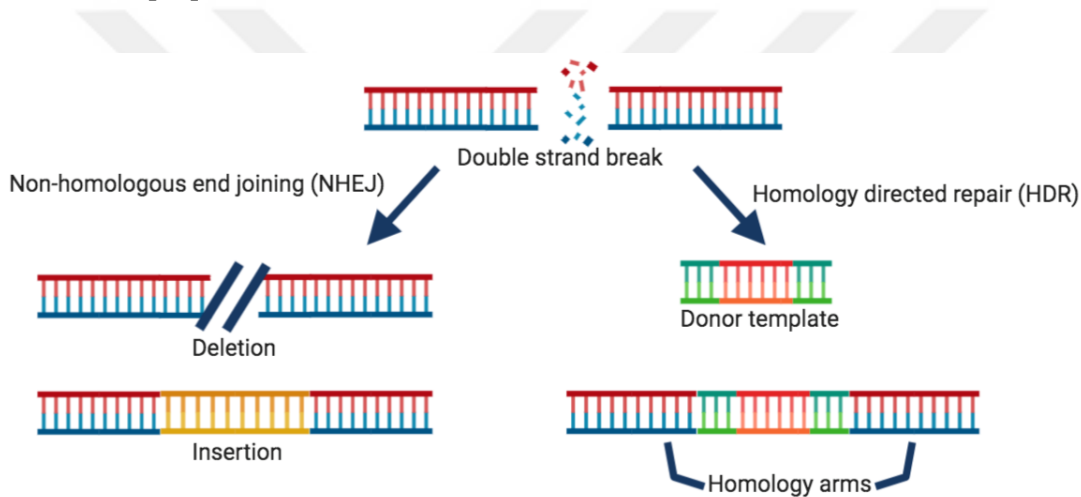


Figure 2.4 : Two endogenous DNA double-strand break repair mechanisms: NHEJ and HDR. In NHEJ, gene disruption occurs by indels without a donor, but in HDR a donor template cause insertion. The figure was modified from [46].

Although targeting of HDR results in highly accurate changes in the gene, the observed recombination events rarely as 1 in 10^6 - 10^9 cells [47]. To overcome this issue, programmable nuclease-based genome editing methods gained importance as the most known breakthroughs in genetic engineering. Meganucleases, Zinc Finger Nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), and RNA-guided Clustered regularly interspaced short palindromic repeats/Cas9 endonucleases (CRISPR/Cas9) mechanisms are based on the introducing site-specific DNA DSBs [48]–[51].

2.1.2.1 Zinc finger nucleases (ZFNs)

ZFN is the gene-editing technique that was firstly discovered in the 2000s [52]. ZFN system consists of two components:

- DNA Binding Zinc Finger Protein Region which binds to DNA with 3 to 6 repeated zinc finger proteins. This region consists of Cys₂-His₂ tandem repeats that bind to the major groove of DNA and recognize 9-18 base-pairs DNA sequences [53]. Each zinc finger protein recognizes 3 base pairs in the DNA sequence.
- DNA Cleaving FokI restriction enzyme-derived nuclease domain: The restriction enzyme FokI binds to DNA sequences and cleaves DNA by dimerization [54] (Figure 2.5). Therefore, ZFN gene-editing requires two monomers binding to the top and bottom strand of DNA [55]. Various methods have been developed to enhance the binding diversities of zinc finger proteins to DNA including the use of a selection of large combinations or oligomerized pool engineering [56]. The natural recognition domain of FokI can be replaced by a designed zinc finger sequence, thus designed ZFN cleaves DNA at a targeted location.

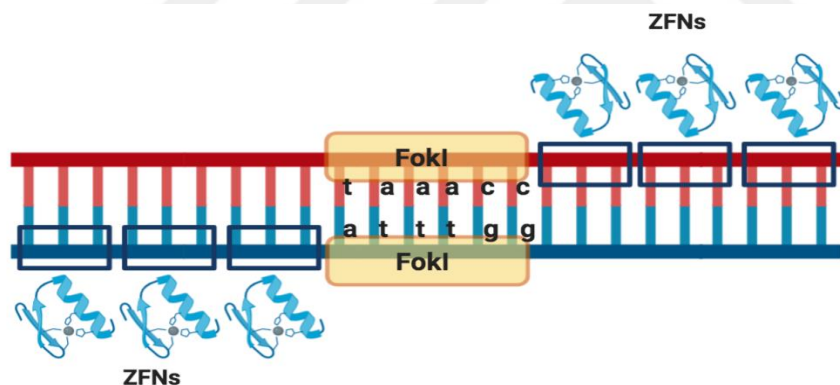


Figure 2.5 : An image of ZFNs system that consists of zinc finger proteins that recognize 3 base pairs in DNA and dimer of FokI restriction enzymes to cleave DNA. The image was modified from [57].

2.1.2.2 Transcription activator-like effector nucleases (TALENs)

Similar to ZFNs, TALENs contain the DNA Binding domain and FokI nuclease domain. DNA Binding Transcription activator-like effector (TALE) proteins derived from *Xanthomonas* bacterium and consist of 33-35 amino acid repeat regions to recognize one base pair of DNA [58]. Four different TALE proteins bind major grooves of DNA and recognize four specific bases; guanine, adenine, cytosine, and thymine. There is a 1:1 binding affinity between TALEs and DNA bases. FokI

restriction endonucleases bind and cleave DNA as similar as in the ZFN gene-editing system (Figure 2.6).

Several methods were generated to enhance the specificity of TALENs by designing customized TALE repeats for more specific DNA binding [59].

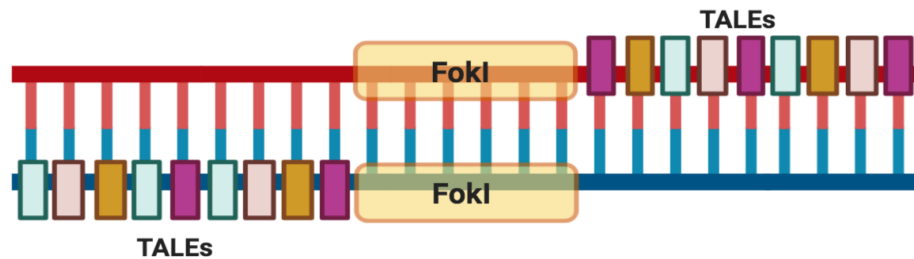


Figure 2.6 : An image of the TALEs gene-editing system. Four different TALE proteins are represented in four different colors and each of them recognizes specific bases in DNA. Each TALE protein binds to one single base pair in DNA. FokI restriction enzyme cleaves DNA. The image was modified from [57].

2.1.2.3 CRISPR/Cas9 system

History of CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided endonuclease Cas9 is the newest genome editing technology that can effectively modify various genomes by short guide RNA targeted DNA recognition via Watson-Crick base pairing.

CRISPRs were first identified as unusual sequences that contained a series of 29-nucleotide repeats that were interspaced by five intervening unique 32-spacer (non-repetitive) sequences in the *E.coli* genome in 1987 [60]. In 2002, CRISPR-associated *cas* genes were identified as a conserved sequence and localized adjacent to CRISPR repetitive sequences [61]. In 2005 systematic studies showed that the sequence of hypervariable spacer sequences showed homology with the genomes of foreign viruses and plasmids, therefore this recognition prevents the virus infection through degradation of the viral transcript by RNAi mechanism or targeting Cas9 enzyme to cleave the CRISPR spacer regions [62], [63]. Therefore, it was confirmed that bacteria and archaea have established adaptive microbial immune responses and memory to survive in the exposure of foreign genetic materials such as bacteriophages.

Since the structural units of the CRISPR system were identified, researchers had begun to use the CRISPR system to investigate its function, such as; generating phage-

resistance dairy cultures, phylogenetic classifications of bacteria [64]. In 2011, the type II CRISPR system was transferred from *Streptococcus thermophilus* into *Escherichia coli* and reconstituted the CRISPR interference system [65]. In 2012, *in vitro* use of the CRISPR/Cas9 system gained a marked as a new field of genome engineering that determining the redirected Cas9 to cleave target DNA by a single-guide RNA (sgRNA) that was constructed by crispr RNA (crRNA) and trans-activating crRNA (tracrRNA). *In vitro* expression of sgRNAs fused with crRNA and tracrRNA within the mammalian genome also stimulated NHEJ or HDR genome editing [66].

Structure of CRISPR/Cas9

The CRISPR loci, consist of clustered set of CRISPR-associated (Cas) genes, repeated DNA sequences, and interspaced by variable spacer sequences (protospacers) that correspond to foreign DNA sequences.

The reconstituted CRISPR/Cas9 system in mammalian cells comprises; Short guide RNA (sgRNA) in 20 nt long CRISPR RNAs, recognizes target DNA sequences through base pairing, in the natural bacterial defense system sgRNA correspond to the virus/phage DNA sequences. The hybridization of CRISPR RNA (crRNA) and auxiliary trans-activating crRNA (tracrRNA) allows targeting effective site-specific CRISPR type II endonuclease *Streptococcus pyogenes* Cas9 (SpCas9) to 20 nt sgRNA within crRNA upstream of 5'-NGG Protospacer adjacent motifs (PAMs) [67]. PAM sequence localized the 3' end of the target DNA sequence that allows the recognition of target DNA sequence by Cas9 for the cleavage. The selection of Cas9 depended on the presence of the PAM site (Figure 2.7). Previous studies showed that the DNA targeting cleavage efficiency of Cas-9 endonucleases based on the length of the tracrRNA that the extension of tracrRNA improved that SpCas9 activity [68].

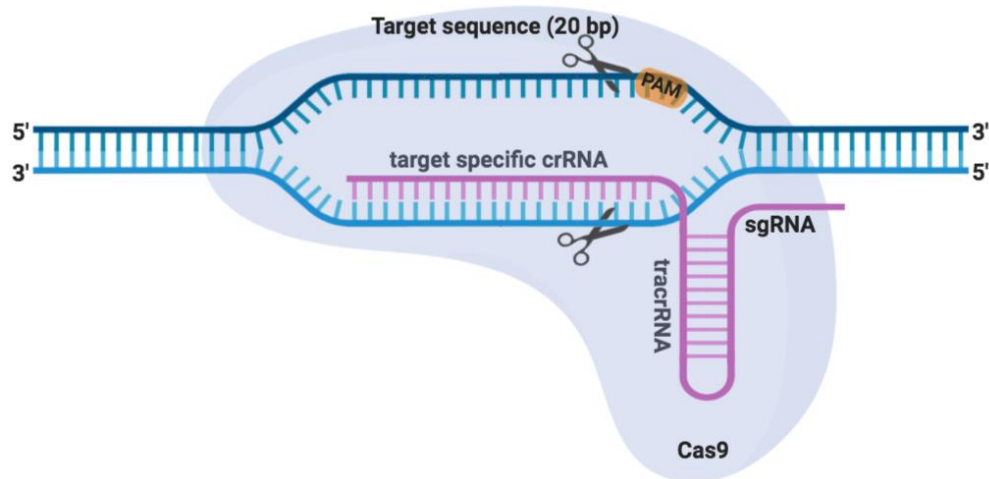


Figure 2.7 : Schematic representation of RNA-guided CRISPR/Cas9 structure, sgRNA consists of a 20 nt-guide sequence with homology to the target DNA sequence. Cas9 creates a DSB 3-bp upstream of the PAM sequence. The representation was modified from [69].

Delivery of CRISPR/Cas9 gene-editing system

To the effective delivery of programmable RNA-guided CRISPR/Cas9, gene-editing systems into cells viral or non-viral methods are used. One of the most favorable viral vectors is a cytomegalovirus adeno-associated virus (AAV) construct that was derived to deliver both Cas9 and sgRNA as essential components of the CRISPR/Cas9 system. The advantage of AAV-derived delivery is that the transfection CRISPR/Cas9 system into targets that are difficult to transfect such as T-cells, and is a less time-consuming strategy [70], [71]. For the non-viral delivery of the CRISPR/Cas9 system direct delivery of Cas9 plasmids or transfection with lipids or nanoparticles were performed [72]. Transfections of the CRISPR/Cas9 system with cationic lipid transfection agents were found highly effective in human cell lines.

Therapeutic applications of CRISPR/Cas9

This natural antiviral immune response is used for the novel genome engineering approaches to make precise modulations in the genome such as; deletion, insertion, or modification in DNA sequences.

According to the therapeutic usage of CRISPR/Cas9 studies, it was observed that mostly used in monogenic disorders and antiviral therapies [73].

In one of the studies, the CRISPR/Cas9 technique was used to correct recessive dystrophic epidermolysis bullosa (RDEB)-causing frameshift loss-of-function mutations in the COL7A1 gene by NHEJ. Targeted deletion of COL7A1 exon in

keratinocytes by retroviral vectors and transplantation of modified keratinocytes into patients was the therapeutic strategy of this *ex vivo* gene editing therapy. To do this, four different sgRNA pairs-guided Cas9 nuclease targeting COL7A1 whole exon 80 deletions transfected to COL7A1-mutated RDEB patient keratinocytes. Then, polyclonal populations of these edited RDEB cells were used to produce bioengineered skin onto immunodeficient mice for and characterized further clinical utility in the future [74]. Moreover, it was observed that correcting the deletion of F508 in the cystic fibrosis transmembrane conductor receptor (CFTR) gene by CRISPR/Cas9 allowed the suppression of cystic fibrosis in intestinal stem cells of patients. Elevated levels of cAMP in human intestinal stem cells caused swelling of CFTR and loss of organoids in cystic fibrosis patients. Therefore, CRISPR/Cas9 system was used to target exon and intron 11 of CFTR locus and correct this locus by homologous recombination and found as a therapeutic model for functional repair for cystic fibrosis patients [75]. CRISPR/Cas9 is also used to treat a global health problem, AIDS. RNA-guided HIV-1 genome disruption within the HIV-1 LTR U3 region showed a suppressed viral gene replication in infected microglial, promonocytic, and T cells and formation of the immune system against HIV-1 in uninfected cells [76].

It is known that CRISPR/Cas9 system has been used in various breast cancer treatment models except for the diseases mentioned above. One of the current studies using the CRISPR/Cas9 system in breast cancer was focused on the characterization of the different subtypes related to its distinct genetic repertoire through finding the factors on the transition of normal cells to neoplasia. P53, Phosphatase, and tensin homologs deleted on chromosome 10 (PTEN), RB1, and NF1 tumor suppressor genes were knocked out in mammary progenitor cells and they showed enhanced survival and proliferation capacities and ER-positive luminal subtype characteristics [77]. Apart from this, the potential role of several other genes and proteins was investigated in the diagnosis and treatment of breast cancer. The potential targets were validated through CRISPR/Cas9-mediated somatic genome editing in invasive lobular breast carcinoma. According to the loss of cell-cell adhesion protein E-cadherin as an invasive lobular breast carcinoma characteristic, the detection of potential candidates was validated. Intraductal injection of lentiviral vectors encoding CRISPR/Cas9 system into female mice carrying conditional alleles of Cdh1 gene which encodes E-cadherin. Injection of Cas9-encoding lentiviral vectors caused the immune response. However, the injection of PTEN disrupting CRISPR/Cas9 lentiviral vectors caused the loss of E-

cadherin expression and induced invasive lobular carcinoma characteristics [78]. In addition, the HER2 oncogene was mutated with CRISPR/Cas9-mediated mutation for the inhibition of cancer cell proliferation and found that exon12 mutated HER2 protein showed dominant-negative phenotype and led to the inhibition of Mitogen-activated protein kinase (MAPK)/ERK pathway [79].

Comparison of ZFNs, TALENs, and CRISPR/Cas9 gene-editing systems

ZFNs and TALENs systems are composed of a sequence-specific protein region that binds DNA and a non-specific restriction enzyme region that cleaves DNA. Both systems performed protein-DNA interactions but the CRISPR/Cas9 system is programmable RNA-guided gene-editing technology. The disadvantages of ZFNs and TALENs require time and investment due to the protein engineering methods. However, CRISPR/Cas9 technique does not require extra protein engineering, it is considered a time-saving method compared to ZFNs and TALENs.

In ZFNs and TALENs gene-editing system FokI endonucleases requires dimerization for the cleavage of DNA however, in the CRISPR/Cas9 system Cas9 endonuclease cleaves DNA as a monomeric form [46].

2.1.3 Signaling pathways for survival and cell death decision

2.1.3.1 Protein kinases

Protein kinases are mostly dysregulated or mutated in several diseases such as diabetes, inflammation, Alzheimer's and Parkinson's diseases, and cancer [80]. The roles of protein kinases in cancer are associated with the progression of cancer, tumorigenesis, and aggressiveness. Protein kinase family phosphorylate their targets (Figure 2.8) and these families are divided into two main groups according to phosphorylation targets: Serine-Threonine kinases and Tyrosine kinases. While Serine-Threonine kinases phosphorylate serine or threonine residues, Tyrosine kinases have a role in the phosphorylation of tyrosine residues. There is also a third group that can phosphorylate both serine-threonine and tyrosine amino acids [81].

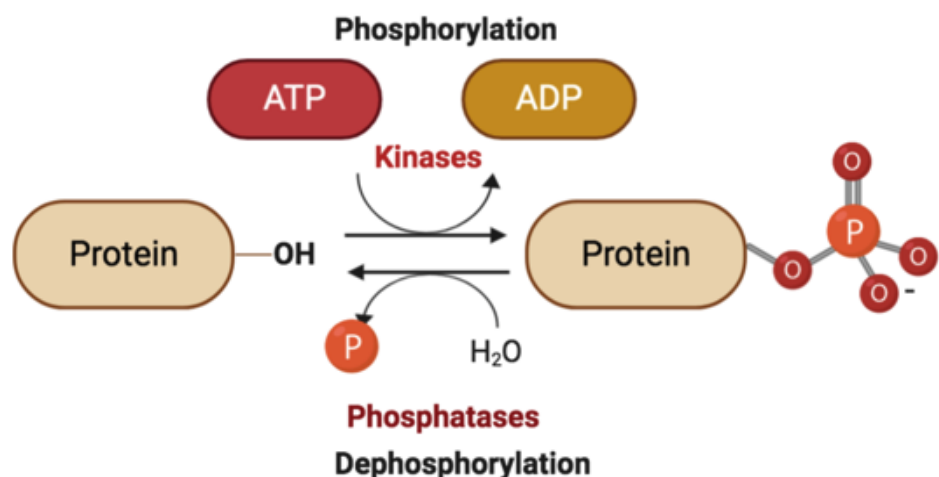


Figure 2.8 : Overview of phosphorylation and dephosphorylation reactions catalyzed by kinases and phosphatases. Protein kinases transfer the phosphate group of ATP to protein. On the contrary, phosphatases transfer the phosphate group from phosphorylated protein to water molecule.

Elevated level or irregular activation of protein tyrosine kinases (PTKs) play an important role in the progression and aggressiveness, differentiation, cytoskeletal reorganization, motility, and apoptosis in breast cancer. PTKs are classified as receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases subgroups. Although all PTKs have conserved catalytic domains they differ in catalytic regions. RTKs are single-pass transmembrane proteins and consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular catalytic tyrosine kinase domain (TKD). Non-receptor kinases (NRTKs) do not have transmembrane domains and reside in the cytosol, nucleus, or plasma [80].

Receptor tyrosine kinases, RTKs

RTKs as a subgroup of PTKs are associated with cell survival, proliferation, differentiation, and metastasis. In addition, elevated levels of RTKs improve the aggressiveness and poor outcome of breast cancer cases [82].

According to the human genome sequencing, 90 tyrosine kinase genes were identified and 58 of the 90 identified tyrosine kinase gene encodes RTK proteins and are classified into 20 subfamilies. Other 32 non-receptor tyrosine kinases are divided into 10 subgroups depending on their kinase domains [83].

The identified 20 different RTK subclasses are: (1) RTK Class I Epidermal Growth Factor Receptors (EGFRs) family, (2) RTK Class II Insulin Receptor (InsR) family, (3) RTK Class III Platelet-Derived Growth Factor Receptors (PDGFRs) family, (4) RTK Class IV Vascular Endothelial Growth Factor Receptors (VEGFRs) family, (5)

RTK Class V Fibroblast Growth Factor Receptors (FGFRs), (6) RTK Class VI Tyrosine-protein kinase like 7 (PTK7)/ Colon Carcinoma Kinase 4 (CCK4) Receptor family, (7) RTK Class VII Nerve Growth Factor Receptors (NGFs) Family/Trk Receptors, (8) RTK Class VIII Hepatocyte Growth Factor Receptors (HGFRs) family/Met Receptors, (9) RTK Class IX Erythropoietin-Producing Human Hepatocellular Receptors (EPHRs) family/ EphA/B Receptors, (10) RTK Class X AXL Receptors family, (11) RTK Class XI Angiopoietin/TIE Receptors family, (12) RTK Class XII Related to Receptor Tyrosine Kinase Receptors (RYKRs) family, (13) RTK Class XIII Discoidin Domain Receptors (DDRs) family, (14) RTK Class XIV RET Receptors family, (15) RTK Class XV ROS Receptor family, (16) RTK Class XVI Leukocyte Receptor Tyrosine Kinase (LTKs) family, (17) RTK Class XVII Receptor Tyrosine Kinase-Like Orphan Receptors (RORs) family, (18) RTK Class XVIII Muscle Specific Kinase Receptors (MuSKs) family, (19) RTK Class XIX LMR Receptors family, (20) RTK Class XX SuRTK106/STYK1 family [83].

RTKs are firstly activated by the binding of ligands such as growth factors, chemokines, or hormones to the extracellular region of RTKs as a receptor [84]. Ligand binding at plasma membrane causes receptor dimerization and conformational change and this conformational change leads to autophosphorylation of the intracellular kinase domain and kinase activation of RTKs. Phosphorylated and active RTKs are docking sites for other downstream molecules for the transduction of intracellular signaling cascades (Figure 2.9) [85].

Phosphorylation-mediated conformational change-inducing activation of RTKs allows the induction of downstream signaling cascades such as; phosphoinositide 3-kinase (PI3K)/AKT, MAPK, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) [86], [87]. Activation of these pathways is associated with breast cancer survival, proliferation, aggressiveness, metastasis, and resistance to conventional therapies [88], [89].

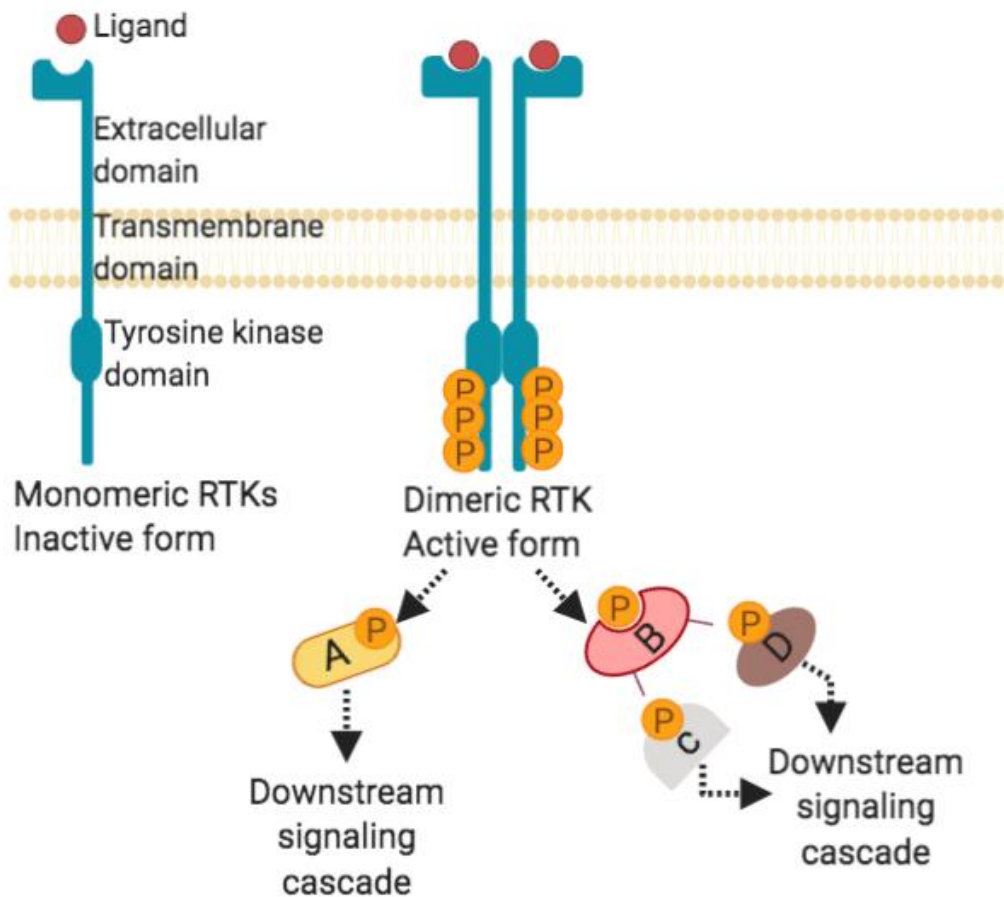


Figure 2.9 : Structure and activation mechanisms of RTKs. RTKs are located at the membrane as a monomeric form. Through the binding of ligands to the receptor, the RTKs are dimerized and activated by autophosphorylation. Active RTKs can directly phosphorylate signaling molecules (A) or be a docking site for adaptor proteins (B). Adaptor proteins further phosphorylate other downstream targets (C, D) for the transducing of the activation cascade. The image was modified from [90].

ErbB/HER family member EGFR (ErbB1/HER1) is associated with breast cancer metastasis and aggressiveness [91]. The members of the EGFR family consist of ErbB/HER-1, ErbB2 (HER-2), ErbB3 (HER-3), and ErbB4 (HER-4). They can translocate into and associate outer and inner parts of mitochondria via a c-Src-dependent manner [92]. As a non-receptor tyrosine kinase, Src is hyperactive in breast cancer and the direct interaction between Src and EGFR led to phosphorylation of receptor at Tyr845 and improves the breast cancer malignancy [93]. Elevated EGFR content in mitochondria is related to cell survival and autophagy [94]. COXII is the mitochondrial downstream target of EGFR, it is inactivated through phosphorylation by EGFR and inactivation causes the decrease in cellular ATP level. EGFR induces downstream signaling pathways such as; Ras, PI3K, and JAK to induce cell survival and progression of breast cancer.

The insulin-like growth factor (IGFs) binds the type I insulin-like growth factor receptor (IGF1R) and activation of IGF1Rs triggers downstream proliferation and metastasis-related signaling cascades through directly binding to estrogen and integrin receptors in breast cancer [95]. Moreover, it was observed that IGFR signaling has a role in the suppression of apoptosis and oncogenic transformation [96].

Fibroblast growth factor receptor (FGFR-1) expression triggers PI3K/Akt and Ras/MAPK pathway to regulate breast cancer survival, proliferation, and apoptosis [97]. FGFR-1, Fms-like tyrosine kinase 3 (FLT-3), and tropomyosin-related kinase (Trk) families are also translocated in the mitochondria and elevated expressions of these proteins are observed in cancer types. Mostly, FGF signaling is important for development, differentiation, and mitogenic and angiogenic responses due to the oncogenic properties of FGFs. They identified as oncogenes in murine mammary cancer and the expression levels were found highly deregulated in mouse-mammary tumor virus (MMTV)-infected mouse strains which the result was associated with the role of FGFs in breast cancer [98].

VEGFs are angiogenic factors that regulate angiogenesis and metastasis through binding their receptors VEGFR1 (Flt1), VEGFR2 (KDR or murine homolog, Flk1). Activation of VEGFRs induces Epithelial-Mesenchymal Transition (EMT)-mediated invasion and metastasis. PDGFRs have a role in the tumor microenvironment and tumor-stroma network. HGFRs family has key roles in cell motility, EMT, angiogenesis, and tumorigenesis through binding the Mesenchymal-epithelial transition (Met) receptor, and elevated levels of Met are shown in several cancers. Met receptors are activated both in the plasma membrane with HGFR and also on endosomes to promote its signal duration. Activation of Met signaling induces PI3K/Akt and MAPK pathways leading to survival, invasion, migration, progression, differentiation, and metastasis profile of cancer cells. EphR, the Eph family has a role in the nervous system regulating cell segregation and axon targeting [99].

Non-receptor tyrosine kinases, NRTKs

The 10 sub-group of non-receptor tyrosine kinases are: (1) ABL, (2) ACK, (3) CSK, (4) FAK, (5) FES, (6) FRK, (7) JAK, (8) SRC, (9) TEC, (10) SYK [82]. The subgroups are classified according to their high conserved catalytic Src Homology 1 (SH1), p-Tyr binding Src Homology (SH2), protein-protein interaction Src Homology 3 (SH3) domains [100].

Src family consists of 3 family members and all members of this family have conserved the SH2 domain and phosphorylate tyrosine residues in other signaling proteins [101]. Src family as the largest group of NRTKs has several regulatory roles in cell survival, proliferation, cytoskeletal organization, motility, differentiation, and apoptosis through regulating important signaling pathways such as Ras/Raf/MEKs/ERKs, PI3K/Akt/mTOR, and STAT3/c-Myc/Cyclin D1 [102]. Elevated levels of the oncogene Src were associated with the tumor formation, migration, and invasion in several cancer types such as breast, lung, melanoma, and colon cancer [103], [104].

The focal adhesion kinase (FAK) family consists of FAK and related adhesion focal tyrosine kinase (Raftk, Pyk2). FAK is reside in the focal adhesion sites in adherent cells, therefore the expression of FAK is found in all tissues. However, Raftk/Pyk2 is localized in the cytoplasm and mainly expressed in the central nervous system in hematopoietic cells [105]. FAK signaling also regulates proliferation, migration, and apoptotic pathways through targeting Src-mediated activation of p130Cas, MAPKs/ERKs, PI3K/Akt pathways [106].

The C-terminal Src kinase (Csk) family phosphorylates the Src family tyrosine kinases and suppresses oncogenic characterization of these proteins [107]. Tec family kinases (TFKs) are associated with hepatocyte proliferation through Ras/Raf/MAPK pathways [108]. Moreover, activation of TFKs by integrins, RTKs, or cytokines led to the regulation of several cellular routes including adhesion, actin remodeling, apoptosis, cell survival, and migration [106]. Abelson tyrosine kinase (Abl) family members are located in the membrane, actin cytoskeleton, cytosol, and nucleus, therefore the members of this family c-Abl and Abl related protein (Arg) regulates actin organization, adhesion, motility processes [109].

2.1.3.2 PI3K signaling

The lipid kinase PI3K signaling is the major downstream pathway of RTKs and functions in cell proliferation, migration, differentiation, and cancer progression with the mutational or differentially expression status in several cancers [110].

PI3K family is divided into classes I, II, and III which phosphorylate lipids, and class IV which are PI3K-related serin-threonine-kinases including Ataxia telangiectasia and Rad3 related (ATR), Mammalian target of rapamycin (mTOR), Ataxia telangiectasia mutated (ATM), and DNA dependent protein kinase (DNA-PK) [111].

The class I family consists of p110 catalytic subunit and p85, p65 or p55 regulatory subunits and hyperactivated in breast cancer and associated with drug resistance and tumorigenesis [112]. PIK3CA, PIK3CB, and PIK3CD encode the subunits of p110 which are p110 α , p110 β , and p110 δ . PIK3R1, PIK3R2, and PIK3R3 encode the subunits of p85 α , p85 β , and p55 γ in class I PI3Ks [110].

Hyperactivation of PI3K pathways is frequently seen in breast cancer. These abnormal activations are associated with mutations on PIK3CA, PIK3CB, and PIK3R1 genes which affect Class I PI3Ks. Elevated levels of RTKs activation by HER2, EGFR, and IGF cause abnormal activation of the PI3K pathway and led to breast cancer progression and aggressiveness [113].

According to the key role of the PI3K pathway, previous studies investigated the expression profiles of PI3K pathway genes in MCF-7 and show that the subunits of PI3K p110 and p85 were expressed in mRNA and protein status with the loss of PTEN protein expression [114]. PTEN mutations affect 5% of patients in breast cancer, while 30-50% of these patients have significantly reduced or lost PTEN expression which results in breast cancer progression, metastasis, and resistance to breast cancer therapies [115]. PTEN expression alternation is observed with a 30% rate in the most aggressive triple-negative breast cancer subtype [116].

The regulation of the PI3K pathway is under the control of tumor suppressor PTEN which negatively regulates this signaling pathway by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2) [117]. PTEN also improves tumorigenic responses of cells through independent PIP3 dephosphorylation wherein localized in the nucleus [118]. Studies identifying PTEN as pivotal the embryonic development showed that homozygotic knockout of PTEN caused lethality, while heterozygous PTEN (+/-) expression developed tumors in prostate and breast tissues in mice [119]. Therefore, loss of PTEN expression was found as an essential factor for cancer initiation [120].

Activation of PI3K Class I proteins occurred by RTKs and active PI3K phosphorylate PIP2 and produce PIP3 which further induces phosphoinositide-dependent kinase 1 (PDK1) activation. PIP3 has a role as a second messenger to localize Akt to the plasma membrane which is further phosphorylated by PDK1 [121]. Akt is the major downstream target of PI3K and activation of Akt occurs through the phosphorylation at Thr308 by PDK1 and at Ser473 by Mammalian target of rapamycin complex 2 (mTORC2) [122]. Phosphorylation of Akt led to the activation of several downstream

molecular targets that induce cell survival, proliferation, progression, apoptosis, autophagy, and migration (Figure 2.10) [123].

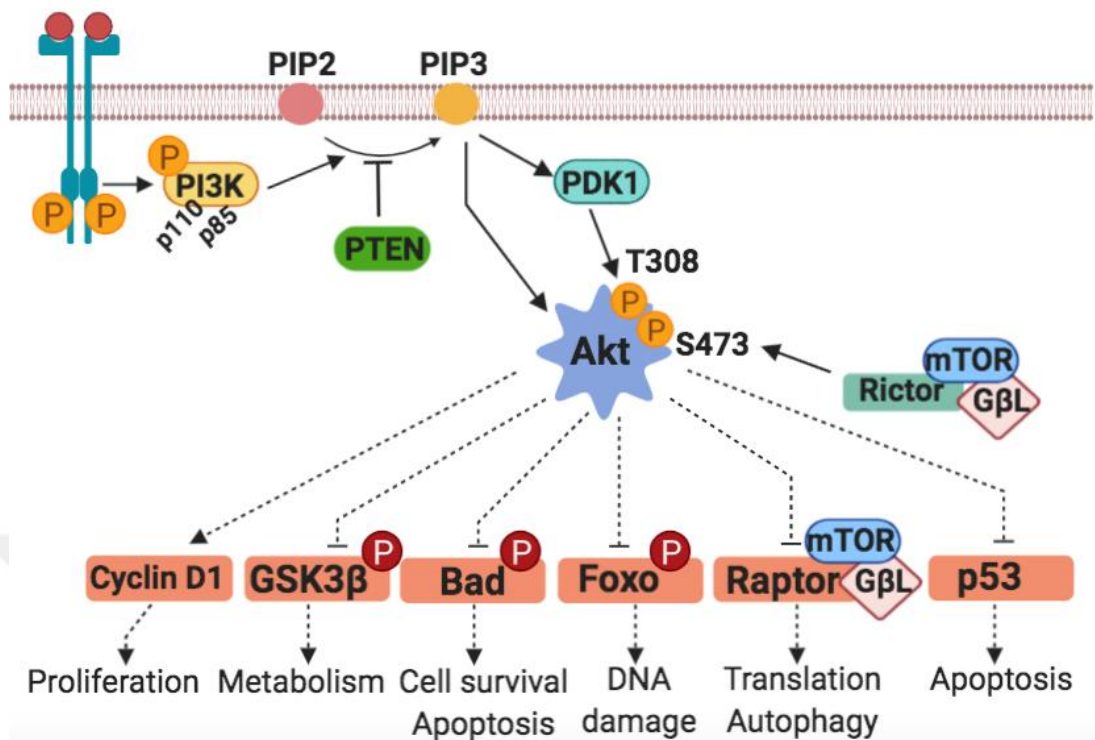


Figure 2.10 : The upstream and downstream members of the PI3K/Akt pathway. Akt is the major downstream member of PI3K signaling, activation of Akt triggers molecular substrates, which regulates cellular responses. The diagram was modified from [111].

Fully active Akt inhibits tuberous sclerosis complex 1 and 2 (TSC1/2) which activates the Mammalian target of rapamycin complex 1 (mTORC1). The Eukaryotic initiation factor 4E binding protein (4EBP1) as a key target for protein synthesis is phosphorylated and inhibited by mTORC1 but the mTORC1 inhibitor Rapamycin analogs activate this protein. The mitogen-activated serine-threonine protein P70S6K is essential for cell cycle progression which is activated through the mTORC1 complex [124]. The Akt signaling pathway regulates protein synthesis, cell cycle, cell growth, and proliferation pathways through altering the mTOR pathway (Figure 2.11).

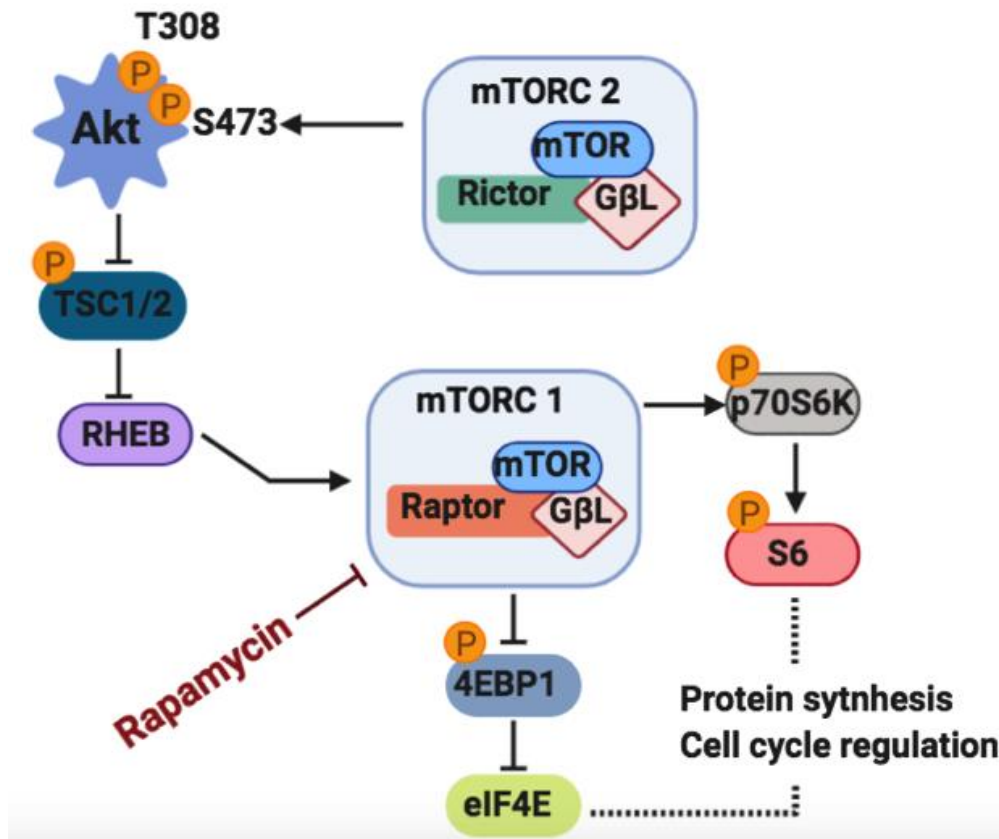


Figure 2.11 : The network of Akt activation and downstream target mTOR pathway. Akt is activated by mTORC2 and active Akt suppressed mTORC1 complex which is an important target for rapamycin. The protein synthesis, cell growth, and cell proliferation pathways are regulated by Akt signaling. The diagram was modified from [124].

Previous studies stated that PI3K and Ras pathways regulate each other in the development of cancer due to the direct physical interaction of Ras to the p110 PI3K subunit. While, this interaction triggered Ras-mediated skin and lung cancer, the disruption of Ras-p110 PI3K interaction by mutations at positions T208D and K227A reduced the Ras-associated lung cancer development [125].

PI3K Class I isoforms are mostly regulated by Met RTK which is induced by hepatocyte growth factor (HGF). Class I PI3K can directly bind Met receptor or indirectly bind Met receptor adaptor proteins such as; growth factor receptor-bound protein 2 (Grb2), Grb2-associated binding protein 1 (Gab1). HGF binds and activates Met which is a critical regulator for cell proliferation, migration, and angiogenesis [126]. Studies on the relation between PI3K, Ras and Met signaling, it was stated that Ras is activated by Met and further Ras-mediated PI3K/Akt signaling regulates cell survival and migration [127].

2.1.3.3 Cell migration targeting signaling pathways

According to the cancer development process cancer cells can detach, move, invade, migrate and metastasize. Cell migration refers to the movement of normal cells, but also is a pivotal cancer progression hallmark that regulates the following cellular processes such as invasion, wound healing, metastasis, embryonic development, and angiogenesis. These are specific properties for the resistance of cancer cells against anti-cancer chemotherapeutic drugs, tumor dissemination, and the recurrence of cancer [128], [129].

Basic steps for migration (Figure 2.12)

1. Cell polarization

To the movement of cells, they become polarized and elongated to extend protrusions at the leading edge via cytoskeleton reorganization. The growth factors or chemokines trigger the movement of cells through the activation of Rho family GTPases. Rho family GTPases including RAC1, RHOA, and CDC42 have different roles in cell motility. RAC1 regulated the movement by locating at the leading edge of the cell, CDC42 has a role in the formation of filopodia and lamellipodia, and RHOA is involved in the formation of stress fibers [130].

2. Formation of cell-extracellular matrix (ECM) adhesion

The formation of focal adhesions between the extended protrusion of cell membrane and ECM proteins are crucial steps for cell migration and metastasis. The interaction between cell-ECM complex consists of adhesion molecules, integrin families, CD44 receptors and syndecans [131]. Integrins, cell surface receptors for extracellular matrix proteins are important signaling molecules for the cytoskeleton re-organization due to the recruitment of FAK and the scaffolding protein paxillin. The adaptor protein paxillin associates with other focal adhesion pathway members such as vinculin, and have a role in the activation of MAPK proteins in growth factor stimulation. FAK becomes activated through auto-phosphorylation on Y397 and interacts with Src migration-associated protein and triggers paxillin and p¹³⁰ Cas [132].

3. ECM degradation by matrix metalloproteinases (MMP)

The ECM degradation metalloproteinases including MT1-MMP, MMP1 and MMP2 have roles in the cleavage of ECM proteins collagen, fibronectin and laminin which restrict tumor cell motility [133].

4. Cell contraction and cytoskeleton organization

The actin cytoskeleton is reorganized for the cell contraction that myosin II binds to actins to form actomyosin. Rho family GTPase members have a role in this process that RHOA regulates the actomyosin contraction and RHO-associated protein kinase (ROCK) inhibits the myosin II inactivation [133].

5. Cell detachment and focal adhesion disassembly

The cytoskeleton is reorganized for cell-ECM detachment and the detachment is initiated at the trailing edge of the moving cell. The focal contact disassembles the polarization and leads the movement of cells through the FAK phosphorylation by Akt [133].

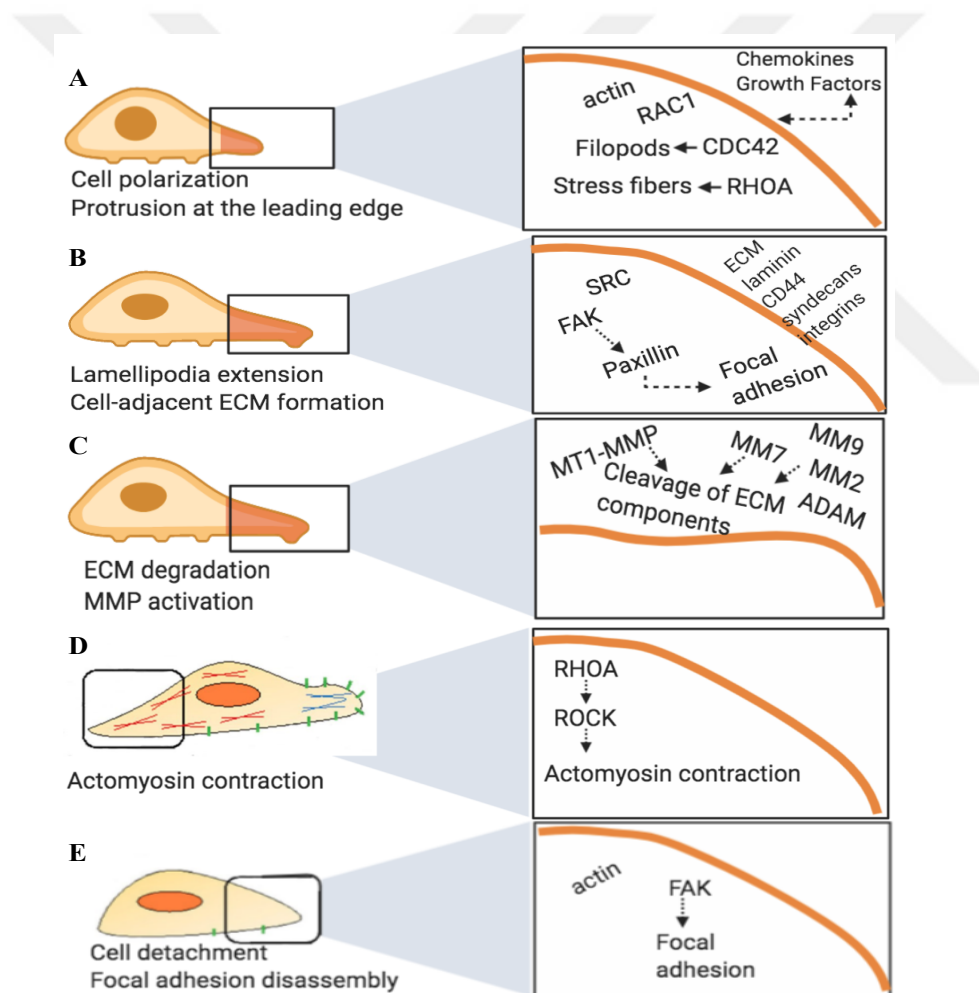


Figure 2.12 : The basic molecular action of cell migration. A) Cell polarization and cytoskeleton reorganization at the leading edge. B) Formation of cell-ECM adhesion complex. C) The newly generated cell-matrix contact is degraded by MMPs. D) Cell contraction and cytoskeleton organization by Rho GTPases. E) Cell movement by focal adhesion disassembly. The figure was modified from [133].

Tumor cells can migrate in different patterns such as collective or single-cell migration. The single-cell migration can be in mesenchymal or amoeboid mode. Cancer cells mostly prefer the mesenchymal migration with the characteristic properties are elongated, spindle-like cell shape with pseudopods. The mesenchymal migration occurs from the focal adhesion and actin-rich protrusion at the leading edge. Rho-family GTPases are the key signaling molecules controlling this process to initiate ECM proteolysis [133].

During amoeboid migration, the round or irregular amoeboid-like cells extend protrusions with actin-myosin-rich interactions. The protrusions of these cells are highly deformable due to their lack of focal contacts [134]. Amoeboid migration is observed in tumor cells with a rounded shape. Cancer cells can transit among mesenchymal and amoeboid migration modes within minutes based on their ECM properties. The ECM around the mesenchymal cells is degraded by proteases at the leading edge during the mesenchymal cell migration process. For the mesenchymal amoeboid transition (MAT), the Rho/ROCK signaling is upregulated and the ECM protease and integrin activities are decreased. Oppositely, an amoeboid mesenchymal transition occurs through the inhibition of Rho/ROCK signaling (Figure 2.13) [135].

The significant difference between mesenchymal and amoeboid cells is based on their cell shapes, that mesenchymal cells are elongated due to their actomyosin positions at the leading and tail end of the cells, while amoeboid cells are observed in a round shape due to the cortical distribution of the actomyosin complex [136]. The advantage of the round shape of amoeboid cells is being compact for the adaptation to gaps and holes in the ECM structure and finding the paths during migration. Moreover, this process is not required receptor-facilitated adhesion to ECM due to the ability of amoeboid cells to deform surrounding ECM [137].

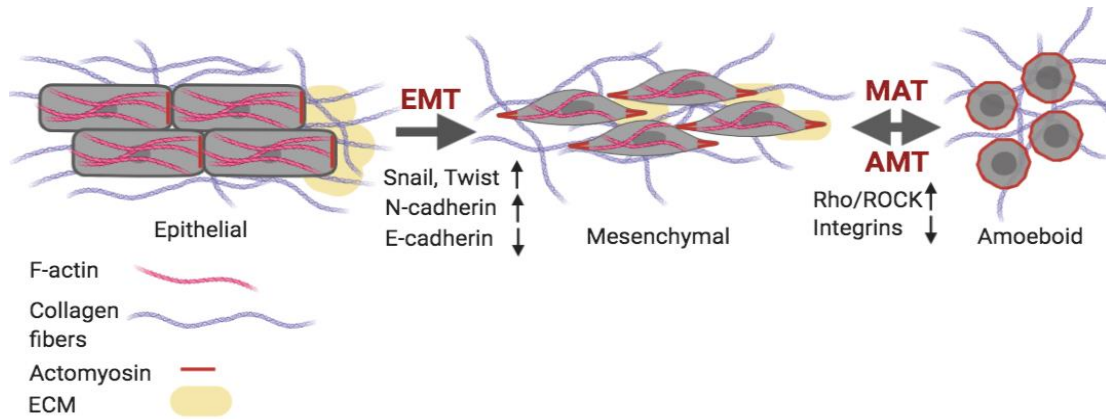


Figure 2.13 : The schematic representation of the transitions among cell migration modes. Epithelial cells migrate toward the leading edge by collective migration. EMT is the conversion of epithelial cells to mesenchymal cells and is dependent on the expression profiles of cadherins and transcription factors. MAT and AMT refer to the transition between amoeboidal and mesenchymal cells due to the expression profiles of Rho family members and the activation of ECM proteases.

2.1.3.4 Epithelial and mesenchymal transition (EMT)

The most important tumor migration process is EMT which refers to the conversion of the epithelial cells to individually migrating cells in the process of cell establishment of cell-ECM contact, and finalization by the degradation of the basement membrane and formation of mesenchymal cells [138]. The biological EMT process is based on the transcriptional and morphological changes of epithelial cells during embryogenic development, tissue remodeling, and metastasis [139].

According to the previous studies, EMT is classified into three distinct subtypes. Type 1 EMTs are linked with embryo and organ development which consists of embryonic gastrulation and formation of mesoderm, endoderm, and neural crest cells. Type 2 EMTs have roles in tissue repair and wound healing processes differs from Type 1 with the enhanced inflammation conditions. Type 3 EMTs are associated with invasive metastases in cancer cells with the genetic and epigenetic changes in tumor development [140]. In type 3 EMT, epithelial cells lose their cell-cell adhesions and have been recognized by spindle-like cell morphologies. Also, the cytoskeletal organization is disrupted to improve the migration and invasion profile of cells. Basal-like triple-negative breast cancer cells, which are known for their most aggressive and poor outcome characteristics, also develop drug resistance as they show high levels of EMT properties, resulting in a worse outcome [141].

There are several important endogenous pathways responsible for the regulation of EMT in breast cancer. Considering the TGF- β signaling pathway, TGF- β act as a

tumor suppressor protein in early tumor tissues while promoting EMT and metastasis in cancer development [142]. Ras/Raf signaling pathways improve the EMT properties of epithelial cells through enhancing TGF- β signaling [143]. The Wnt pathway also plays a promoting role in the development and metastasis of breast cancer through EMT induction.

The members of EMT families are Cadherins, Vimentin, Claudins, Occludin, β -Catenin, fibronectin, collagen, and transcription factors; ZO-1, Snail, Slug and (Zinc finger E-box binding homeobox 1) ZEB1.

Cadherins are calcium-dependent cell-cell adhesion proteins and have roles in tissue remodeling and development [144]. Epithelial cell-cell adhesion in the structural organization is maintained by the adherent junction protein E-cadherin and tight junction proteins claudins and occludin. These proteins have roles in the suppression of invasion in epithelial cells. Invasion and metastasis are regulated by the loss of E-cadherin and elevated levels of mesenchymal cell-cell adhesion protein N-cadherin, Vimentin, Fibronectin, collagen, and smooth muscle actin [145]. The induction of EMT occurs by the repression of the transcription factors Snail and Twist families and follows by the downregulation of E-cadherin and loss of intercellular junctions [146].

The induction of EMT is initiated with the growth factor signaling including EGF, PDGF, HGF, TGF- β , follows by the activation of transcription factors Slug, Snail, ZEB1, and Twist and continues in the downstream signaling pathways such as PI3K/Akt, MAPK/ERK, Rho family and Ras signaling [147], [148].

Previous studies showed that the tumor-suppressive role of PTEN is based on the interaction with β -catenin and E-cadherin through MAGI-2 protein [149].

Previous mouse studies showed that mesenchymal cells have vimentin, desmin, FSP-1, and α -SMA markers [150].

Mesenchymal-epithelial transition (MET) is the reverse process of EMT which is based on the transition of mesenchymal cells to epithelial characteristics. Although several studies were observed in EMT regulation but MET process has not been detailed investigated. Previous studies showed MET is regulated by paired box 2 (Pax2) gene in kidney development [151]. In addition, EMT product fibroblasts were reset into induced pluripotent stem cells through activating the MET process. It was shown that Sox2/Oct4 suppressed the Snail transcription factor, Klf-4 which enhanced the E-cadherin level during the suppression of EMT and activating MET [152].

2.1.4 Bag-1

The Bag-1 protein was initially identified in 1995 as a novel physically and functionally Bcl-2-binding protein showing no homology with Bcl-2 or other Bcl-2 family members. Functional analysis showed that Bag-1 prolonged the survival of cells alone and enhanced the anti-apoptotic function of Bcl-2 in the resistance to cell death. Therefore, Bag-1 is considered an anti-cell death protein [153].

2.1.4.1 Structure

Bag-1 gene and mRNA organization

Following the identification studies of Bag-1, the genomic location and the protein structure of Bag-1 were identified. The *Bag-1* (Bcl-2 associated athanogene-1) gene is located on human chromosome 9p12, about 10 kb in size comprising 7 exons and 9pter chromosomal region 9p34 in the mice. Comparison of human and mouse Bag-1 protein studies showed that the amino acid sequences of Bag-1 proteins were 90% identical except that 55 amino acid residue longer due to a unique NH₂-terminal extension in the human Bag-1 protein. In addition, human Bag-1 protein was found to be more acidic than mouse Bag-1 protein [154].

The initial chromosomal location studies of Bag-1 revealed that chromosome 9 was associated with many different functions in the cell. The tumor suppressor locus on the short arm of chromosome 9, where the Bag-1 gene is also encoded was found frequently homozygously deleted in breast cancer tumor formation [155]. In addition, regions adjacent to the 9q12 have also been associated with rare disorders and defects in cell death [156]. The results of these studies on chromosome 9 provided clues to the functional role of Bag-1 in the cell.

To investigate the transcriptional regulation of the Bag-1 gene, 5' the flanking region of the human Bag-1 gene was sequenced. Sequenced 893 bp of the Bag-1 gene upstream of the first translation start codon (CTG) was comprised of 272 bp CpG island located between -472 to -200 which provided Bag-1 expression can be regulated by methylation/demethylation process. Also, Bag-1 promoter consisted of transcription binding sites for transcription factors including Sp1, WT1, Ets, transforming growth factor- β (TGF- β) receptor, GATA-1, GCN4, and YY1. Besides, the luciferase studies showed the maximum promoter activity of Bag-1 gene was

observed at -353 and -54 regions and Bag-1 promoter was transactivated by mutant p53 [157].

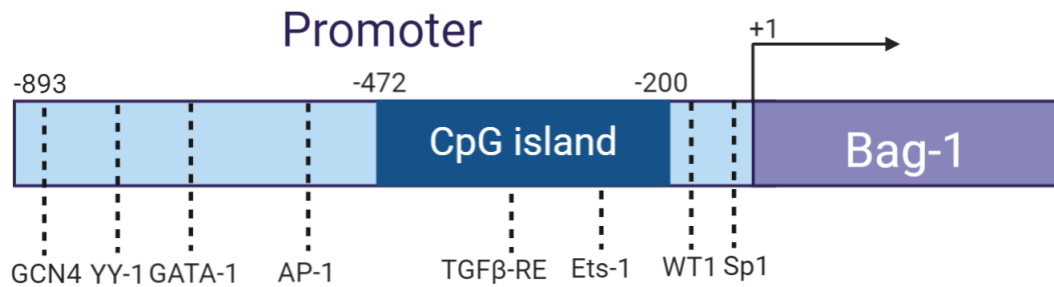


Figure 2.14 : The schematic representation of the 5' flanking region of the human Bag-1 gene. +1: The first nucleotide of translation initiation site CTG. This representation was modified from [157].

Bag-1 protein isoforms

Bag-1 has three different isoforms translated from one single mRNA through alternative translation initiation sites. The Bag-1 consists of isoforms named Bag-1L as p50, Bag-1M as p46, and Bag-1 as p36. Bag-1L isoform has 71 aa extensions at N terminal, consisting of 345 aa and about 50 kDa, translated from upstream CUG non-canonical start codon, based on cap-dependent translation and starts with leucine. Bag-1L includes positively charged residues because of its nuclear localization signal (NLS), therefore resides within the cell nucleus [153]. The 46 kDa form of Bag-1, Bag-1M isoform translated from the first AUG codon and starts with methionine. Bag-1M isoform consists of 274 aa and includes basic amino acids in N terminal regions which are important for interaction networks and presented in both cytosol and nucleus. The 230 aa and 36 kDa Bag-1S were translated from the second in-frame AUG by internal ribosome entry site (IRES) and predominantly localized in the cytoplasm and most abundant in the cell [158]. The smallest and fourth isoform of Bag-1 is 29 kDa and has been recognized by its lowest expression profile in humans.

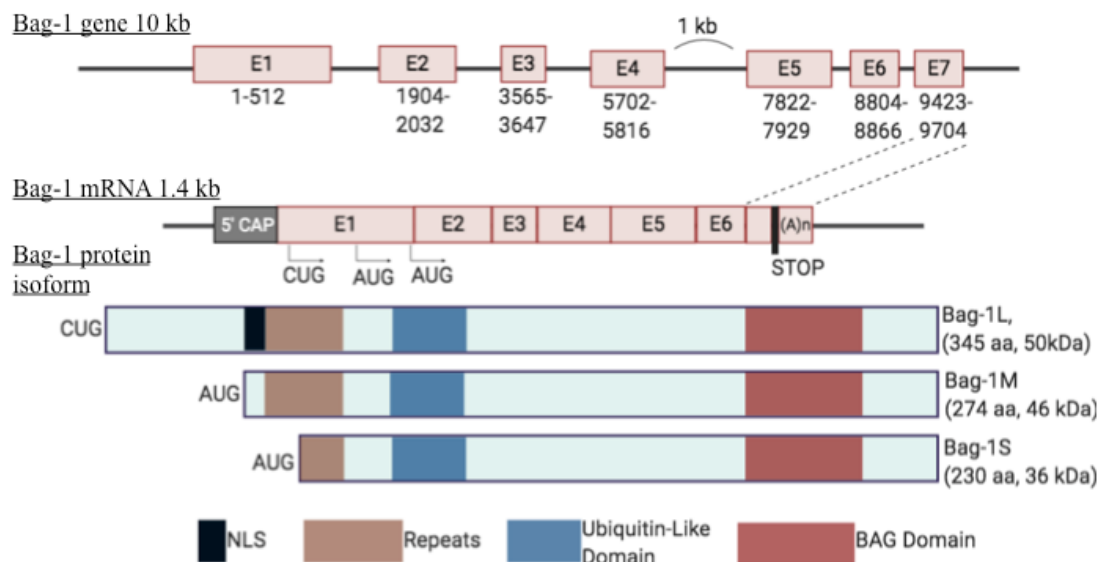


Figure 2.15 : Structure of human Bag-1 gene, Bag-1 mRNA, and Bag-1 protein isoforms. E1, E2, E3, E4, E5, E6, and E7 represented the 7 different exons of the Bag-1 gene. Bag-1 gene is localized 9p12 and consists of 7 exons spanning 10 kb of DNA. In Bag-1 mRNA exon, 7 consisted of polyadenylation site. Three different isoforms of Bag-1 protein were translated by alternative translation initiation sites. The specific NLS, Repeats, ULD, and BAG domains were shown in different Bag-1 L, M, and S isoforms. The translation initiation sites were located within exon 1.

Domains within Bag-1 protein

Different isoforms have the same carboxyl-terminal sequences therefore the distinct bioactivities of these isoforms occur as a result of different amino-terminal residues. The 70 aa 'BAG Domain' located at the carboxyl-terminal of all isoforms and consisted of a pair of anti-parallel amphipathic α -helices and upstream helix. The amino acid positions of this region are located between 246-326 and the sequence of this domain are 'QLEELNKELTGIQQGFLPKDLQAEALCKLDRRVKATIEQFMKILEEIDTLILPENKDSRLKRKGLVKKVQAFLAECDTVE'. The terminal COOH-BAG domain is required for mostly Hsc70 and Hsp70 chaperone and other protein-protein interactions including Raf-1 [159], [160]. Competitive binding of Hsp70 and Raf-1 to Bag-1 has played an important role in regulating cell survival because the high levels of Hsp70 suppress Raf-1 activation and Raf-1 mediated Bag-1 survival role [161].

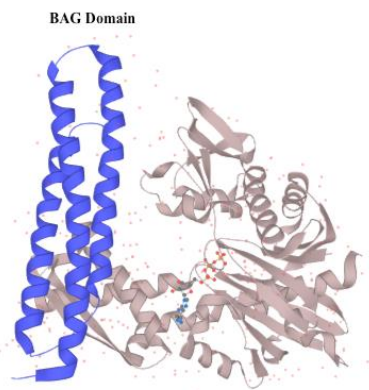


Figure 2.16 : The basic α -helices structure of BAG Domain in different Bag-1 isoforms. The structure and sequences of the Bag-1 domain were obtained from UniProt, PDB entry 1HX.

The ‘Ubiquitin-Like Domain (ULD)’ is required for the binding to the proteasome. Initial studies showed that the amino acid sequence of Bag-1 did not similar to other proteins exception of a ubiquitin-like domain sequence and therefore termed as a novel anti-cell death gene [153].

According to the UniProt database of Bag-1 PDB entry 1WXV, the aa sequence of the ULD domain is ‘LTVTVTHSNEKHDLHVTSSQGSSEPVVQDLAQVVEEVIGVPQS FQKLIFKG KSLKEMETPLSALGIQDGCRVMLIGKKNSP’, and this domain is located at a position between 144-224 [162]. The ‘Repeat’ domain was located at the amino-terminal of all Bag-1 isoforms, while Bag-1L and Bag-1M contain nine copies of six aa repeats, Bag-1S contains three copies. Although the exact function of the repeats was not known but might regulate glucocorticoid receptor-dependent transcription [162]. The ‘Nuclear Localization Signal (NLS)’ domain located at the amino terminus at Bag-1L isoform that regulated nuclear translocation of this isoform [162].

2.1.4.2 Interaction partners and biological functions of Bag-1

The multifunctional Bag-1 protein regulates diverse growth control pathways including cell survival, apoptosis, metastasis, proliferation, transcription, stress responses, and protein degradation via multiple direct or indirect interaction partners. The cellular interaction partners of Bag-1 include Bcl-2, the Hsc70, and Hsp 70 heat shock proteins, glucocorticoid, estrogen, and androgen hormone receptors, hepatocyte and platelet-derived growth factor receptors, the RAF-1 kinase, CHIP, Rb, ubiquitin/proteasome system, and DNA [163]–[166]. Studies to investigate the regulatory role of the retinoblastoma protein (Rb) in colorectal cancer progression

showed that colorectal cancer cells express high levels of Rb, contradicting the tumor suppressor role of Rb in other cancer cells. It was established that Rb interacts with Bag-1 to enhance its nuclear localization and anti-apoptotic function. Moreover, the interaction between Rb and Bag-1 was required for the protection of cells against radiation-induced apoptosis not only in colon tumor-derived cells but also in osteosarcoma cells [167].

Considering the role of Bag-1 in breast cancer, Bag-1 was detected in 100% of normal breast tissues but was mostly found in benign lesions in ductal carcinoma in situ (DCIS) with the detection of 90% in ER-positive tumors. Bag-1 expression was found low levels in CAL51, CAMA1, SK-BR-7, and BT20 cells but highly expressed in ZR-75-1, MCF7, MDA-MB-453, and GI-101 cells. The different isoforms were highly expressed in breast cancer and this condition was associated with positive ER status. It has been observed that cells expressing highly functional ER also showed high levels of Bag-1 isoforms [6]. The clinical relevance of Bag-1 was shown as a prognostic biomarker in Oncotype DX and PAM50 assay, which highlighted its role in breast cancer [3]. The immunohistochemical studies showed that Bag-1 cytoplasmic positive staining scores were correlated with breast cancer progression in estrogen receptor-positive tissue samples [168]. The elevated levels of Bag-1 prevent cell death through association with hepatocyte growth factor (HGF) in liver progenitor cells [164], and also with glucocorticoid receptor interaction of Bag-1 were found in mouse thymoma cells [169]. Interaction between Bag-1 and cytoplasmic tail domain of heparin-binding EGF-like growth factor has functioned in the suppression of apoptosis [170]. However, downregulation of Bag-1 expression sensitized tamoxifen-resistant cells to tamoxifen and enhanced the apoptotic potential of cisplatin and paclitaxel in MCF-7 cells [171].

Bag-1 protein has been identified as a cochaperone role for Hsp70/Hsc70, by binding to the ATPase domain of Hsp70. It functions as a nucleotide exchange factor to stimulate ADP release and enhance the ATPase activity of Hsp70 by binding to the nucleotide-binding cleft of the Hsp70 ATPase domain [172]. Considering the two controversial findings of the association with Bag-1 and Hsp70 showed that some of the previous researches suggested that Bag-1 is an inhibitor of ATP-dependent Hsp70 activity, while some studies opposed that Bag-1 is a stimulatory cochaperone [173], [174]. Isoform-specific Bag-1 studies determined that the Bag-1S isoform enhances but Bag-1M and Bag-1L isoforms negatively regulate the cochaperone foldase activity [175]. The interactome analysis showed that Bag-1 has been identified in endoplasmic

reticulum-associated protein degradation (ERAD) through interacting VCP/p97; which is a translational endoplasmic reticulum ATPase, Rad23B; a shuttling factor ubiquitinated proteins [176].

In addition to the role of Bag-1 in cancer development, several studies were established to show the critical function of Bag-1 in the regeneration of neural cells due to its limited regeneration capacity after traumatic injuries. To investigate the effect of Bag-1 on regeneration of retinal ganglion cells (RGC), the adeno-associated virus (AAV)-mediated Bag-1 overexpression was performed in optic nerve transection models *in vivo* through virus injection. The survival ratio of RGC after optic nerve transection and the axonal regeneration in optic nerve crush site promoted by AAV-mediated Bag-1 overexpression in mouse models. Moreover, AAV-mediated Bag-1 overexpression was also induced neurite outgrowth in RGC cells *in vitro* [177]. Bag-1 knockout mouse models showed enhanced pluripotency markers such as Oct4, Sox2, and Nanog [178]. The survival role of Bag-1 in differentiating neurons and hematopoietic cells showed enhanced apoptotic parameters in Bag-1 knockout conditions [179].

These well-established studies indicate that Bag-1 is a potential target for investigating cellular homeostasis and development. Therefore studying the function of Bag-1 attracts considerable interest for future therapeutic approaches.

2.2 Hypothesis

Bag-1 protein modulates several different pathways due to its distinct interaction partners in the cell. Previous studies showed that overexpression of Bag-1 has been identified in pro-survival anti-apoptotic function in several cancers, whereas downregulation of Bag-1 enhanced the chemotherapeutic-induced apoptosis. Therefore, the first part of the thesis hypothesized that deficiency of Bag-1 significantly alters the growth conditions and cellular response of MCF-7 breast cancer cells targeting significant survival-targeted pathways.

2.3 Materials and Methods

2.3.1 Materials

2.3.1.1 Equipments

Types of equipment that are used in this study are listed in APPENDIX A.

2.3.1.2 Chemicals, media components, antibodies, and kits

Chemicals, media components, antibodies, and molecular biology kits that were used in this study are shown in APPENDIX B.

2.3.1.3 Buffers and solutions

Buffers and solutions that were used in this study are shown in APPENDIX C.

2.3.2 Methods

2.3.2.1 Cell culture

Our generated MCF-7 Human breast adenocarcinoma cells (ATCC, USA) were grown in high glucose DMEM (Dulbecco's Modified Eagle Medium, Pan Biotech, Germany) supplemented with 10% FBS (Fetal Bovine Serum, Pan Biotech, Germany) and 1% penicillin/streptomycin (Pan Biotech, Germany). Cells were maintained at 37°C in a humidified 5% CO₂ incubator. Akt inhibition experiments were performed by the treatment of 500 nM MK-2206 for 24h.

2.3.2.2 Generation of CRISPR/Cas9-based Bag-1 knockout MCF-7 cells (Bag-1 KO cells)

In this thesis, MCF-7 cells were selected for knockout experiments due to their intermediate level of endogenous Bag-1 expression. Bag-1 CRISPR/Cas9 Knockout plasmid (sc-417179), Bag-1 Homology Directed Repair plasmid (sc-417179-HDR), and Control CRISPR/Cas9 Knockout plasmid (sc-418922) was purchased from Santa Cruz Biotechnology, USA.

The strategy of Bag-1 knockout cells was based on the transfection of two different types of plasmids, which have 3 different sgRNA sequences for the Bag-1 gene. Bag-1 CRISPR/Cas9 Knockout (KO) and Bag-1 HDR (HDR) plasmids were co-transfected into MCF-7 breast cancer cells, using UltraCruz Transfection Reagent (Santa Cruz Biotechnology, USA). Meanwhile, the Control Knockout plasmid was transfected into MCF-7 cells using the same transfection agent to confirm the transfection efficiency. After transfection, puromycin (Santa Cruz Biotechnology, USA) selection was performed for 48h, and cells were selected by single-cell dilution in 96-well plates.

2.3.2.3 Single-cell dilution

Following the transfection of CRISPR/Cas9 plasmids, cells were seeded in 96-well plates through modifying the “A single cell dilution protocol for obtaining CRISPR targeted cell clones” protocol. Briefly, 1×10^4 cells were diluted in 220 μ l media, and 110 μ l of the suspension was added into 890 μ l fresh media. 5 cell/ml cell culture media was seeded at 100 μ l into 96 well plates. Following the attachment of cells, light microscopy was used for colony selection. Expanded monoclonal cells were used for further experiments.

2.3.2.4 Generation of stable Bag-1 overexpressed MCF-7 cells (Bag-1 OE cells)

For the comparison of the Bag-1 expression profile, Bag-1 overexpressed cells (Bag-1 OE) were used in further experiments. Bag-1 overexpression was performed by TAP (tandem affinity purification) tagged Bag-1L with C-terminal DNA insert plasmid transfection into MCF-7 cells, which was described in our previous study [180]. Bag-1L transfected stable cells were selected using G418. The map of the Bag-1 OE plasmid vector was shown in Appendix D.

2.3.2.5 Generation of transient Bag-1 isoform-specific plasmid transfections

For the recovery of Bag-1 expression in an isoform-specific manner, we transfected MCF-7 wt and Bag-1 KO cells with isoform-specific TAP-tagged Bag-1S, M, and L with N-terminal DNA insert plasmid that was cloned in pEZ-M02-plasmid (CS-L0198-M02; Capital Biosciences, Rockville, MD, USA).

For further studies, the following abbreviations were used to demonstrate selected stable MCF-7 cell lines. wt: wild-type, Control KO: Control Knockout plasmid transfected, Bag-1 KO: Bag-1 Knockout and Bag-1 HDR plasmid co-transfected, Bag-1 OE: Bag-1L C-Tap-tagged plasmid transfected stable Bag-1 overexpressed cells.

2.3.2.6 Genomic polymerase chain reaction (PCR) analyses and Sanger sequencing of different exons of MCF-7 Bag-1 KO cells

Genomic DNA of MCF-7 wt, Control KO, and Bag-1 KO cells (1×10^6) was extracted using Quick DNA/RNA Kit (Zymo Research). Primers are designed for exon 2, exon 3, and exon 6 which have the binding sites of sgRNAs. To detect the off-target effects also 3 different primers were designed according to the most possible off-target sites. Primers designed by CRISPOR and Primer3Plus, synthesized by Thermo Scientific,

USA. The most possible 3 different off-target sequences for Bag-1 KO determined by the CRISPOR web tool were mentioned in Appendix E.

Amplification of specific regions of Bag-1 from MCF-7 cells was performed using Taq DNA Polymerase (Thermo Scientific, USA). Amplicons were run on 2% agarose gel. PCR products were Sanger sequenced using specific primers with Applied Biosystems 3500XL Genetic Analyzer for Human Identification (Thermo Scientific, USA).

2.3.2.7 Immunoblotting assay

To detect the expression profiles of selected proteins, MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells were seeded at 1×10^6 /well on a 60 mm petri dish. Then, the cells were washed with ice-cold 1x PBS (Phosphate-buffered saline, PAN Biotech, Germany) then lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific, UK) with protease inhibitor cocktail (cOmplete, Mini, Sigma Aldrich, USA) according to the manufacturer's instructions. The protein concentration was determined by the Bradford assay. Lysates were boiled with 5 min in 4x Laemmli Sample Buffer at 95°C. Equal concentration of protein lysates per sample was fractionated on a 12% SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, USA). Membranes were blocked with 5% BSA in TBS-Tween 20 for 1h at room temperature and incubated with appropriate primary antibodies (Cell Signaling Technologies, USA) overnight at 4°C, and anti-rabbit or anti-mouse IgG-horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies, USA) overnight at 4°C. Following a gentle washing step with TBS-Tween 20, the membranes were treated with enhanced chemiluminescence detection reagent and analyzed with Chemidoc MP Imaging System (Bio-Rad, USA). Densitometric analysis of immunoblots was performed using ImageJ. All proteins were quantified relative to the loading control. GAPDH was used as an internal loading control.

2.3.2.8 Green fluorescent (GFP)/red fluorescent (RFP) fluorescence detection

MCF-7 wt, Control KO, and Bag-1 KO cells were seeded at 5×10^4 cells were seeded in 12-well plates, and following their attachments cells were stained with 5 mg/ml DAPI for 10 min and GFP density of Control KO cells, GFP and RFP density of Bag-1 KO cells and DAPI density of wt, Control KO and Bag-1 KO cells were visualized by fluorescent microscopy option of Olympus 1X71 Inverted fluorescent microscope according to excitation and emission wavelengths. (GFP Excitation: 488 nm, and

Emission: 525 nM, RFP Excitation: 561 nM, and Emission: 575 nM, DAPI Excitation: 350 nM, and Emission: 570 nM).

2.3.2.9 MTT assay

MTT Cell viability assay was assessed to determine the viability of MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells, to investigate the cytotoxic efficiencies of Akt inhibitor (MK-2206) and the survival role of Bag-1 isoform-specific plasmid transfected cells. Cells were seeded at 1×10^4 /well in 96 well plates and maintained for their attachments. Then the cell viability was determined by the addition of 5 mg/ml MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) to the media and cells were incubated at 37°C for 4 h. After removal of MTT reagent included media, 100 μ l DMSO (dimethylsulfoxide) was added to each well and incubated for 5 min at dark conditions. Absorbance was determined at 570 nM using the iMark Microplate Absorbance Reader (Bio-Rad).

2.3.2.10 Trypan blue dye exclusion assay

To determine the cell proliferation ratios of MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells and to investigate the effect of MK-2206, cells were seeded at 5×10^4 cells/well density in 6 well plates. According to the time points (24 h, 48 h, 72 h), cells were trypsinized and counted every 24 h by staining with 0.4% trypan blue dye with a Neubauer dual-chamber hemocytometer under the light microscope. Trypan blue-stained cells were considered non-viable and were excluded from the calculation.

2.3.2.11 Colony formation assay

Investigation the role of Bag-1 expression on colony-forming potentials of wt, Control KO, Bag-1 KO, and Bag-1 OE cells, cells were seeded at 500 cells/well density in 6 well plates maintained for 14 days to form colonies. Following this period, cells were trypsinized and fixed with methanol: acetic acid (3:1 ratio) for 5 min. Then, the fixing agent was removed and cells were stained with 0.5% crystal violet in methanol for 15 min following the washing step, morphological images were taken under light microscopy.

2.3.2.12 (3,3'-dihexyloxacarbocyanine iodide) DiOC6 staining

MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells were seeded at 1×10^5 density at 6- well plates, and following their attachments cells were stained with 4nM DiOC6

for 15 min and visualized by fluorescent microscopy option of Olympus 1X71 Invert fluorescent microscope at Excitation: 488 nM, and Emission: 525 nM.

2.3.2.13 Receptor tyrosine kinase signaling antibody array

To determine the Bag-1 expression level on kinase phosphorylation profiles and downstream signaling nodes of MCF-7 cells, we used PathScan RTK Signaling Antibody Array Kit (#7982, Cell Signaling Technology) according to the manufacturer's instructions. Briefly, this slide-based antibody array kit allows simultaneously detecting 39 proteins when phosphorylated at tyrosine or other residues. Total protein lysate (1 mg/ml) was incubated on an array slide. Then Horseradish Peroxidase-linked streptavidin was used before the chemiluminescence detection. LumiGLO® and Peroxide Reagent were used to detect bound phosphorylated proteins by chemiluminescence. The slide was visualized by ChemiDoc (Bio-Rad) imaging system. The densitometry analysis was performed according to the previous studies. Images were analyzed by ImageJ. Each spot was selected manually and the average pixel density of two replicate spots was calculated. Subtracting the pixel density of the background from each spot was performed. The signal intensity was normalized with the pixel density of the positive control. Significance was determined using a cut-off point of density signal higher than 0.4.

2.3.2.14 Measurement of cellular ROS generation

The measurement of the cellular Reactive Oxygen Species (ROS) is based on the oxidation of a ROS indicator 2',7'-dichlorodihydrofluorescein (H2-DCFHDA). The non-fluorescent H2-DCFHDA is oxidized and converted into 2',7'-dichlorofluorescein (DCF) by intracellular ROS generation, and the DCF intensity was evaluated by FACS flow. Cells were seeded at 3×10^5 cells/well density in 6-well plates. Then, cells were treated with MK-2206 (500 nM) for 24 h and incubated with H2O2 (0.1 mM) for 20 min for positive control. Cells were trypsinized and stained 0.1 μ M H2-DCFHDA for 30 min at 37 °C. The intensity was determined using a 488 nm excitation filter and detected at 535 nm.

2.3.2.15 Immunofluorescence experiment

To compare the organization of actin cytoskeleton in MCF-7 wt, Bag-1 KO, Bag-1 OE, and Bag-1 isoform-specific S, M and L plasmid transfected wt and Bag-1 KO cells, Actin Cytoskeleton and Focal Adhesion Staining Kit (FAK100, Merck, USA)

were performed according to the manufacturer's instructions. Briefly, cells were seeded at 50% confluency in 6 well plates and fixed with 4% paraformaldehyde then permeabilized with 0.1% Triton X-100. Following permeabilization, cells were blocked with 1% BSA and incubated with fluorescent-labeled Phalloidin (TRITC-conjugated Phalloidin) to observe the F-actin monomers, which are the major constituent of microfilaments. After incubation, cells were stained with DAPI for 5 min and fluorescent images were visualized with a fluorescent microscopy option of Olympus 1X71 inverted fluorescent microscope.

2.3.2.16 Statistical analysis

All the statistical analysis of experiments was performed using GraphPad Prism version 8, <https://www.graphpad.com/>. MTT Cell viability assays were repeated three times and statistically analyzed using Two-Way ANOVA Sidak's multiple comparison test. Trypan Blue Dye Exclusion Assay analysis was performed Two-Way ANOVA Dunnett's multiple comparison test. Path Scan analysis was performed by Two-Way ANOVA, Tukey's multiple comparison tests. The densitometric calculation of three replicated immunoblotting images was performed using Image Lab Software (Bio-Rad) and analyzed by Two-Way ANOVA, Tukey's multiple comparison test. Significance levels are represented as following: ns $p > 0.05$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent $\pm$ standard deviation values.

2.4 Results

2.4.1 Generation of Bag-1 knockout MCF-7 cell model

2.4.1.1 Informations about Bag-1 CRISPR/Cas9 KO, Bag-1- HDR, and Control KO plasmids

To generate Bag-1 KO MCF-7 breast cancer cells, Bag-1 CRISPR/Cas9 KO plasmid (sc-417179) was co-transfected with Bag-1 HDR Plasmid (sc-417179-HDR) and Bag-1 gene was inactivated through HDR mechanism. In addition, Control CRISPR/Cas9 plasmid was used as a negative control of experimental procedures.

Bag-1 CRISPR/Cas9 KO plasmid

To knockout, the Bag-1 gene, the CRISPR/Cas9 KO plasmid cassette includes the sequences required for expression of both Cas9 and target gRNA sequences were used. The overview of the CRISPR/Cas9 KO plasmid cassette is shown in Figure 2.17. Red

labeled 20 nt sequences in the plasmid refer to the gRNA sequences that guide Cas9 to target DNA sequences. The gRNA scaffold is required for the binding of Cas9 to target the genomic DNA sequence. U6 is a typical gRNA promoter and the Termination signal is for the termination of transcription. Chicken β -actin hybrid (CBh) promoter is a Cas enzyme promoter for the high-level expression of Cas9 in the mammalian expression vector. The amino acid sequence NLS directs Cas9 into the nucleus after production in the cytoplasm for the formation of the Cas9-gRNA complex in the nucleus. 2A is a peptide that led the production of both Cas9 and GFP from the same CBh promoter. GFP is for the verification of Bag-1 CRISPR/Cas9 KO plasmid transfection into mammalian cells. The Bag-1 CRISPR/Cas9 KO plasmid consists of three different Bag-1 exon-specific 20 nt guide RNA sequences derived from GeCKO (v2) library, with high modification efficiency and low off-target effects. These guide RNAs direct the Cas9 protein to induce a site-specific DSB in Bag-1 gene 3-5 base pairs downstream of the 3' end of the target sequence. The pool of three sgRNAs enhances the efficient gene disruption. The sequences of three different sgRNAs on Bag-1 CRISPR/Cas9 KO plasmid were provided from Santa Cruz, and the regions were determined from comparing the human nucleotide sequences through Basic Local Alignment Search Tool (BLAST) and checked at Bag-1 genomic sequence (NG_029018.1). The sequence of sgRNA A was “TGAACCAGTTGTCCAAGACC” targeted exon 2, sgRNA B sequence was “GGTTGCCGGGTCATGTTAAT” targeted exon 3 and sgRNA C sequence was “AGGCTTGGTAAAAAAGGTTC” targeted exon 6 (Figure 2.17).

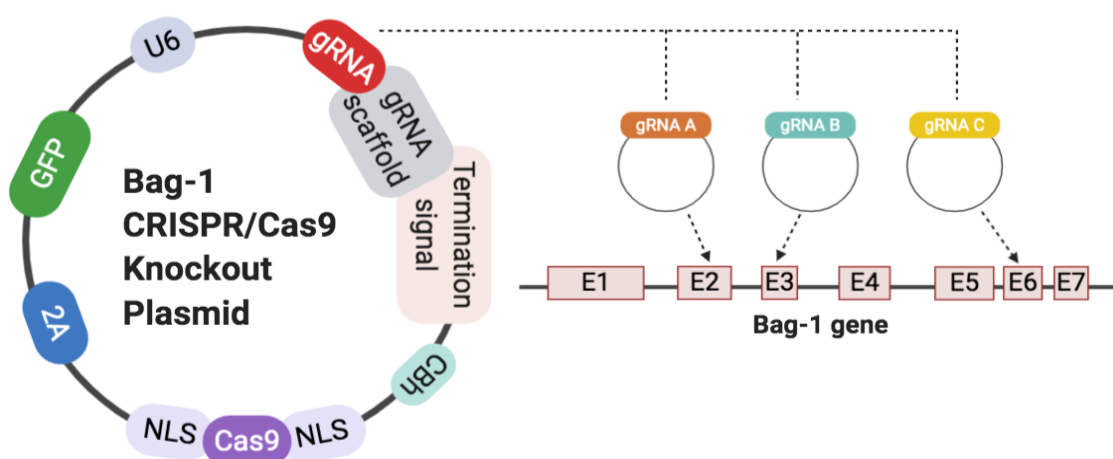


Figure 2.17 : Map of Bag-1 CRISPR/Cas9 Knockout plasmid modified from Santa Cruz.

Bag-1 HDR plasmid

Bag-1 HDR plasmid contains an HDR template for a DSB corresponding to the cut sites generated by the Bag-1 CRISPR/Cas9 KO plasmid. HDR plasmid integrates puromycin resistance gene following co-transfection with Bag-1 CRISPR/Cas9 KO plasmid for the selection of stable Cas9-induced DNA-cleaved knockout cells. This plasmid also consists of a pool of three HDR template-included plasmids, which are specific for corresponding different Bag-1 gRNAs. HDR templates contain 800 bp homology arms that bind to the target Bag-1 genomic DNA surrounding the corresponding Cas9-induced DSB site. In addition, HDR plasmid also included RFP for the visual verification of plasmid transfection and Elongation Factor 1a (EF1a) promoter for efficient expression of RFP. LoxP sites are required for the recognition of Cre recombinase which cuts the two LoxP sites and allows the excise of only targeted DNA regions between two loci of X-over of P1 (LoxP) sites as a circular DNA and a selective step for generation of knockout cell line (Figure 2.18).

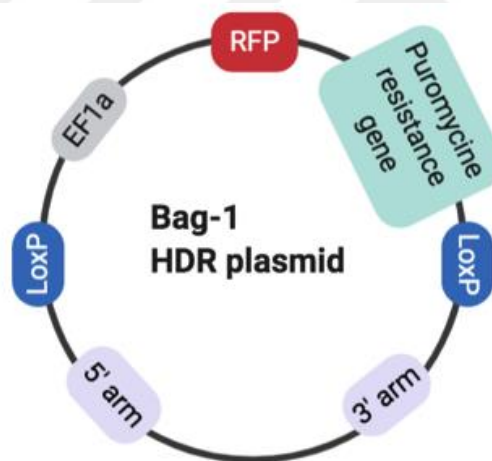


Figure 2.18 : The map of Homology-Directed Repair Plasmid provided from Santa Cruz.

Control CRISPR/Cas9 plasmid

In addition to Bag-1 KO MCF-7 cell model, generated Control CRISPR/Cas9 plasmid transfected MCF-7 cells for the negative control of our study. Control CRISPR/Cas9 plasmid encodes Cas9 nuclease and consists of non-specific 20 nt scrambled guide RNA that can not bind to genomic DNA. The transfection verification was performed due to the GFP region of this plasmid (Figure 2.19). The main differences between the Bag-1 CRISPR/Cas9 KO plasmid and Control CRISPR/Cas9 plasmid are that the gRNAs are Bag-1-exon specific in Bag-1 CRISPR/Cas9 KO plasmid and co-transfection of HDR plasmid to cause gene disruption.

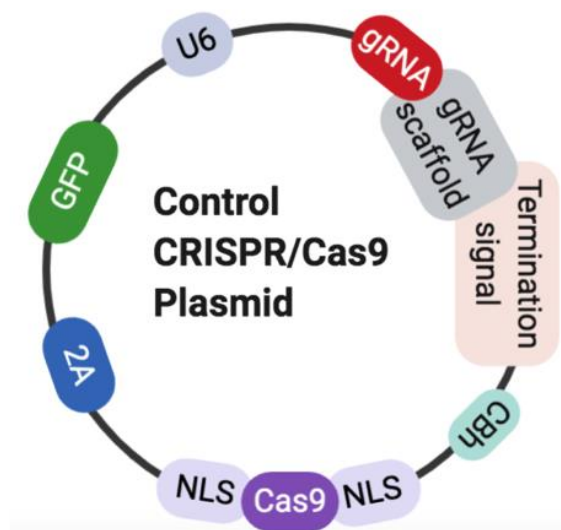


Figure 2.19 : The map of Control CRISPR/Cas9 plasmid provided from Santa Cruz.

The generated MCF-7 cell models obtained after transfection were indicated by the following abbreviations for further studies. Bag-1 KO: Bag-1 CRISPR/Cas9 KO and Bag-1 HDR plasmid co-transfected MCF-7, stable cell model. Control KO: Control CRISPR/Cas9 plasmid transfected MCF-7 stable cell model.

2.4.1.2 The general workflow of generation of stable Bag-1 KO and Control KO MCF-7 breast cancer cells

MCF-7 cells were co-transfected with Bag-1 CRISPR/Cas9 KO and Bag-1 HDR plasmid for Bag-1 KO and transfected with Control CRISPR/Cas9 plasmid for the control of transfection. Cells were incubated for 48h and generated Bag-1 KO and Control KO MCF-7 cells were selected through puromycin selection (1-10 µg/ml) for a minimum of 2 weeks, which allows the assortment of cells with successful plasmid transfection. Then, living cells were further diluted through a single-cell dilution process to obtain a clonal population. Bag-1 KO and Control KO MCF-7 cells were single-cell diluted in 96-well plates at 1 cell per well. Following single-cell dilution, 12 different clones from generated Bag-1 KO cells and 6 distinct clones from Control KO cells were selected concerning plasmid transfection. The general strategy for generating Bag-1 KO and Control KO cells through a single-cell dilution process was shown in Figure 2.20.

Following maintaining of single-cell clones from Bag-1 KO and Control KO MCF-7 cells, validation and characterization of CRISPR/Cas9-mediated Bag-1 KO efficiency were performed through genomic PCR, immunoblotting, and DNA sequencing analysis.

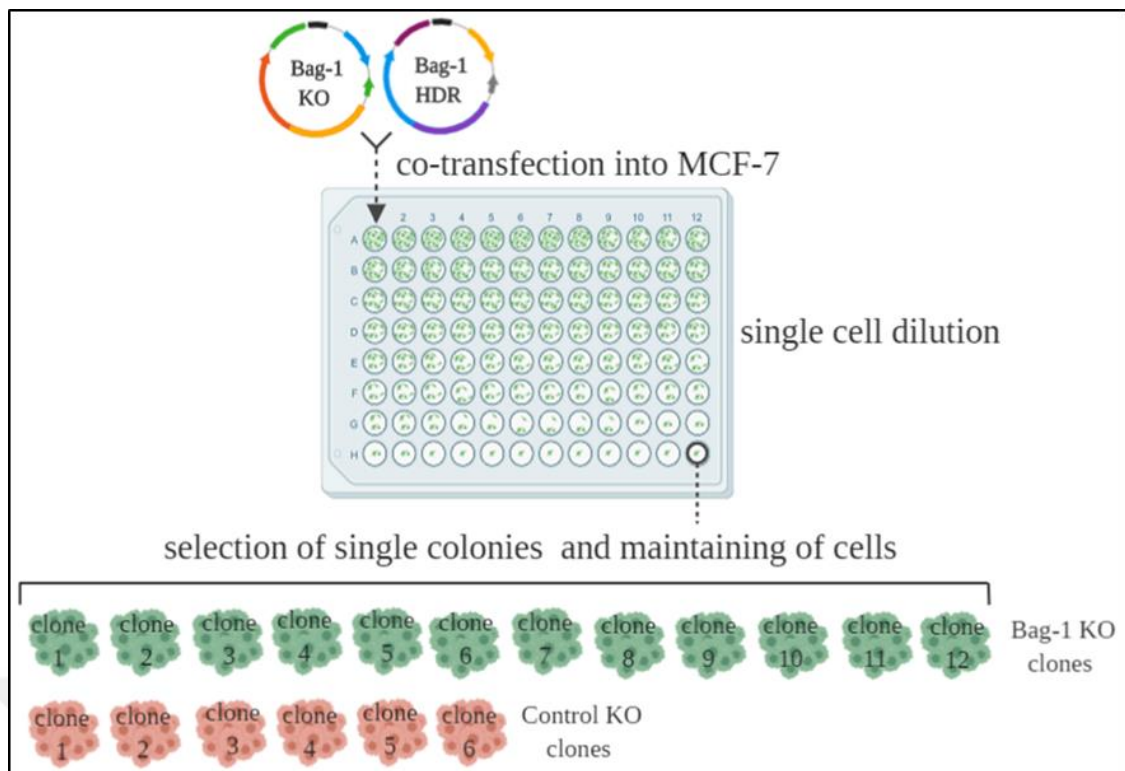


Figure 2.20 : General strategy for generation of Bag-1 KO and Control KO MCF-7 cells. Bag-1 KO plasmid was co-transfected with Bag-1 HDR plasmid into MCF-7 cells. Following transfection, cells were seeded according to the single-cell dilution process until diluted at 1 cell per well in 96-well plates as mentioned 2.4.2.3. Single-cell dilution process. Then 12 different colonies for Bag-1 KO and 6 different colonies for Control KO cells were selected and maintained and visualized periodically from 7-10 days under a microscope.

2.4.1.3 Validation and characterization of Bag-1 KO MCF-7 cells

To validate the Bag-1 KO effect in MCF-7 cells, we performed genomic PCR, Western Blot, and DNA sequencing analysis. Several different primers have been designed for genomic PCR analysis. The 3 primers were designed for specific Bag-1 exons such as exon 2, exon 3, and exon 6. In addition, to investigate the off-target effects of Bag-1 KO, the most possible 3 off-target sequences were determined corresponding to different sgRNAs including sgRNA A, B, and C, and primers were designed as mentioned 2.4.2.6. The designed primers for different Bag-1 exons and off-target regions were shown in Table 2.3.

Table 2.3 : Designed primers and expected base pair sizes to validate Bag-1 knockout in MCF-7 cells.

Primers for Bag-1 KO					
Name	Target region	gRNA sequences	Primer sequences	Expected product size	PCR
Guide A	Exon 2	TGAACCAGTTGTCCAAGACC	F: TTTCTTTCTCCCCAGGCAAT R: TGTGGAACCCCTATGACCTC	118 bp	
Guide B	Exon 3	GGTTGCCGGGTCATGTTAAT	F: GGAAACACCGTTGTCAGCAC R: ACAGGAGCAGGAGGGTATGA	252 bp	
Guide C	Exon 6	AGGCTTGGTAAAAAAGGTTTC	F: ACAGGATACAGACCCAGGGA R: ACCACGCTCCTACACTACCT	337 bp	
Primers for off-targets					
Name	Target region	Target gRNA	Primer sequences		
Off-target 1	CTD-2183H9.7 F8_chrX chrX:154126403-154126425	gRNA A Exon 2	F: TCGTCGGCAGCGTCAACACTGCAGCTGCGTATCT R: GTCTCGTGGGCTCGGAGAAGGCCTGAAGAGTGCAC		
Off-target 2	AC007682.1 AC097463.2_chr2 Chr 2: 52130030-52130052	gRNA B Exon 3	F: TCGTCGGCAGCGTCCAGTCAGTCATCTCAGAGAAGACA R: GTCTCGTGGGCTCGGTGTGCTCTGCCAACTTTGTT		
Off-target 3	RPL31P12 RNU6-1246P_chr1 – Chr1:73056651-73056673	gRNA C Exon 6	F: TCGTCGGCAGCGTCACCTGGCAATATTCTCAGCCA R: GTCTCGTGGGCTCGGACATGAACTCTTCTGTGGCA		

2.4.1.4 Optimization of the annealing temperatures of designed primers

To perform genomic PCR reactions using wt, Bag-1 KO, and Control KO MCF-7 genomic DNAs with designed primers for further validation processes, the exact annealing temperatures of these primers were optimized by gradient PCR reactions using wt MCF-7 genomic DNA template as an initial step. The specific sequences corresponding to the designed primers were amplified with different annealing temperatures (65 °C- 50 °C). According to the optimized bant patterns 62 °C was decided as an annealing temperature of designed primers for Guide A, C, off-target-1, off-target-2, off-target-3, and 55,9 °C was selected for Guide B primer of annealing temperature (Figure 2.21).

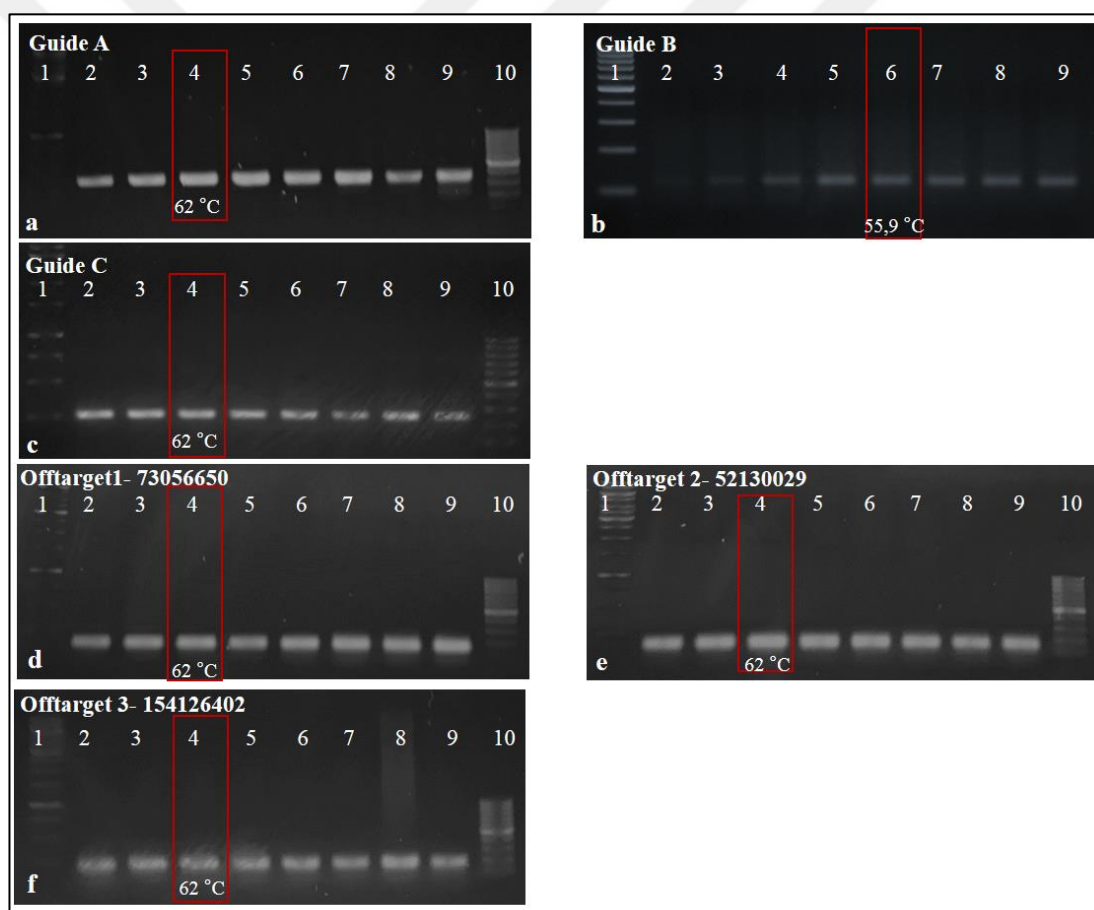


Figure 2.21 : Agarose gel electrophoresis of gradient PCR to optimize annealing temperature of different primers in MCF-7 wt cells. RT-PCR reaction was performed with with 2X PCR Master Mix at conditions; Denaturation 95 °C- 30 sec/95 °C- 5 min, Annealing 65 °C- 50 °C -30 sec, Extension 72 °C- 45 sn/72 °C-10 min. 1: 1kb marker, 2: 65 °C, 3: 64 °C, 4: 62 °C, 5: 59.3 °C, 6: 55.9 °C, 7: 53.2 °C, 8: 51.1 °C, 9:50 °C, 10: 50bp marker.

2.4.1.5 Analysis of amplification profiles of gRNA A, gRNA B, and gRNA C regions targeting Bag-1-specific exons 2,3 and 6 in wt and different Bag-1 KO MCF-7 clones

Following annealing temperature determination of the designed primers, standard PCR was performed using the Guide A, Guide B, and Guide C primers which targeted exon 2, 3, and 6 with DNA template from MCF-7 wt and 12 different Bag-1 KO clones. The results showed that the majority of Bag-1 KO clones produced smaller PCR products compared to wt cells (Figure 2.22).

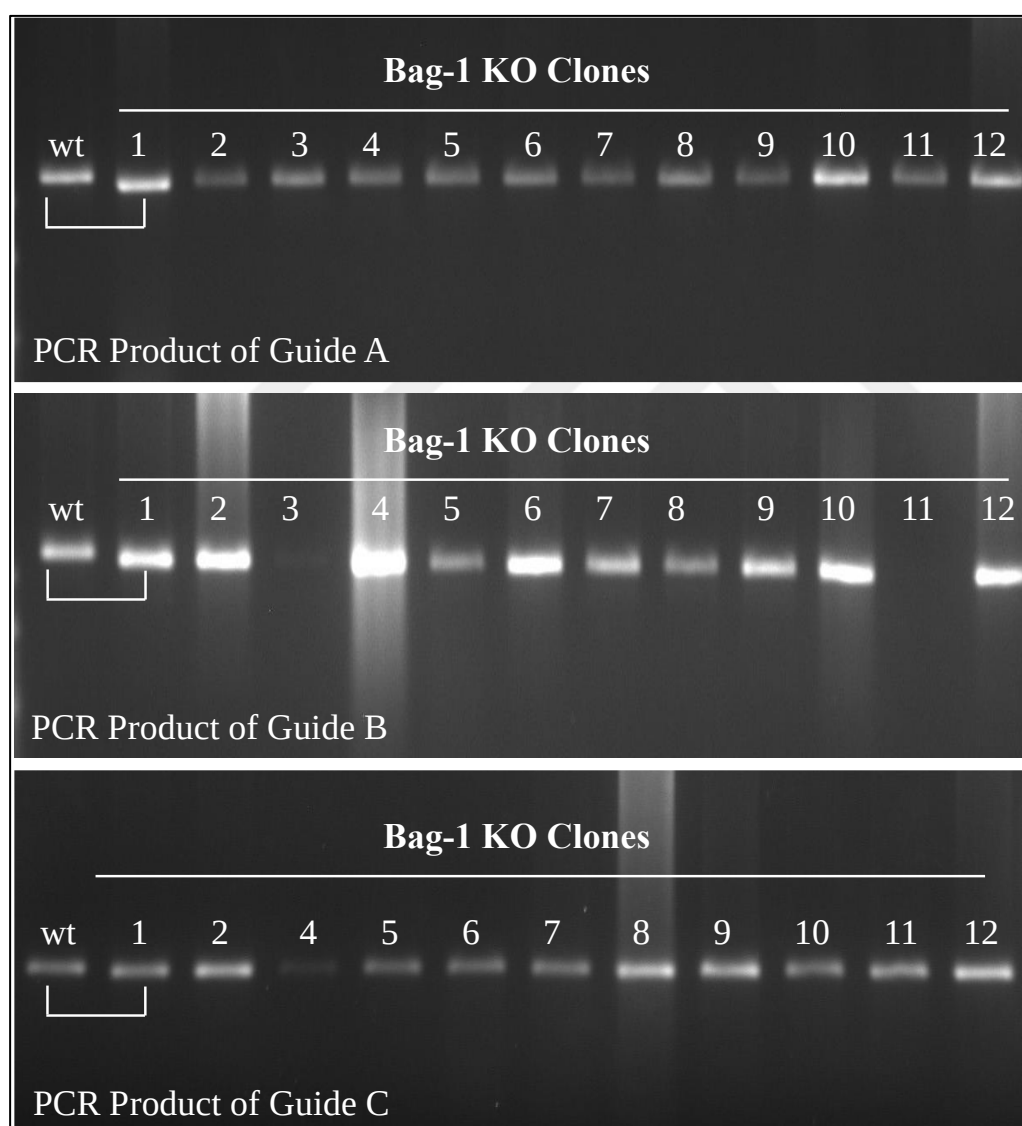


Figure 2.22 : The genomic PCR analysis of exons 2, 3 and 6 targeting PCR products of MCF-7 wt and different Bag-1 KO clones. RT-PCR reaction was performed with 2X PCR Master Mix at conditions; Denaturation 95 °C- 30 sec/95 °C- 5 min, Annealing 62 °C- 30 sec, Extension 72 °C- 45 sn/72 °C- 10 min.

2.4.1.6 Investigation Bag-1 expression profile in different Bag-1 KO clones comparing wt MCF-7 cells

To determine the loss of Bag-1 expression level in generated Bag-1 KO MCF-7 clones compared to wt cells, total protein isolation was performed from MCF-7 wt and 12 different Bag-1 KO cell lysates in further passages. Significant Bag-1 expression loss did not observe in selected 1-6 Bag-1 KO clones; although, clones 7-12 showed significantly lower Bag-1 expression profile compared wt cells in passage number 2. In further passage 3, the complete loss of Bag-1 expression was obtained in clones 7-12 but not in clone 1 (Figure 2.23). Considering the results of genomic DNA PCR assay and immunoblotting results, Clone 12 with completely none Bag-1 expression was selected as a Bag-1 KO cell for further experiments.

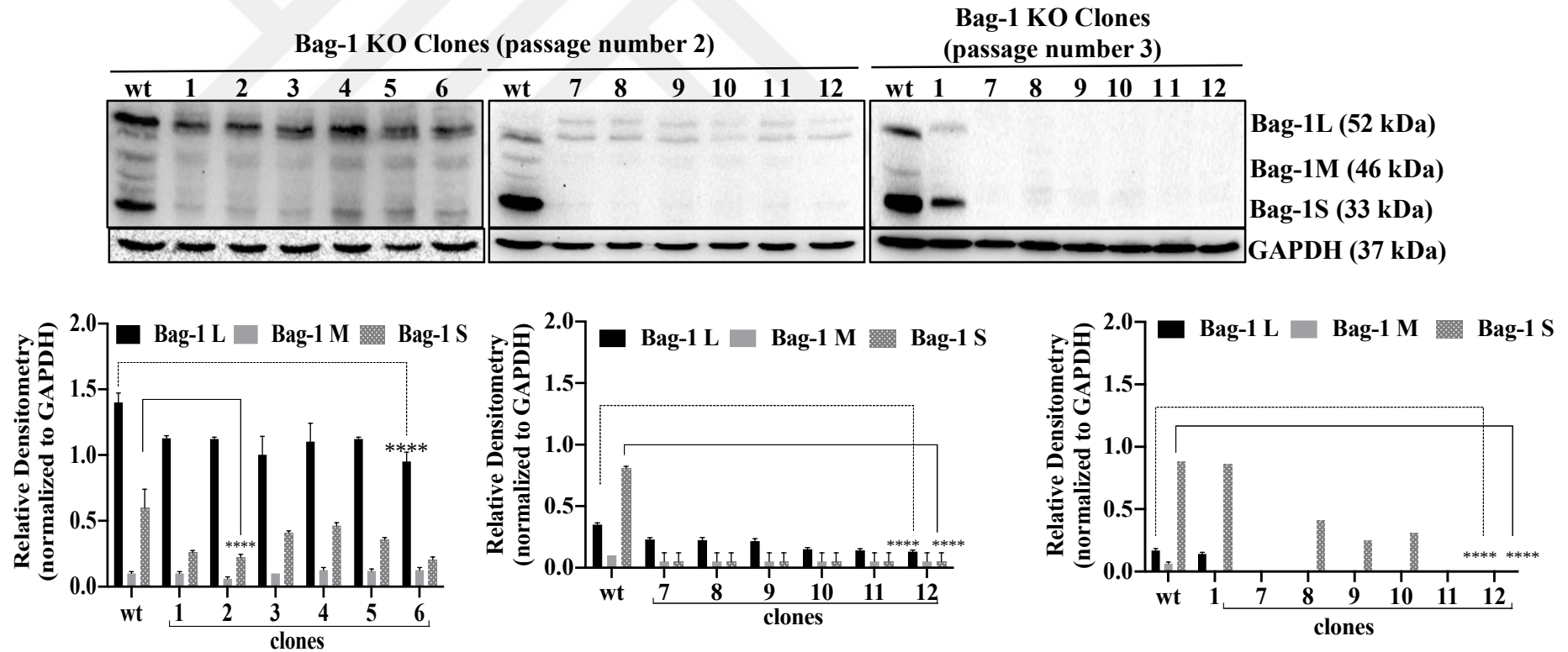


Figure 2.23 : Identification of selected clones that exhibit loss of Bag-1 expression by immunoblot analysis of cellular Bag-1 KO cell lysates. GAPDH was used as a loading control. Equal protein concentration of wt and Bag-1 KO clones were loaded on 12% SDS-PAGE. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. (** $p = 0.0044$, *** $p = 0.0005$, **** $p < 0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

2.4.1.7 Determination of the band profiles of three guide RNA and off-target regions targeting specific Bag-1 exons in selected Bag-1 KO, Control KO colonies, and wt MCF-7 cells

Following the selection of one individual clone from Bag-1 KO cells (Colony 12) and Control KO cells as a negative control, a standard PCR reaction was performed to compare the Bag-1 KO effect on specific Bag-1 exons and off-targets. Genomic DNA of MCF-7 wt, Control KO, and Bag-1 KO cells was used as a template for PCR reaction, and specifically designed primers for different exons targeted by distinct sgRNAs in Bag-1 CRISPR/Cas9 plasmid were used to compare PCR product bp lengths. The size of the PCR products using genomic DNA from Bag-1 KO clone 12 was found to be smaller than the size of the PCR product using the DNA of wt and Control KO clones on an agarose gel (Figure 2.24A). We also analyzed the off-target effect of selected sgRNA sequences of Bag-1 gene comparing the PCR product size between wt and selected Control KO, Bag-1 KO clones, and no size change determined (Figure 2.24B).

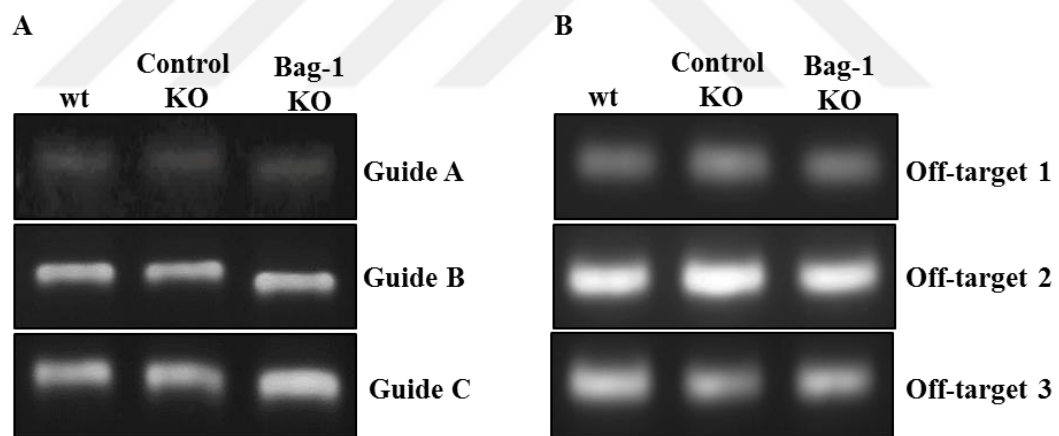


Figure 2.24 : Characterization of base pair differences in wt, selected Bag-1 KO and Control KO cells by genomic PCR analysis. Standard PCR reaction was performed with 2X PCR Master Mix (Thermo Fisher) at conditions; Denaturation 95 °C- 30 sec/95 °C- 5 min, Annealing 62 °C and 55.9 °C -30 sec, Extension 72 °C- 45 sn/72 °C-10. min.

2.4.1.8 Visualization of GFP or RFP densities in Control KO and Bag-1 KO MCF-7 cells

Transfection of Control CRISPR/Cas9 and Bag-1 CRISPR/Cas9 KO plasmids into MCF-7 cells was verified by fluorescence microscopy. Bag-1 KO MCF-7 cells were co-transfected with Bag-1 KO CRISPR/Cas9 plasmid, which has a GFP region, and

Bag-1 HDR plasmid, which includes the RFP region. Therefore, the transfection efficiency of both plasmids into MCF-7 cells was verified by the intensities of both proteins. The Control KO MCF-7 cells were generated by the transfection of the Control CRISPR/Cas9 Knockout plasmid that has only the GFP region. Thus, in transfected Control KO cells, only GFP intensities were determined. Nuclear formations of cells were visualized by DAPI staining (Figure 2.25).

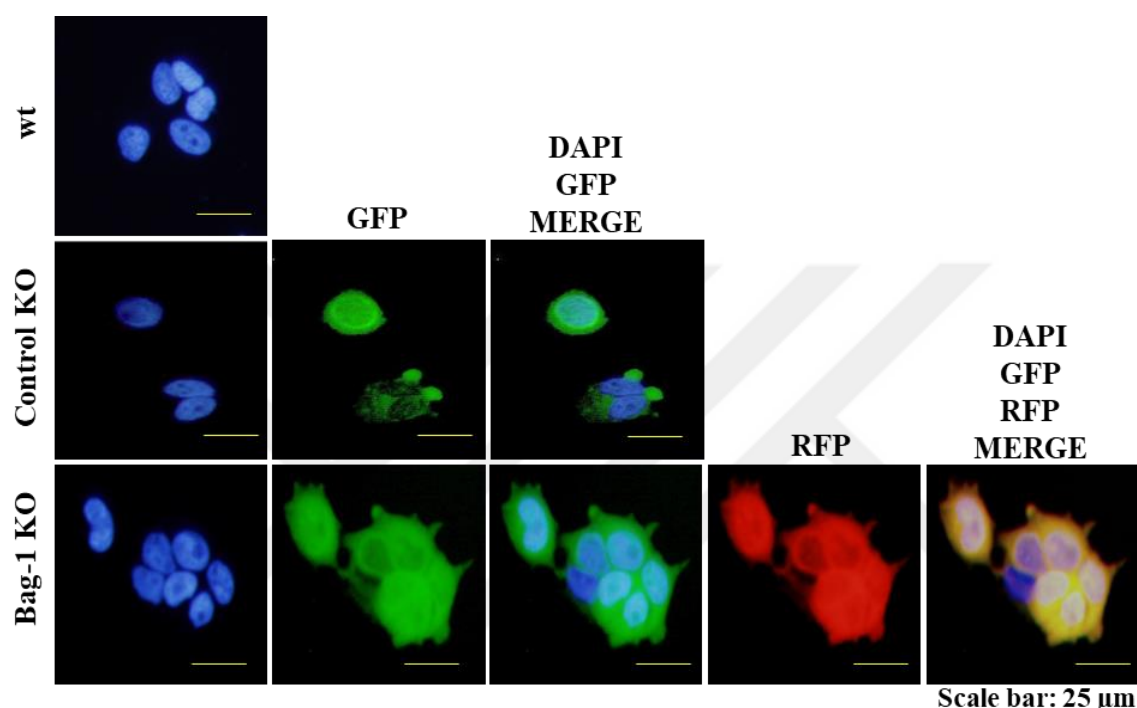


Figure 2.25 : Verification of selected Control KO and Bag-1 KO clones by GFP and RFP fluorescent protein intensity. DAPI was used for the nuclear formation of cells. Scale bar is 10 μm and magnification 40x.

2.4.1.9 Investigating gene-editing efficiency of different gRNA-guided Cas9 in Bag-1 KO MCF-7 cells

To confirm the deletion function of sgRNA A, B, and C, genomic DNA of Bag-1 KO cells were used as a template for PCR reaction, and differently designed primers for sgRNA A, B, and C were used. Bidirectional Sanger was performed for sequencing of three different PCR products. DNA sequencing results were aligned with the wt DNA sequence for exon 2, 3, and 6, respectively. The target sequences, PAM sequences, and deleted regions were highlighted to show the deletions on targeted sites. The sequencing result of the PCR product obtained as a result of the PCR reaction using Guide A primer showed 7bp deletion in forward strand (Figure 2.26 A) and 6 bp deletion in reverse strand (Figure 2.27 A) which were in the same sites. The sequenced

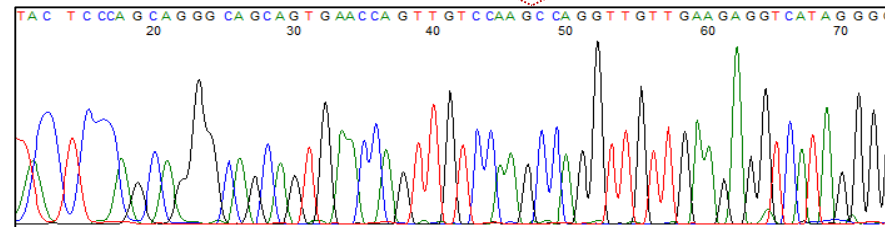
nucleotides were compared using BLAST and found that the regions were matched with the Bag-1 gene (NG_029018.1) (Figure 2.26 B, Figure 2.27 B). The PCR product of Guide B primer showed 6bp deletion in forward strand (Figure 2.28 A) and 5 bp deletions in reverse strand (Figure 2.29 A) which the sequences were aligned with Bag-1 gene (NG_029018.1) and observed the same nucleotides were deleted (Figure 2.28 B, Figure 2.29 B). The sequence result of Guide C-used PCR product showed 15 bp in each strand at the corresponding nucleotide sequences (Figure 2.30 A, Figure 2.31 A). The BLAST comparison was confirmed the deleted sequences in Bag-1 genomic sequence (NG_029018.1) (Figure 2.30 B, Figure 2.31 B).



A Bag-1 KO Guide A Forward Sequencing (targeting exon 2)

Sanger sequencing result

wt 5'-TACCTCCCAGCAGGGCAGCAGTGAACCAGTTGTCCAAGACCTGGCCCAGGTTGTTGAAGAGGTCATAGGGG-3'
 Bag-1 KO 5'-TAC--TCCCAGCAGGGCAGCAGTGAACCAGTTGTCCAAG-----CCAGGTTGTTGAAGAGGTCATAGGGG-3' (-7 bp)



B

BLAST result

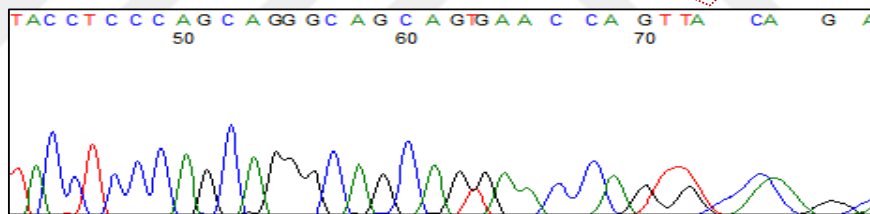
Score	Expect	Identities	Gaps	Strand
99.0 bits(53)	1e-24	76/85(89%)	9/85(10%)	Plus/Plus
wt 1951	TCATG-TTACCTCCCAGCAGGGCAGCAGTGAACCAGTTGTCCAAGACCTGGCCCAGGTTG	2009		
Bag-1 KO	TCATGATTA-CTCCCAGCAGGGCAGCAGTGAACCAGTTGTCCAAG-----CCAGGTTG	55		
wt 2010	TTGAAGAGGTCATAGGGGTTCCACA	2034		
Bag-1 KO	TTGAAGAGGTCATAGGGGTTCCACA	80		

Figure 2.26 : A) Displaying of the target sequence and PAM sequence of sgRNA A which targeted exon 2 and the forward read of Sanger sequence. **B)** The Nucleotide BLAST result of the forward read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide A primer.

A Bag-1 KO Guide A Reverse Sequencing (targeting exon 2)

Sanger sequencing result

wt 5'-TACCTCCCAGCAGGGCAGCAGTGAACCAAGTTGTCCAAGACCTGGCCCAGGTTGTTGAAGAGGTCATAGGGG-3'
 Bag-1 KO 5'-TACCTCCCAGCAGGGCAGCAGTGAACCAAGTTGTCCAAGACCTGGCCCAGGTTGTTGAAGAGGTCATAGGGG-3' (-6 bp)



B

BLAST result

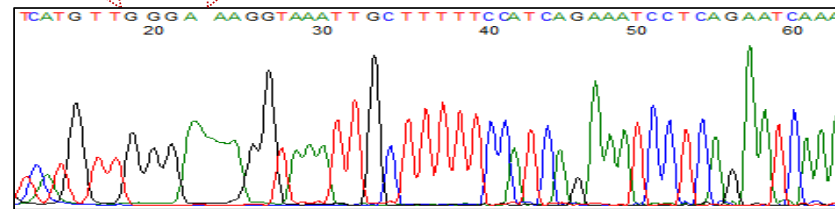
	Score	Expect	Identities	Gaps	Strand
	99.0 bits(53)	1e-24	66/71(93%)	5/71(7%)	Plus/Plus
wt	1957	TACCTCCCAGCAGGGCAGCAGTGAACCAAGTTGTCCAAGACCTGGCCC			
Bag-1 KO		TACCTCCCAGCAGGGCAGCAGTGAACCAAGTTGTCCAAGACCTGGCCC			
wt	2017	GGTCATAGGGG	2027		
Bag-1 KO		GGTCATAGGGG	66		

Figure 2.27 : A) Displaying of the target sequence and PAM sequence of sgRNA A which targeted exon 2 and the reverse read of Sanger sequence. B) The Nucleotide BLAST result of the reverse read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide A primer.

A Bag-1 KO Guide B Forward Sequencing (targeting exon 3)

Sanger sequencing result

Target sequence PAM
 wt 5'-ATGGTTGCCGGGTCATGTTAATGGGAAAAAGGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAA-3'
 Bag-1 KO 5'-ATGGTTGCCGGGTCATGTT-----GGGAAA---GGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAA-3' (-6 bp)



B

BLAST result

	Score	Expect	Identities	Gaps	Strand
	91.6 bits(49)	2e-22	64/70(91%)	6/70(8%)	Plus/Plus
wt	3644	ATGGTTGCCGGGTCATGTTAATGGGAAAAAGGTAAATTGCTTTTCCATCAGAAATCCT	3703		
Bag-1 KO		ATGGTTGCCGGGTCATGTT---GGG---AAAGGTAAATTGCTTTTCCATCAGAAATCCT	54		
wt	3704	CAGAATCAAA	3713		
Bag-1 KO		CAGAATCAAA	64		

Figure 2.28 : A) Displaying of the target sequence and PAM sequence of sgRNA B which targeted exon 3 and the forward read of Sanger sequence. **B)** The Nucleotide BLAST result of the forward read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide B primer

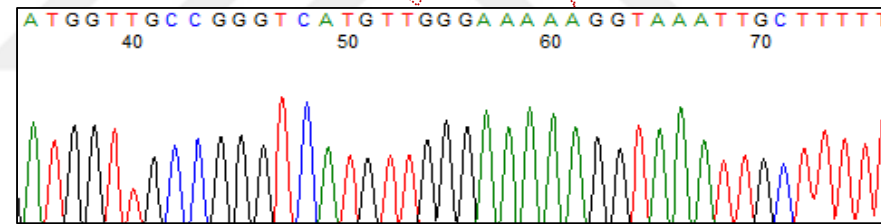
A Bag-1 KO Guide B Reverse Sequencing (targeting exon 3)

Sanger sequencing result

Target sequence PAM

wt 5'-ATGGTTGCCGGGTCATGTTAATGGGAAAAAGGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAA-3'

Bag-1 KO 5'-ATGGTTGCCGGGTCATGTT-----GGGAAAA---GGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAA-3' (-5 bp)



B

BLAST result

	Score	Expect	Identities	Gaps	Strand
	348 bits(188)	4e-99	201/206(98%)	5/206(2%)	Plus/Minus
wt	3612	GGAAACACCGTTGTCAGCACTTGAATACAAGATGGTTGCCGGGTCATGTTAATTGGGAA	3671		
Bag-1 KO		GGAAACACCG-TGTCAGCACTTGAATACAAGATGGTTGCCGGGTCATGTT----GGGAA	158		
wt	3672	AAAGGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAATGACTGAAGTCTTTTATA	3731		
Bag-1 KO		AAAGGTAAATTGCTTTTCCATCAGAAATCCTCAGAATCAAATGACTGAAGTCTTTTATA	98		

Figure 2.29 : A) Displaying of the target sequence and PAM sequence of sgRNA B which targeted exon 3 and the reverse read of Sanger sequence. **B)** The Nucleotide BLAST result of the reverse read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide B primer.

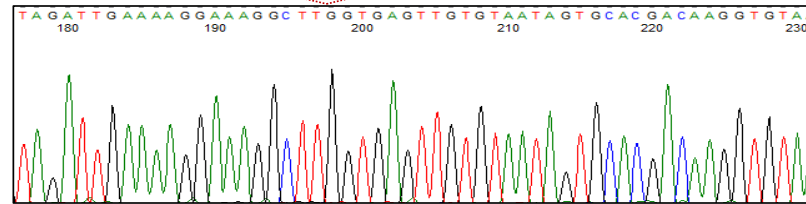
A Bag-1 KO Guide C Forward Sequencing (targeting exon 6)

Sanger sequencing result

Target sequence PAM

wt 5'-TAGATTGAAAAGGAAAGGCTTGGTAAAGGTTTCAAGTGAGTTGTGTAATAGTGCACGACAAGGTGTAA-3'

Bag-1 KO 5'-TAGATTGAAAAGGAAAGGCTT-----GGTGAGTTGTGTAATAGTGCACGACAAGGTGTAA-3'(-15bp)



B

BLAST result

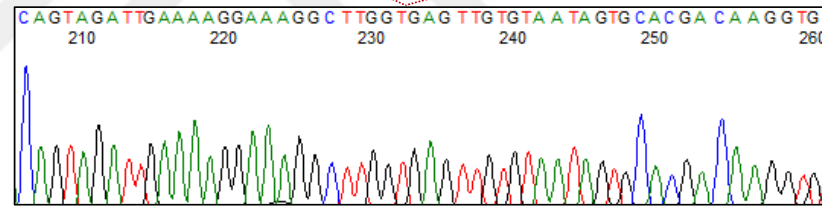
Score	Expect	Identities	Gaps	Strand
449 hits(243)	1e-129	281/296(95%)	15/296(5%)	Plus/Plus
wt	TCACATTTACATTGGTGACCTGTGACCTGTTTTGTGTACTGTGGGTGTTTCTTATGTGCT	8755		
Bag-1 KO	TCACATTTACATTGGTGACCTGTGACCTGTTTTGTGTACTGTGGGTGTTTCTTATGTGCT	71		
wt	CTCATGTCAATTTTCACTGAGGATTATTTGTTACATGTTTACTATTTACTAACTTGATTA	8815		
Bag-1 KO	CTCATGTCAATTTTCACTGAGGATTATTTGTTACATGTTTACTATTTACTAACTTGATTA	131		
wt	TCAACTTACTCTTCCATAGATCCTGCCAGAAAAATTTCAAAGACAGTAGATTGAAAAGGAA	8875		
Bag-1 KO	TCAACTTACTCTTCCATAGATCCTGCCAGAAAAATTTCAAAGACAGTAGATTGAAAAGGAA	191		
wt	AGGCTTGGTAAAAAAGGTTTCAGGTGAGTTGTGTAAATAGTGCACGACAAGGTGTAAATATGT	8935		
Bag-1 KO	AGGCTT-----GGTGAGTTGTGTAAATAGTGCACGACAAGGTGTAAATATGT	236		
wt	TAATGTGTAGGTATGTGTGTTTATCTGTTCAAATCAGGTAGTGTAGGAGCGTGGT	8991		
Bag-1 KO	TAATGTGTAGGTATGTGTGTTTATCTGTTCAAATCAGGTAGTGTAGGAGCGTGGT	292		

Figure 2.30 : A) Displaying of the target sequence and PAM sequence of sgRNA C which targeted exon 6 and the forward read of Sanger sequence. B) The Nucleotide BLAST result of the forward read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide C primer.

A Bag-1 KO Guide C Reverse Sequencing (targeting exon 6)

Sanger sequencing result

wt 5'-CAGTAGATTGAAAAGGAAAGGCTTGGTTAAGGTTGAGTTGTGTAATAGTGCACGACAAGGTG-3'
 Bag-1 KO 5'-CAGTAGATTGAAAAGGAAAGGCTTGGTT-----GAGTTGTGTAATAGTGCACGACAAGGTG-3'(-15bp)



B BLAST result

	Score	Expect	Identities	Gaps	Strand
	446 bits(241)	2e-128	281/297(95%)	16/297(5%)	Plus/Minus
wt	8654	TACAGGATACAGACCCAGGGAAAGGGTTCCTGAAAATGGATATCACATTTACATTGGTGA	8713		
Bag-1 KO	289	TACAGGATACA- ACCCAGGGAAAGGGTTCCTGAAAATGGATATCACATTTACATTGGTGA	231		
wt	8714	CCTGTGACCTGTTTTGTGTACTGTGGGTGTTTCTTATGTGCTCTCATGTCATTTTCACTG	8773		
Bag-1 KO	230	CCTGTGACCTGTTTTGTGTACTGTGGGTGTTTCTTATGTGCTCTCATGTCATTTTCACTG	171		
wt	8774	AGGATTATTTGTTACATGTTTACTATTTACTAACTTGATTATCAACTTACTCTTCCATA	8833		
Bag-1 KO	170	AGGATTATTTGTTACATGTTTACTATTTACTAACTTGATTATCAACTTACTCTTCCATA	111		
wt	8834	GATCCTGCCAGAAAAATTTCAAAGACAGTAGATTGAAAAGGAAAGGCTTGGTAAAAAAGGT	8893		
Bag-1 KO	110	GATCCTGCCAGAAAAATTTCAAAGACAGTAGATTGAAAAGGAAAGGCTTGGT-----	60		
wt	8894	TCAGGTGAGTTGTGTAATAGTGCACGACAAGGTGTAATATGTTAATGTGTAGGTATG	8950		
Bag-1 KO	59	-----GAGTTGTGTAATAGTGCACGACAAGGTGTAATATGTTAATGTGTAGGTATG	9		

Figure 2.31 : A) Displaying of the target sequence and PAM sequence of sgRNA C which targeted exon 6 and the reverse read of Sanger sequence. **B)** The Nucleotide BLAST result of the reverse read of MCF-7 wt sequence with Bag-1 KO PCR product sequence obtained with Guide C primer.

2.4.1.10 Confirmation of stable Bag-1 overexpressed MCF-7 cells

To investigate the function of Bag-1 deficiency in breast cancer survival, we designed our studies by comparing the effect of different Bag-1 expression levels in MCF-7 cells. As mentioned in previous results, wt MCF-7 cells have the endogeneous Bag-1 level and generated Bag-1 KO cells lack the Bag-1 expression level. In addition, Bag-1 OE MCF-7 cells have stable Bag-1 overexpression through transfection of TAP-tagged Bag-1L with C-terminal DNA insert into MCF-7 cells. The expression profile of Bag-1 in wt and Bag-1 OE MCF-7 cells was confirmed by immunoblotting assay (Figure 2.32). While wt cells showed expression of only three isoforms of Bag-1, Bag-1L-TAP tag expression was also observed in addition to Bag-1 isoforms in Bag-1 OE cells.

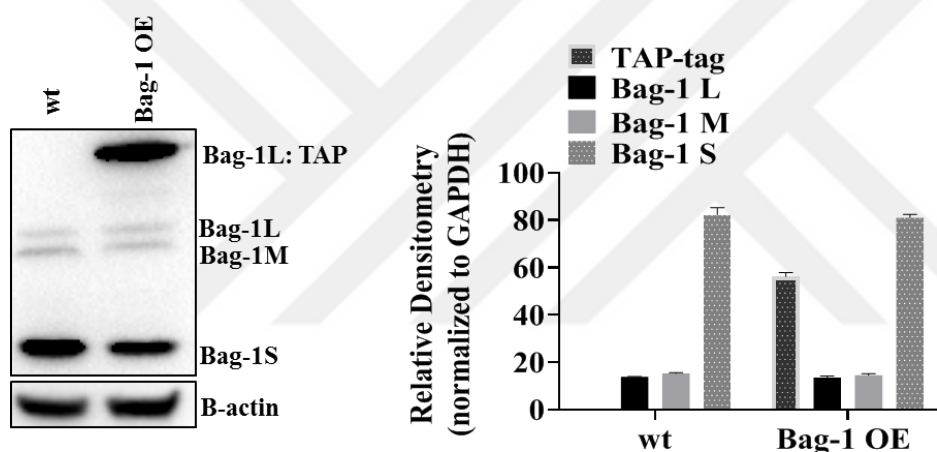


Figure 2.32 : Immunoblotting assay confirmed the Bag-1 expression in TAP-tag containing Bag-1L transfected stable MCF-7 cells.

2.4.1.11 Investigation of the knockout effect of Bag-1 on cell survival, proliferation, and colony formation potential of MCF-7 cells

To investigate the knockout effect of Bag-1 on cell viability, MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells were seeded according to the MTT Cell Viability assay procedure for 24 h and 48 h. Based on the 24 h results, Bag-1 KO cells showed the lowest cell viability ratio with 44% compared to wt cells (**** $p < 0.0001$). However, Control KO and Bag-1 OE cells did not show significant alteration for cell viability within 24 h. The longer plating of Bag-1 KO cells till 48 h increased the cell viability ratio from 44% to 73% compared to that of wt MCF-7 cells. This 29% increased cell viability ratio was the highest increment compared to 48 h cell viability ratio for each wt, Control KO and Bag-1 OE cells, respectively (**** $p < 0.0001$) (Figure 2.33). We

concluded that maintaining Bag-1 KO cells for 24 h was not sufficient for the adherence of cells to petri dishes.

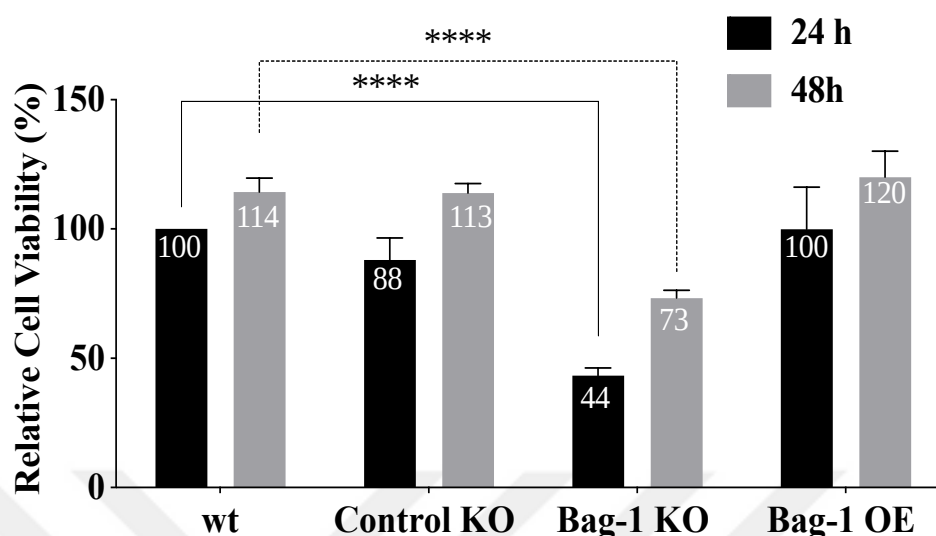


Figure 2.33 : Cell survival assay of wt, Control KO, Bag-1 KO, and Bag-1 OE cells was performed by MTT assay. The bar histograms represent the mean $\pm$ SD of three independent experiments with at least four replicates. (* $p=0.0162$, ** $p=0.0021$, *** $p=0.0001$, **** $p<0.0001$ by Two-way ANOVA, Sidak's multiple comparison test).

Following cell survival assay, we also investigated the proliferation ratios of wt, Control KO, Bag-1 KO, and Bag-1 OE MCF-7 cells in a time-dependent manner (0-72 h). Our results showed the viable cell number of wt and Control KO cells doubled in each time period. Bag-1 KO model showed a cytostatic effect within 24 h, but longer plating of Bag-1 KO cells showed an increased proliferation ratio (** $p=0.0020$, **** $p<0.0001$), which was slower than wt or Control KO cells. On the contrary, the proliferation ratio of Bag-1 OE cells was the highest compared to that of wt MCF-7 cells (**** $p<0.0001$). When morphological shapes and sizes of the cells were compared; Bag-1 OE cells were highly capable of growth in size and forming elongated shapes in contrast to Bag-1 KO cells compared to wt and Control KO cells within 72 h, as observed by light microscopy (Figure 2.34). We decided that maintaining Bag-1 KO cells for 24 h was not sufficient for the adherence of cells to petri dishes. After this step, we performed our experiments by starting 48 hours after the cell seeding procedure.

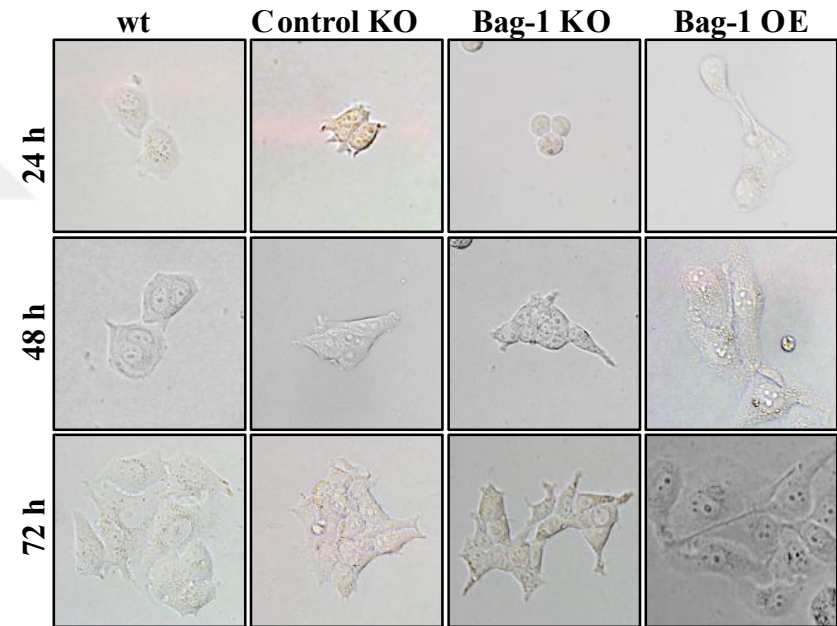
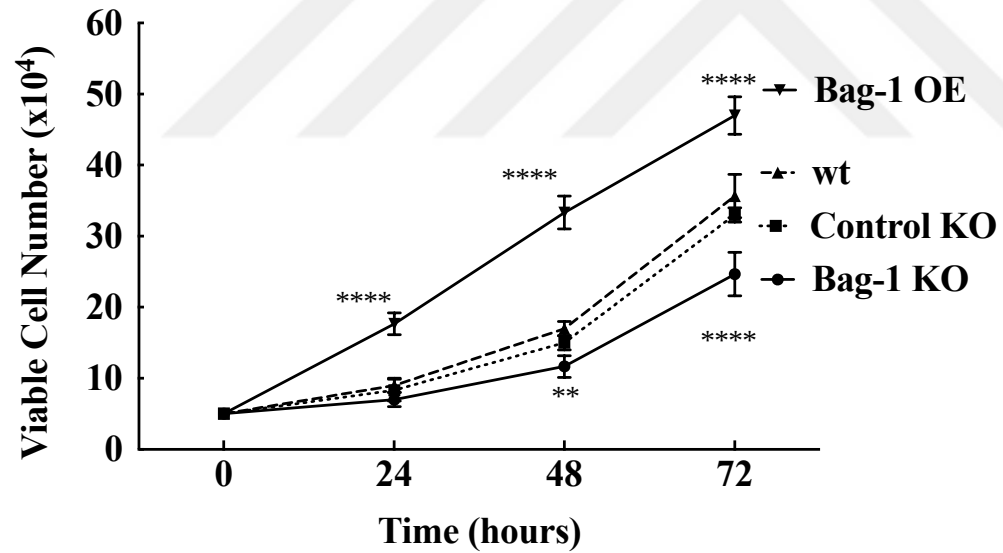


Figure 2.34 : Cell proliferation of wt, Control KO, Bag-1 KO, and Bag-1 OE was determined by Trypan Blue Dye Exclusion assay in a time-dependent manner (0-72 h). The mean $\pm$ SD of three repetitive experiments were analyzed by Two-way ANOVA, Tukey's multiple comparison tests. (**, $p=0.0020$, **** $p<0.0001$). Morphological images of wt, Control KO, Bag-1 KO, and Bag-1 OE cells were obtained by light microscopy in a time-dependent manner. Magnification 400x.

To investigate the effect of Bag-1 KO on the colony formation potential of MCF-7 cells, a clonogenic cell survival assay was performed. The result indicated that loss of Bag-1 expression slightly decreased the number of colonies compared to that of wt and Control KO cells whereas; Bag-1 overexpression significantly increased the colony-forming potential of MCF-7 cells. The magnified colony images (purple ones) of each MCF-7 cell model also confirmed the size variation results, so that the sizes of knockout of Bag-1 colonies were significantly small compared to that of wt, Control KO, and Bag-1 OE cells (Figure 2.35).

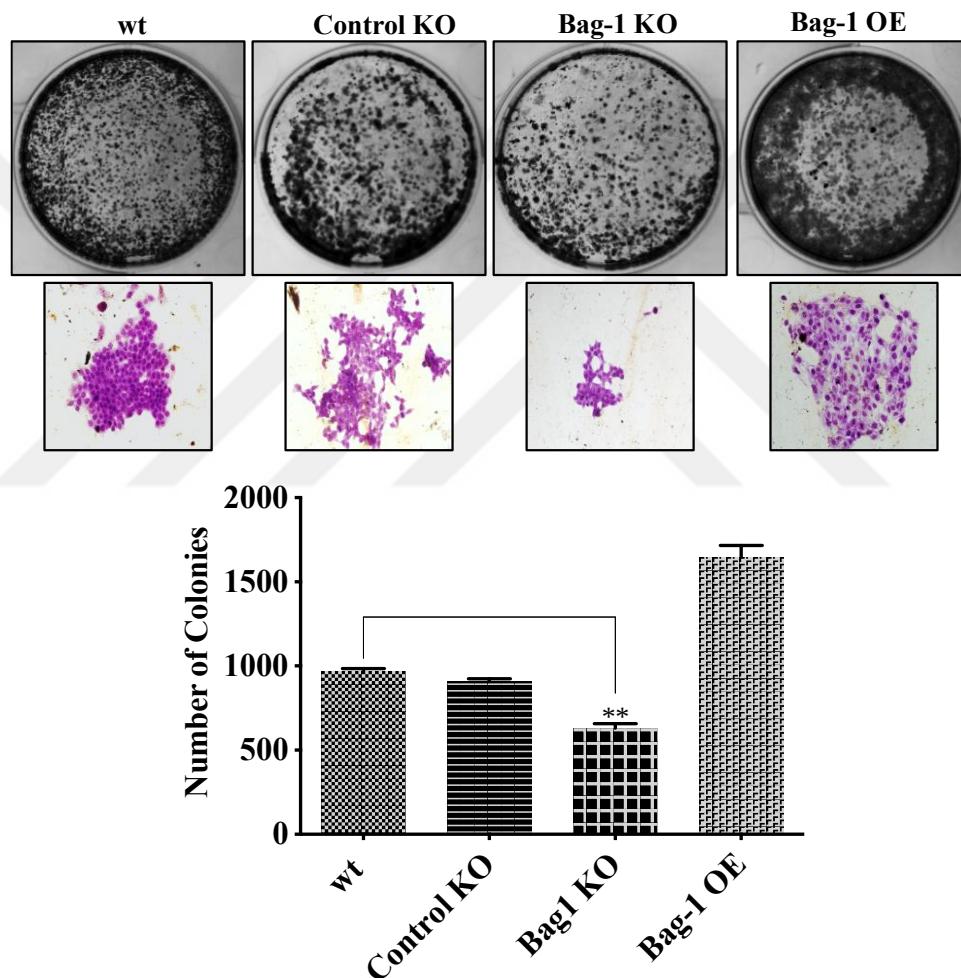


Figure 2.35 : The colony formation potentials of wt, Control KO, Bag-1 KO, and Bag-1 OE cells were determined by colony formation assay. The black images showed the colonies of wt, Control KO, Bag-1 KO, and Bag-1 OE cells and purple ones showed the magnification of one colony of each cell model. The numbers of colonies represented the mean $\pm$ SD of three repetitive experiments and were analyzed by Two-way ANOVA, Tukey's multiple comparison tests. Scale bar is 10 μ m and magnification 10x. (**, $p=0.0012$, *** $p=0.0002$).

2.4.1.12 Determination Bag-1 function on RTK expression profile in MCF-7 breast cancer models with different Bag-1 expressions

Considering the important roles of RTKs in breast cancer development, we determined the effect of Bag-1 expression on the phosphorylation status of RTKs in MCF-7 cells by using the PathScan RTK Signaling Antibody Array kit. This slide-based array allowed for the detection of the phosphorylated 28 receptor tyrosine and 11 important signaling proteins at tyrosine or other residues. The array was nitrocellulose-coated glass slide and consists of 8 part-array each coated with 39 protein antibodies with 2 replicates. As shown in Figure 2.36 we used wt, Bag-1 KO, and Bag-1 OE cell lysates with 2 replicate. The list of proteins whose signals were detected when they were phosphorylated by this array kit was given in Figure 2.37.



Figure 2.36 : Representative image of PathScan RTK Signaling Antibody Array Kit (<https://www.cellsignal.com/products/elisa-kits/rtk-signaling-antibody-array-kit-chemiluminescent-readout/7982>).

According to this array results, a wide variety of RTKs and related signaling pathway members including EGFR, FGFR, IRS1, Ephrins, and IRS-1 were found to increase in both Bag-1 KO and Bag-1 OE cells compared to that of wt MCF-7 cells (Figure 2.38).

Receptor Tyrosine Kinases			
	Target	Phosphorylation Site	Family
1	EGFR/ErbB1	pan-Tyr	EGFR
2	HER2/ErbB2	pan-Tyr	EGFR
3	HER3/ErbB3	pan-Tyr	EGFR
4	FGFR1	pan-Tyr	FGFR
5	FGFR3	pan-Tyr	FGFR
6	FGFR4	pan-Tyr	FGFR
7	InsR	pan-Tyr	Insulin R
8	IGF-IR	pan-Tyr	Insulin R
9	TrkA/NTRK1	pan-Tyr	NGFR
10	TrkB/NTRK2	pan-Tyr	NGFR
11	Met/HGFR	pan-Tyr	HGFR
12	Ron/MST1R	pan-Tyr	HGFR
13	Ret	pan-Tyr	Ret
14	ALK	pan-Tyr	LTK
15	PDGFR	pan-Tyr	PDGFR
16	c-Kit/SCFR	pan-Tyr	PDGFR
17	FLT3/Flk2	pan-Tyr	PDGFR
18	M-CSFR/CSF-1R	pan-Tyr	PDGFR
19	EphA1	pan-Tyr	EphR
20	EphA2	pan-Tyr	EphR
21	EphA3	pan-Tyr	EphR
22	EphB1	pan-Tyr	EphR
23	EphB3	pan-Tyr	EphR
24	EphB4	pan-Tyr	EphR
25	Tyro3/Dtk	pan-Tyr	Axl
26	Axl	pan-Tyr	Axl
27	Tie2/TEK	pan-Tyr	Tie
28	VEGFR2/KDR	pan-Tyr	VEGFR
Signaling Nodes			
	Target	Phosphorylation Site	Family
29	Akt/PKB/Rac	Thr308	Akt
30	Akt/PKB/Rac	Ser473	Akt
31	p44/42 MAPK (ERK1/2)	Thr202/Tyr204	MAPK
32	S6 Ribosomal Protein	Ser235/236	RSK
33	c-Abl	pan-Tyr	Abl
34	IRS-1	pan-Tyr	IRS
35	Zap-70	pan-Tyr	Zap-70
36	Src	pan-Tyr	Src
37	Lck	pan-Tyr	Src
38	Stat1	Tyr701	Stat
39	Stat3	Tyr705	Stat

Figure 2.37 : List of 39 different proteins of PathScan RTK signaling antibody array kit.

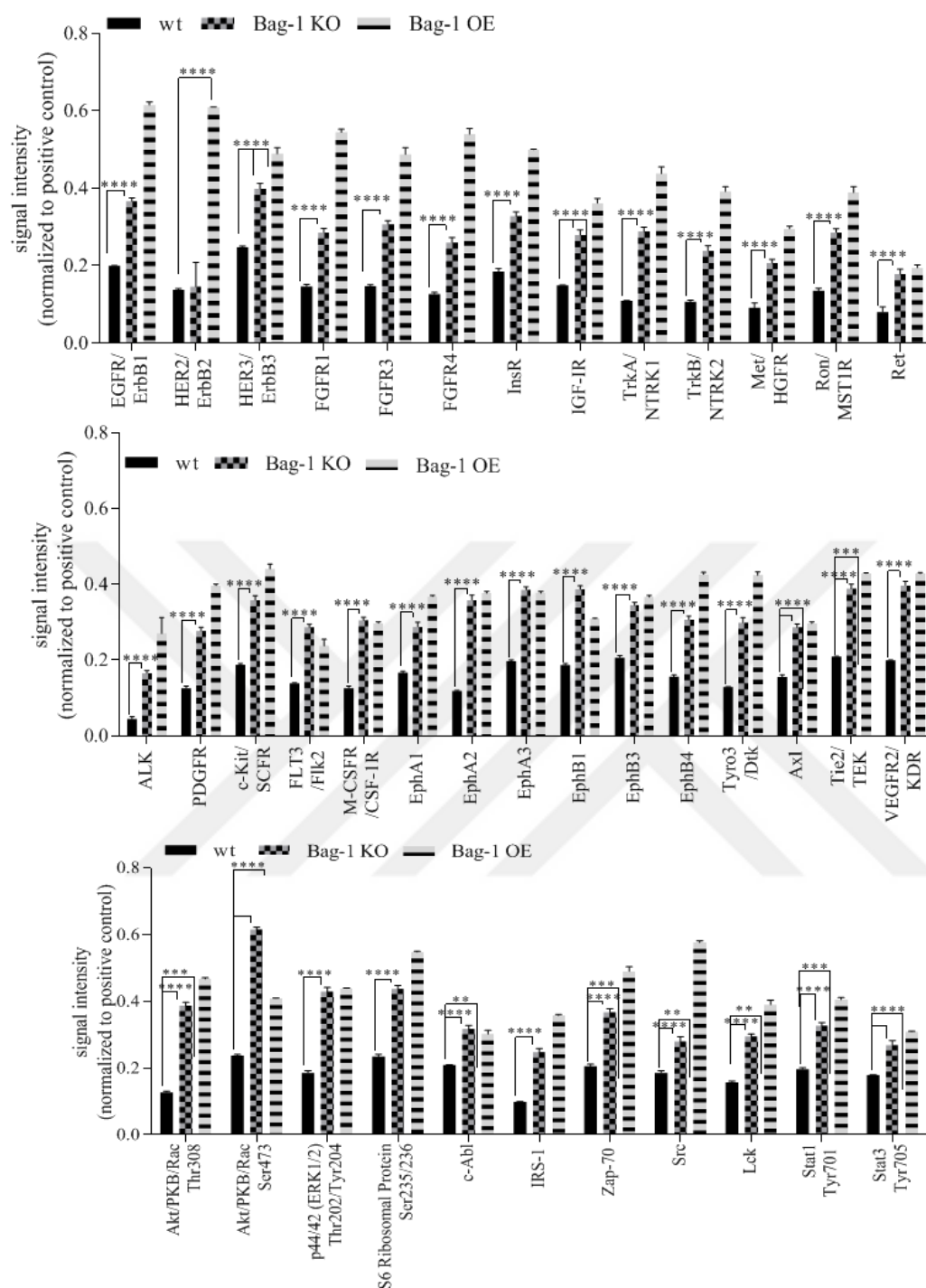


Figure 2.38 : The expression profiles of 39 different RTKs that were obtained by Path Scan RTK assay. The signal intensity of these proteins was normalized to positive control and analyzed GraphPad Prism version 8.

According to normalized densitometry calculations of signals, we found that phospho-Akt Thr308 and phospho-Akt Ser473 were determined to be the most altered targets in Bag-1 KO cells compared to that of wt MCF-7 cells (**** $p < 0.0001$) (Figure 2.39).

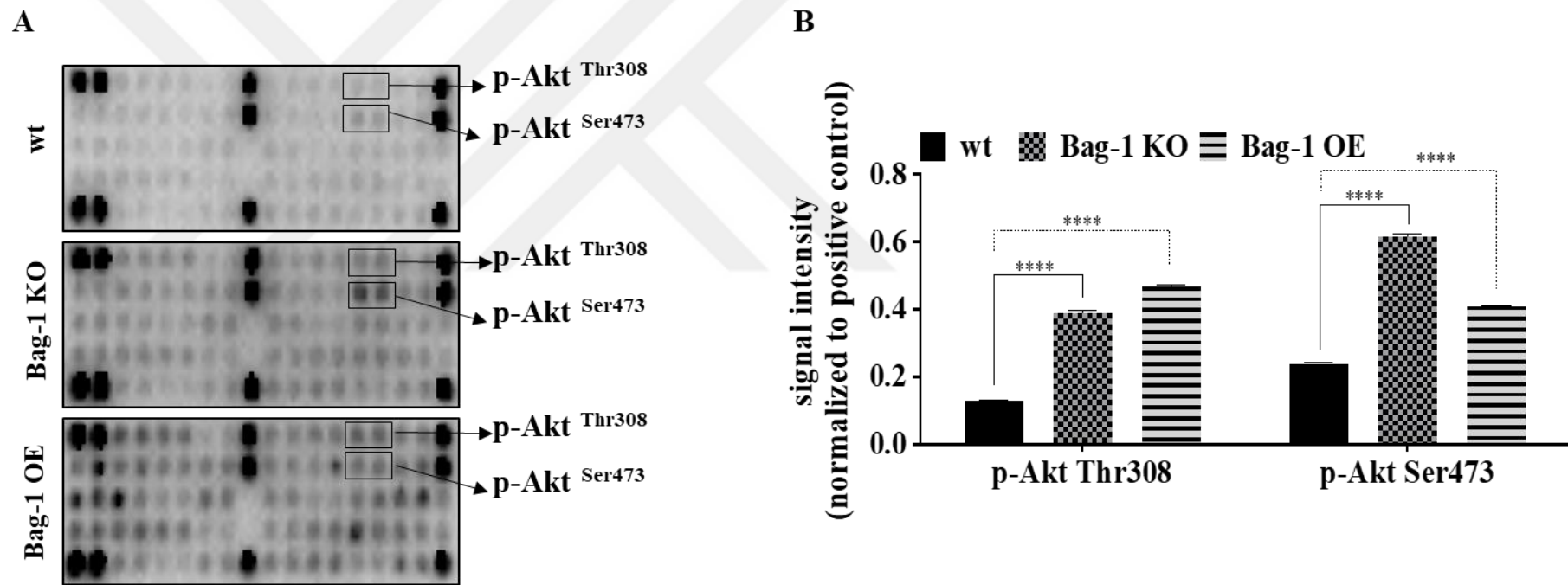


Figure 2.39 : The representative image of PathScan® RTK Signaling Antibody Array panel that showed the expression of activated RTKs in wt, Bag-1 KO, and Bag-1 OE MCF-7 cells. The bar graphs showed the quantification of the signal intensity of each two replicated proteins. The mean $\pm$ SD of three repetitive experiments were analyzed by Two-way ANOVA, Tukey's multiple comparison tests. (*** $p=0.0004$, **** $p<0.0001$).

We confirmed the expression profile of these targets by immunoblotting assay and found that the level of phospho-Akt Ser473 was highly increased (**** $p < 0.0001$) in Bag-1 KO cells compared to that of wt MCF-7 cells. Similarly, a remarkable increase was also detected in phospho-Akt Thr308 (**** $p < 0.0001$). According to the β -actin (*** $p = 0.0003$) and pan-actin (**** $p < 0.0001$) downregulation in Bag-1 KO cells, GAPDH was selected as a loading control (Figure 2.40).

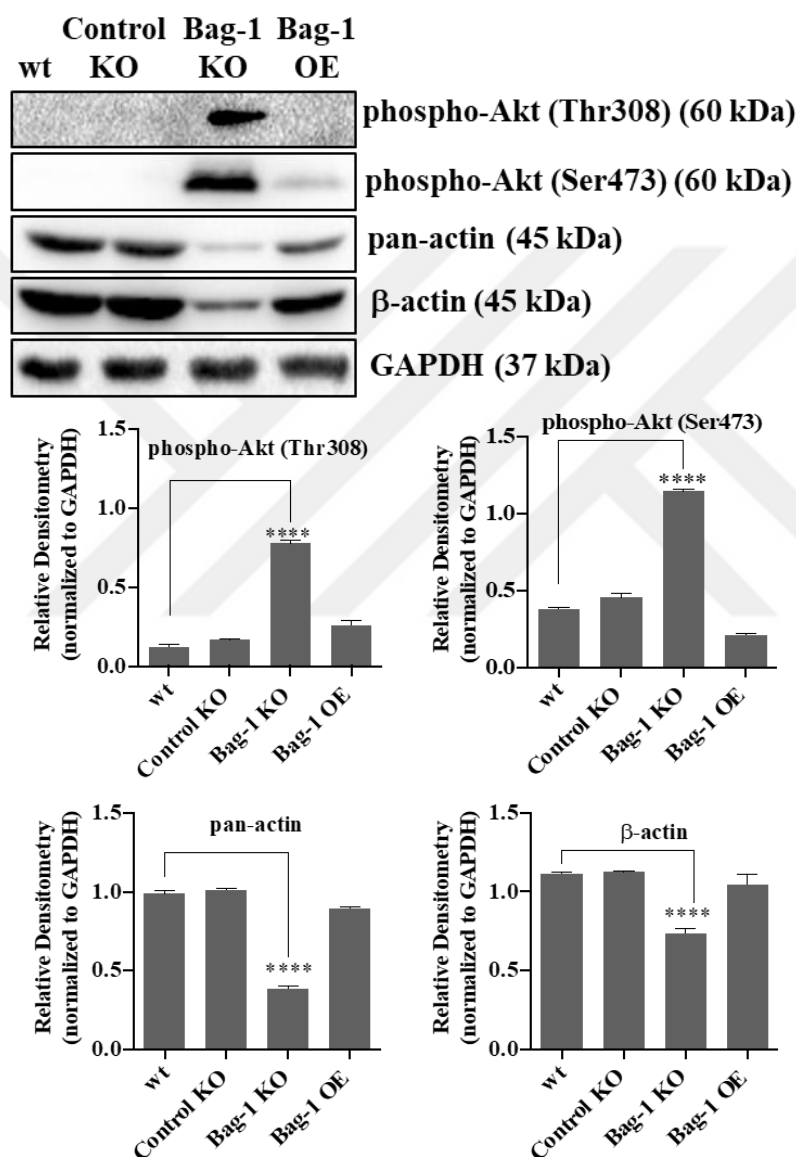


Figure 2.40 : Expression profiles of selected phosphorylated proteins were determined by immunoblotting assay. GAPDH was used as a loading control. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. (*** $p = 0.0003$, **** $p < 0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

2.4.1.13 Investigation of the hyperphosphorylation of Akt associated with increased ROS and p38 activation in Bag-1 KO cells

To understand the relationship between loss of Bag-1 expression and Akt hyperactivation related to cell survival mechanism, we utilized a cell viability assay in the presence of MK-2206, a potent Akt inhibitor in wt, Control KO, Bag-1 KO, and Bag-1 OE MCF-7 cells, respectively. For this purpose, all cell models were plated for 48 h, and then cells were treated with 500 nM MK-2206 Akt inhibitor for 24h. Akt inhibition led to decreased cell viability ratio compared to untreated control cells for each cell line. In Bag-1 KO cells, inhibition of Akt decreased cell viability by 14% compared to Control KO cells (**** $p < 0.0001$), while Bag-1 OE protected cells against Akt inhibition-mediated cell viability loss (** $p = 0.004$) (Figure 2.41).

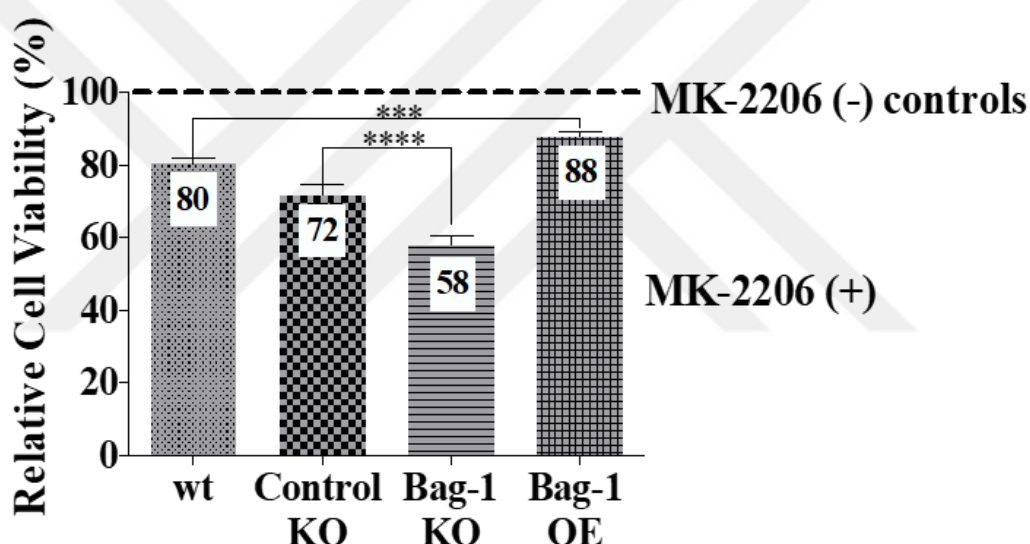


Figure 2.41 : The effect of 24 h treatment of 500 nM MK-2206 on cells was determined by MTT Cell Viability assay. The bar histograms represent the mean $\pm$ SD of three independent experiments with at least four replicates. (** $p = 0.0003$, **** $p < 0.0001$ by Two-way ANOVA, Sidak's multiple comparison test).

Following MTT Assay, we also investigated the colony formation potentials of each cell model in the presence of MK-2206 Akt inhibitor for 24h. Similar to MTT results, Akt inhibition decreased the number of colonies from 738 to 547 in wt cells, from 698 to 462 in Control KO cells, from 410 to 283 in Bag-1 KO cells, and from 882 to 676 in Bag-1 OE cells (**** $p < 0.0001$). Akt-inhibited Bag-1 KO cells showed reduced colony formation compared to MK2206 treated Control KO cells (Figure 2.42).

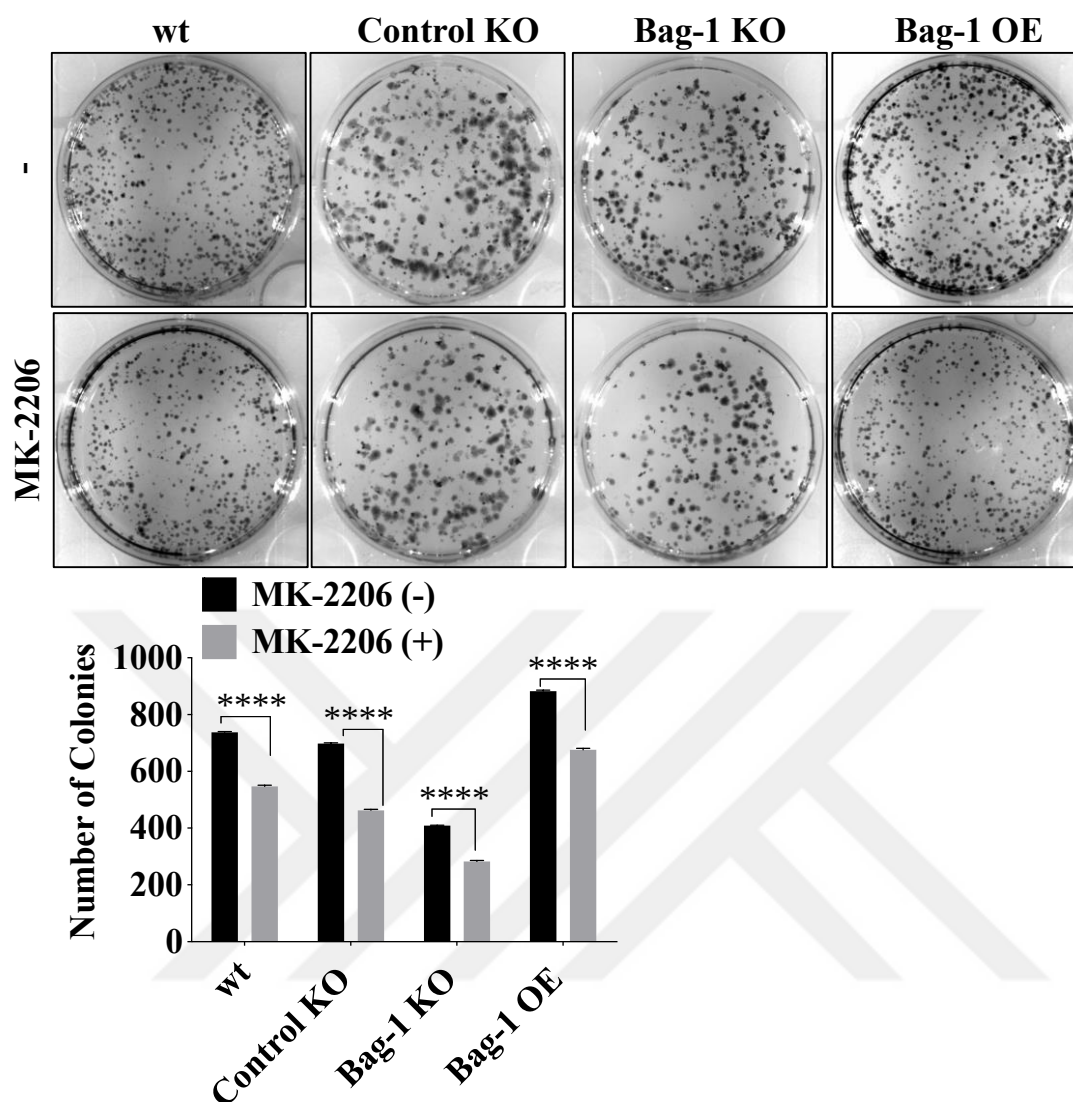


Figure 2.42 : The effect of MK-2206 on colony formation potentials of wt, Control KO, Bag-1 KO, and Bag-1 OE cells was determined by colony formation assay. The number of colonies represented the mean $\pm$ SD of three repetitive experiments and was analyzed by Two-way ANOVA, Tukey's multiple comparison tests (**** $p < 0.0001$).

Then, we measured the cellular Reactive Oxygen Species (ROS) level, which is a main stress indicator in the cells. As the experimental confirmation of assay, we treated cells with (0.1 mM) H_2O_2 for 24 h after the plating of cells for 48 h and found that a 95.75% increase in ROS generation was apparent in wt MCF-7 cells compared to untreated cells (**** $p < 0.0001$). We found that Akt inhibition reduced basal ROS level in wt MCF-7 by 1.36% and Bag-1 OE cells by 2.77% (* $p < 0.0241$). The excess ROS generation was detected in Bag-1 KO cells and Akt inhibition by the co-treatment of MK-2206 further increased ROS generation (** $p = 0.0021$) (Figure 2.43).

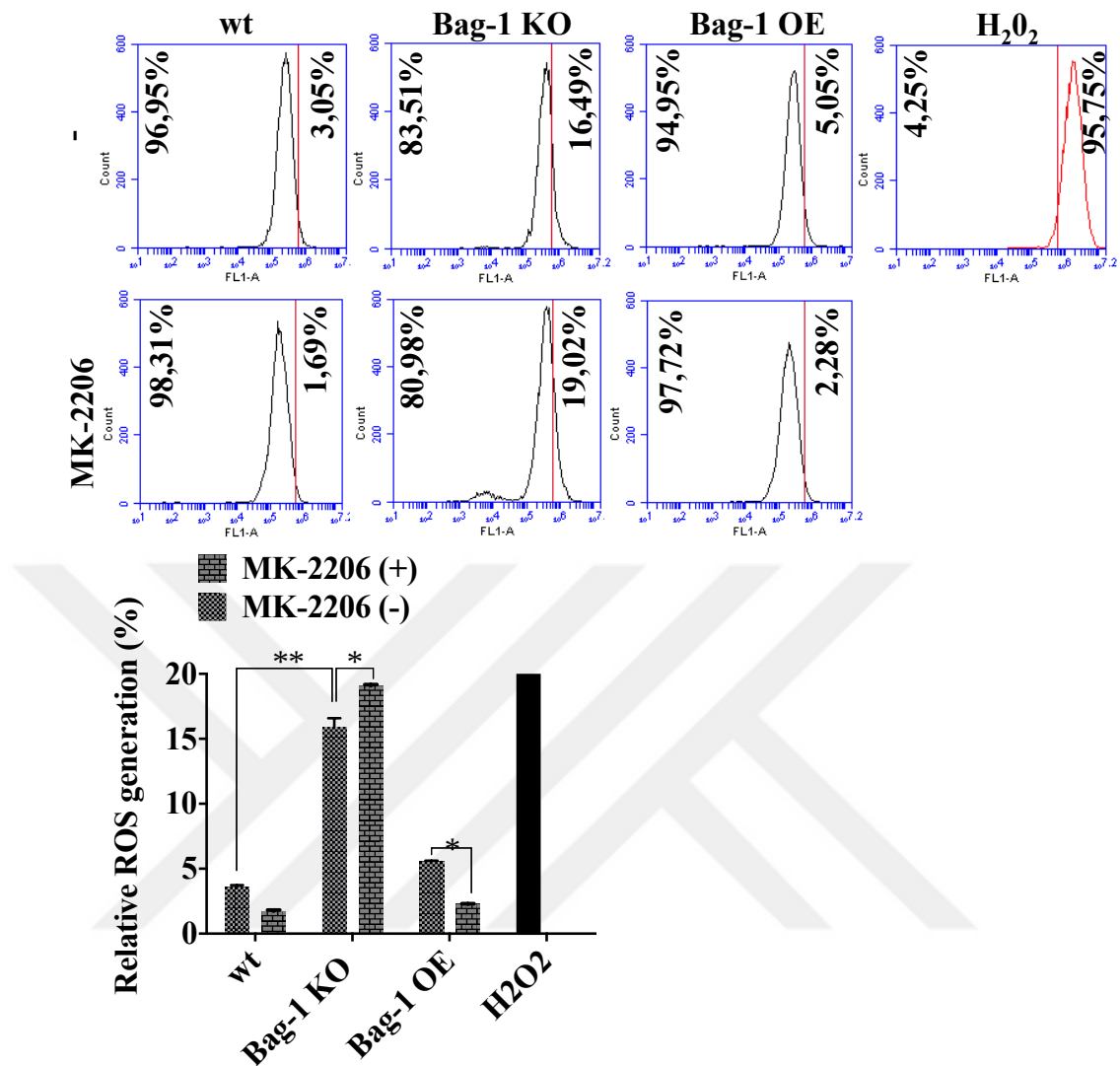


Figure 2.43 : Cellular ROS generation of wt, Control KO, Bag-1 KO, and Bag-1 OE cells was measured by DCFH-DA staining and analyzed by BD Accuri C6 flow cytometer (* $p < 0.05$ and ** $p < 0.01$, **** $p < 0.0001$).

In addition, to confirm the relationship between Bag-1 expression profile and cellular stress-response in MCF-7 cells, we checked the activation of stress-activated protein kinase p38 Thr180/Tyr182, which was significantly increased in Bag-1 KO (** $p = 0.0011$) and Bag-1 OE (**** $p < 0.0001$) cells compared to that of Control KO or wt MCF-7 cells, which was suppressed by Akt inhibition. GAPDH was used as a loading control (Figure 2.44).

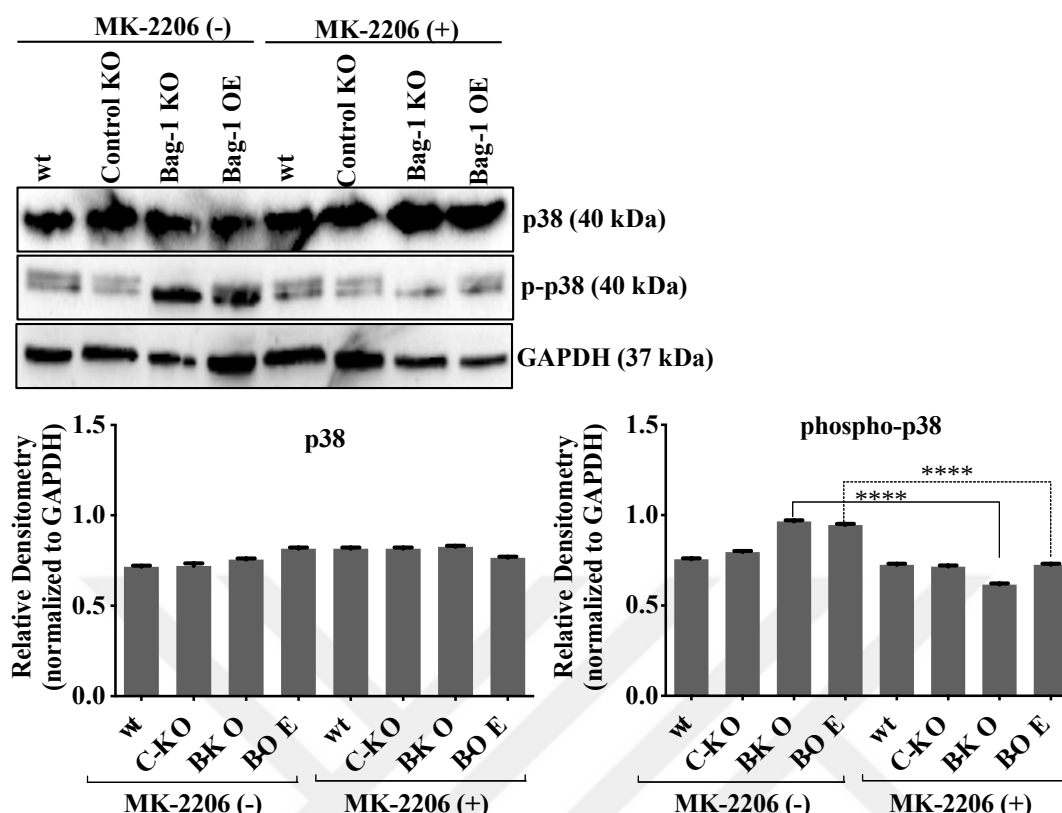


Figure 2.44 : The expression of p38 and phospho-p38 stress markers was determined by immunoblotting assay. GAPDH was used as a loading control. (**p=0.0011, ****p< 0.0001). The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. (****p< 0.0001 by Two-way ANOVA, Tukey's multiple comparison test).

2.4.1.14 Investigation of the expression profile of PI3K/Akt signaling members in Akt inhibitor (MK-2206) treated or untreated wt, Control KO, Bag-1 KO, and Bag-1 OE cells

Considering the significant Akt phosphorylation both in Ser473 and Thr308 residues in Bag-1 KO cells, we checked the expression profile of upstream and downstream members of Akt signaling pathways through MK-2206. In the presence of MK-2206, PI3K expression levels were not altered compared to untreated samples in wt, Control KO and Bag-1 KO cells, respectively. However, the remarkable PI3K overexpression was detected in Bag-1 OE (****p<0.0001) cells and the Akt inhibitor did not significantly change Bag-1-mediated PI3K expression.

Total PTEN and phosphorylated PTEN expression levels were similarly downregulated in Bag-1 KO and Bag-1 OE cells (****p<0.0001, **p=0.0028). Akt inhibitor treatment did not change functional PTEN activity in all cell models.

Similarly, Akt inhibition did not lead to expressional alterations at the total Akt level in the cells (** $p=0.0056$), but MK-2206 prevented hyperphosphorylation of Akt at Ser473 and Thr308 in Bag-1KO cells (**** $p<0.0001$). We confirmed that Akt inhibitor treatment led to significant Akt dephosphorylation in all cell lines. These results suggested that the MK-2206 treatment is potent to prevent Bag-1 KO-mediated Akt hyperactivation due to excess stress conditions (Figure 2.46).

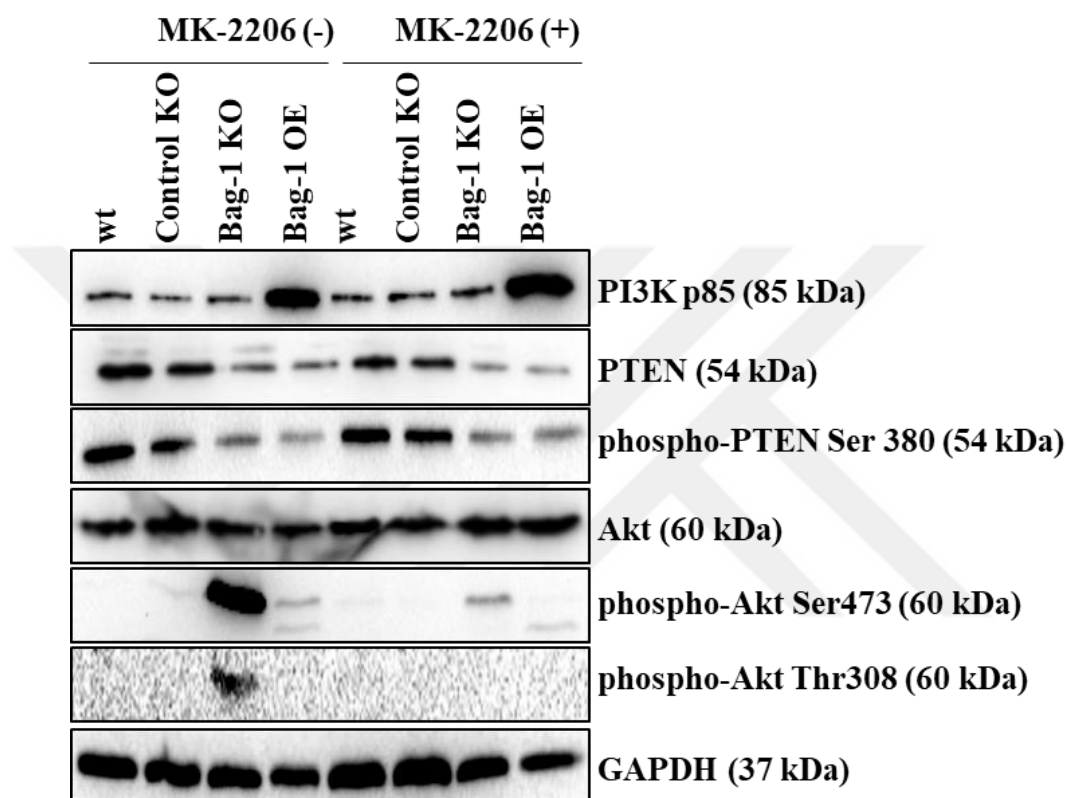


Figure 2.45 : Expression profiles of Akt signaling pathway members were investigated by MK-2206 treatment in MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control. (* $p=0.0114$, ** $p=0.0013$, *** $p=0.0006$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

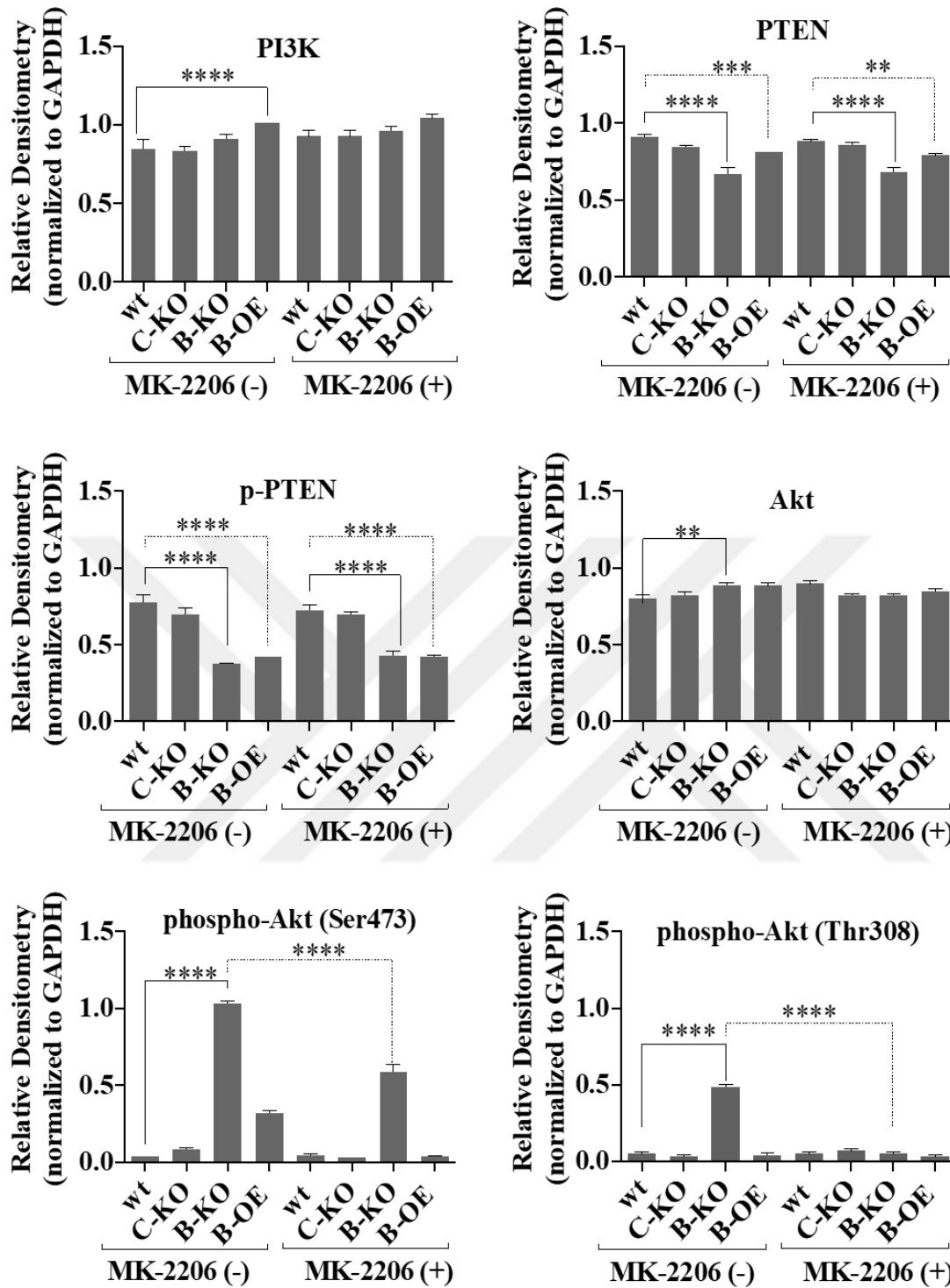


Figure 2.45 (continued): Expression profiles of Akt signaling pathway members were investigated by MK-2206 treatment in MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control. (* $p=0.0114$, ** $p=0.0013$, *** $p=0.0006$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

2.4.1.15 Determination of the effect of Bag-1 deficiency on mesenchymal transition and actin organization of MCF-7 cells

Due to Akt-mediated actin filament regulation in the cells, we questioned diminished β -actin expression levels in Bag-1 KO cells compared to wt MCF-7 and Control KO cells. We investigated the potential role of Akt on actin-related cell differentiation profile in Bag-1 KO cells by immunoblotting assay.

Next, we determined the epithelial marker E-cadherin expression levels, which were upregulated when wt and Control KO cells were exposed to Akt inhibitor (**** $p < 0.0001$). Interestingly, we found that E-cadherin expression levels were dramatically downregulated in both Bag-1 KO and Bag-1 OE cells regardless of Akt activation in the cells. By these results, we found that N-cadherin expression levels were upregulated in Bag-1 KO and Bag-1 OE cells (**** $p < 0.0001$).

Although N-cadherin 140 kDa fragment was detected in Bag-1 OE cells, the N-cadherin upper band profile was about above 15-kDa normal band profiles in Bag-1 KO cells. This band profile indicated an inactive form of N-cadherin due to irregular cleavage of pro-domain of N-cadherin [181]. Following MK-2206 treatment, we determined the diminished expression levels for N-cadherin at 140 kDa band profiling level in Bag-1 KO cells. The upregulation for N-cadherin was detected in Bag-1 OE cells. Concomitantly, we determined that Bag-1 loss led to upregulation of N-cadherin, which was prevented by Akt inhibitor treatment in knockout cells compared to wt or Control KO MCF-7 cells.

The EMT signaling transcription factor ZEB1 expression was significantly enhanced in Bag-1 KO cells (**** $p < 0.0001$) compared to Control KO cells. On the contrary, the tight junction protein ZO-1 was downregulated in Bag-1 KO cells, which was upregulated further by MK-2206 treatment (**** $p < 0.0001$). The transcription factor TWIST1 was upregulated in untreated Bag-1 KO cells, whereas was downregulated by MK-2206 treatment (**** $p < 0.0001$).

phospho-FAK at Tyr397 expression was observed in Bag-1 OE cells and downregulated with MK-2206 treatment (**** $p < 0.0001$). FAK expression level was significantly downregulated in Bag-1 KO cells (**** $p < 0.0001$) and Akt inhibitor treatment did not alter Bag-1 KO mediated FAK downregulation profile significantly (* $p = 0.0264$). On the contrary, MK-2206 treatment led to diminished FAK expression

levels in Bag-1 OE cells compared to untreated Bag-1 OE (**** $p < 0.0001$). A similar expression pattern was determined in paxillin expression (**** $p < 0.0001$).

Related to the cell cytoskeleton and EMT progression network, Akt activation-dependent diminished levels of β -actin and α -actinin (**** $p < 0.0001$) were detected in Bag-1 KO cells, which their expressions enhanced through Akt inhibition in Bag-1 KO cells compared to untreated profiles. Phosphorylated cofilin level at Ser3 was significantly higher in Bag-1 KO cells compared to other cell models (**** $p < 0.0001$).

All of these results highlighted that Bag-1 expression-dependent alterations in MCF-7 breast cancer cells led to a sharp change in the expression profile of cytoskeleton proteins, which then affect EMT. Therefore, the cytoskeleton regulatory potential of Bag-1 in an Akt-dependent manner is a novel understanding of the molecular role of Bag-1 in cell physiology. In addition, the loss of Bag-1 expression differs from Bag-1 OE due to the dysregulation of N-cadherin cleavage potential in MCF-7 cells (Figure 2.46).

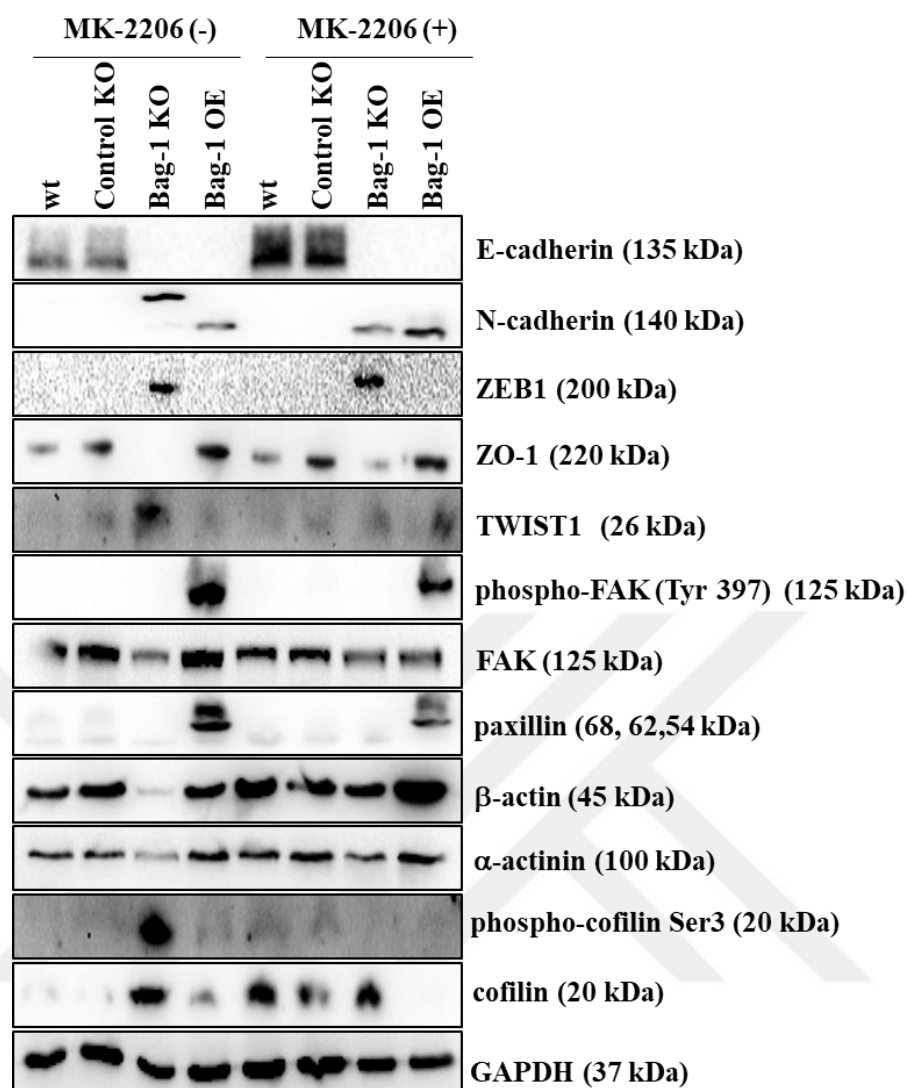


Figure 2.46 : Expression profiles of EMT and focal adhesion kinase pathway members were investigated by MK-2206 treatment in MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control. (* $p=0.0114$, ** $p=0.0013$, *** $p=0.0006$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

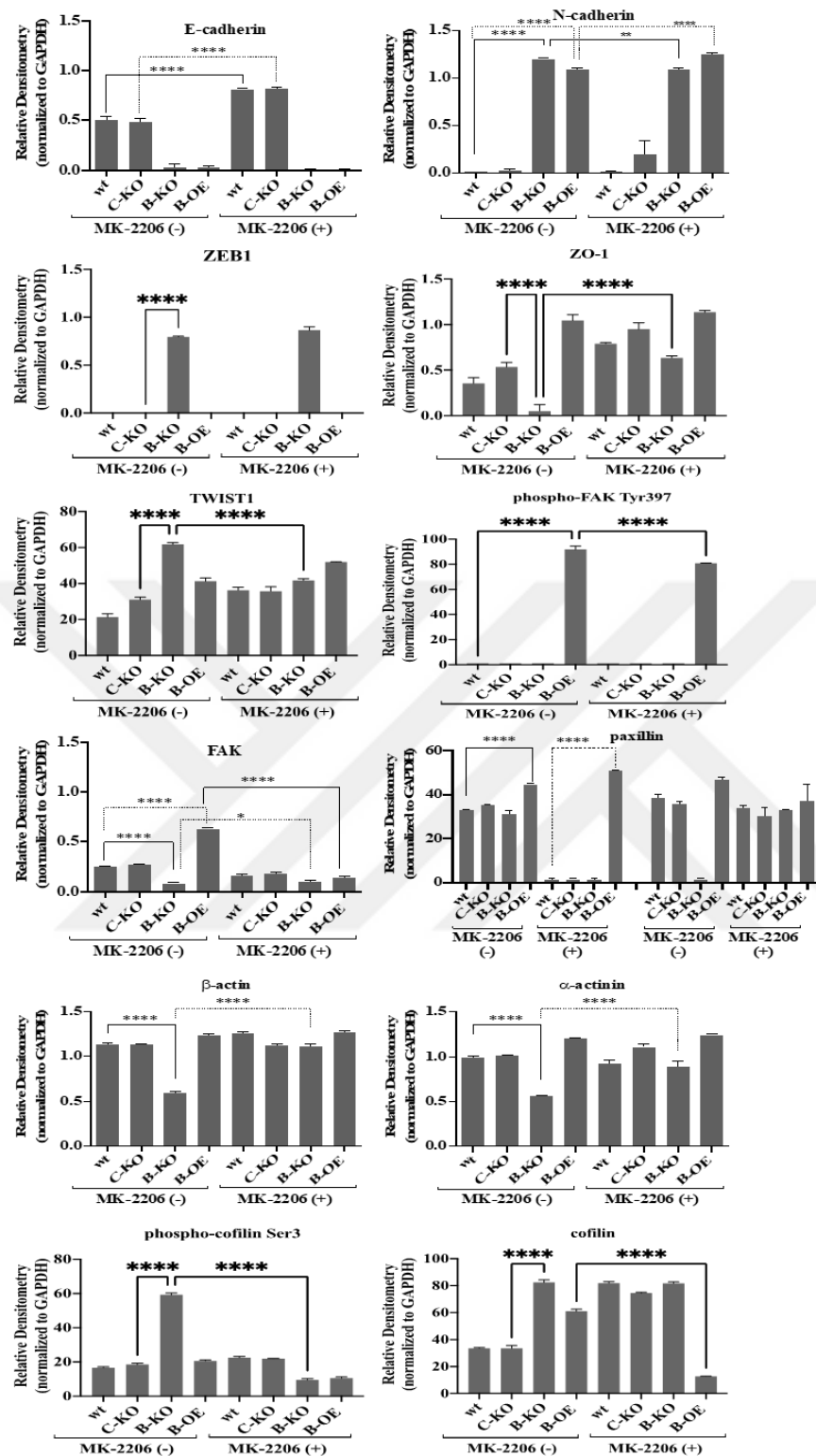


Figure 2.46 (continued): Expression profiles of EMT and focal adhesion kinase pathway members were investigated by MK-2206 treatment in MCF-7 wt, Control KO, Bag-1 KO, and Bag-1 OE cells determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control. (* $p=0.0114$, ** $p=0.0013$, *** $p=0.0006$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test).

Moreover, we examined the relative mRNA expression of E-cadherin, TWIST1, Snail1, Snail2, β -actin, and Akt by qRT-PCR. MK-2206 treatment decreased the Akt mRNA expression in all cell models. E-cadherin mRNA expression increased in Bag-1KO and Bag-1 OE cells (* $p=0.0126$, **** $p<0.0001$). The relative Snail 1 and Snail 2 mRNA expressions were lower in Bag-1 KO cells compared to wt and Bag-1 OE cells, and Akt inhibition further reduced their expressions (** $p=0.0021$, *** $p=0.0002$). TWIST1 mRNA expression was higher in Bag-1 KO and Bag-1 OE cells, whereas significantly decreased by inhibition of Akt (** $p=0.0089$). β -actin mRNA level was increased through Akt inhibition in Bag-1 KO cells (* $p=0.0114$) (Figure 2.47). The designed primer sequences were shown in Table 2.4.

Table 2.4 : Designed primers for EMT Markers.

Target	Sequence
E-cadherin Forward	ATTTTCCCTCGACACCCGAT
E-cadherin Reverse	TCCCAGGCGTAGACCAAGA
Snail1 Forward	ACTGCAACAAGGAATACCTCAG
Snail1 Reverse	GCACTGGTACTTCTTGACATCTG
Snail2 Forward	TGTGACAAGGAATATGTGAGCC
Snail2 Reverse	TGAGCCCTCAGATTTGACCTG
Twist1 Forward	GTCCGCAGTCTTACGAGGAG
Twist1 Reverse	GCTTGAGGGTCTGAATCTTGCT
β -actin Forward	AGAGCTACGAGCTGCCTGAC
β -actin Reverse	AGCACTGTGTTGGCGTACAG

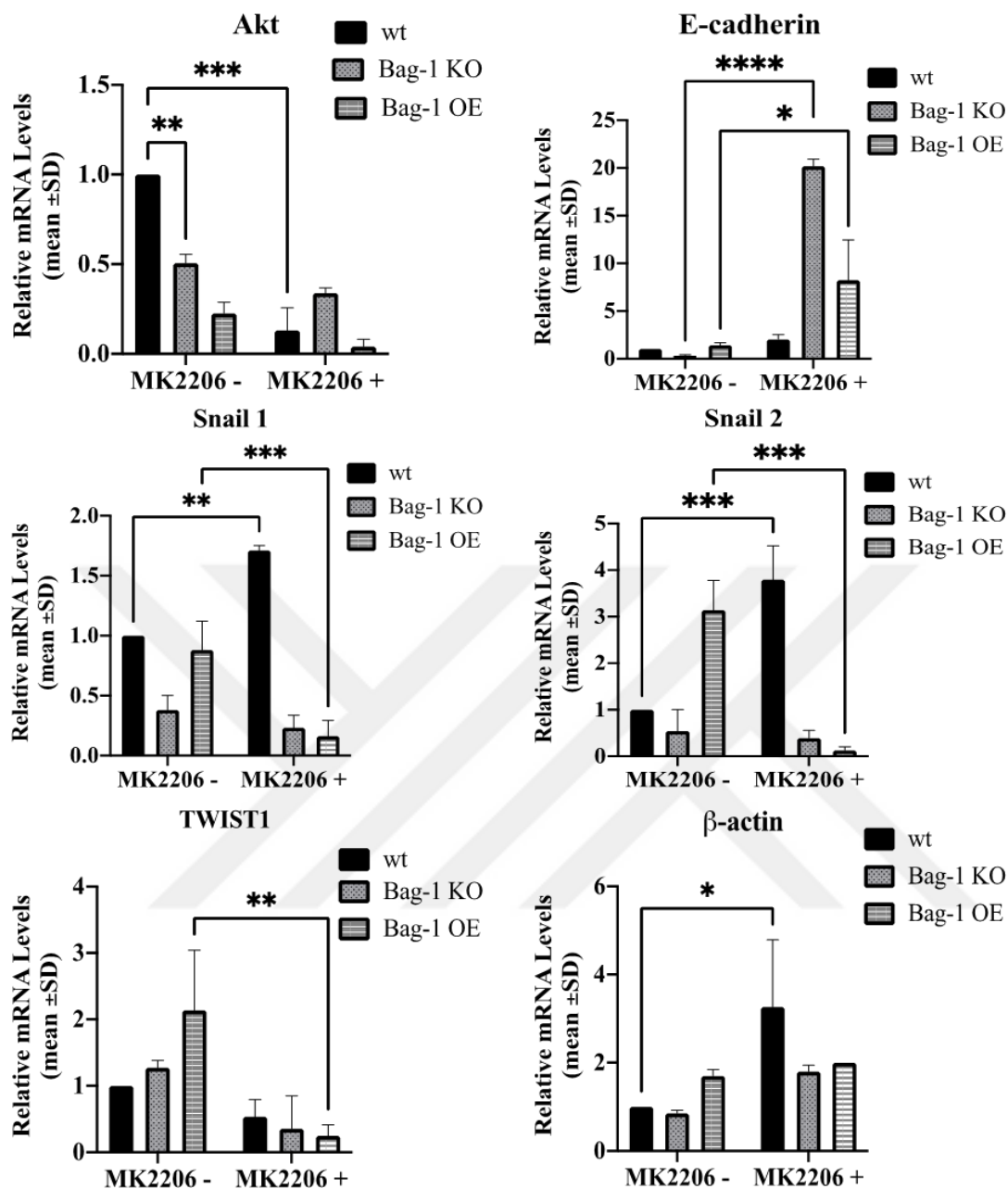


Figure 2.47 : The transcriptional level of Akt, E-cadherin, Snail1, Snail2, TWIST1, and β -actin were detected in qRT-PCR analysis. All data collected from three independent experiments (Akt **= $p=0.0040$, *** $p=0.0002$, E-cadherin **** $p<0.0001$, * $p=0.0317$, Snail1 ** $p=0.0021$, *** $p=0.0007$, Snail2 *** $p=0.0002$, TWIST1 ** $p=0.0089$, β -actin * $p=0.0114$).

2.4.1.16 Investigating the role of Bag-1 knockout on the migratory and metastatic profile of MCF-7 cells

Considering the high activation of Akt and increased expression profiles of EMT markers in Bag-1 KO cells, the potential association of Bag-1 expression on cell migration was determined by performing a wound-healing assay. Bag-1 KO was more effective to repair *in vitro* wound closure compared to wt and Control KO MCF-7 cells after 48 h growth of cells. Bag-1 OE cells showed significant wound closure in 48 h. Akt inhibition suppressed the migration of cells into wound area significantly in Bag-1 KO cells and other cell models. Time-dependent (0-24 and 48 h) wound healing images were captured under light microscopy and fluorescence microscopy after staining with DiOC6. We suggest that the knockout of Bag-1 enhanced the migration potential of MCF-7 cells (Figure 2.48).

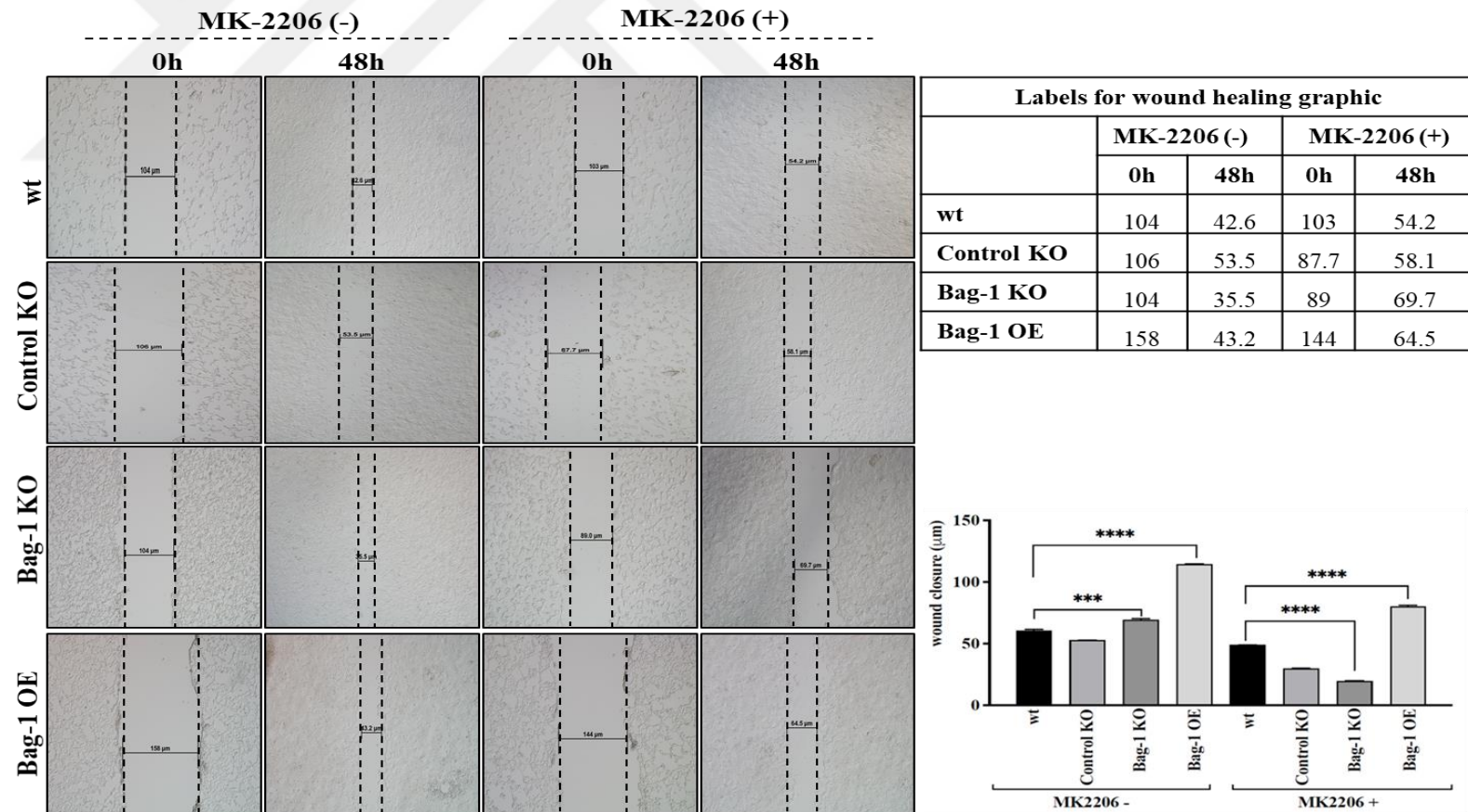


Figure 2.48 : The effect of Bag-1 expression on cell migration of MCF-7 cells was assessed by wound healing assay, which was repeated by two-independent assays. Wound closure was imaged by light microscopy (magnification 4x, scale 10 μ m), and analyzed by Two-way ANOVA, Tukey's multiple comparison test, **** p< 00001.

In addition, we also investigated the invasion potential of MCF-7 cells with different Bag-1 expression levels using the Boyden Chamber assay. Both wt, Bag-1 KO, and Bag-1 OE cells were migrated into the lower chamber, but Bag-1 OE cells showed higher migration capability compared to wt and Bag-1 KO cells (**** $p < 0.0001$). It was noticeable that Bag-1 KO cells showed distinct morphology, smaller cell size, and colony-formed cell population compared to wt and Bag-1 OE cells (Figure 2.49).

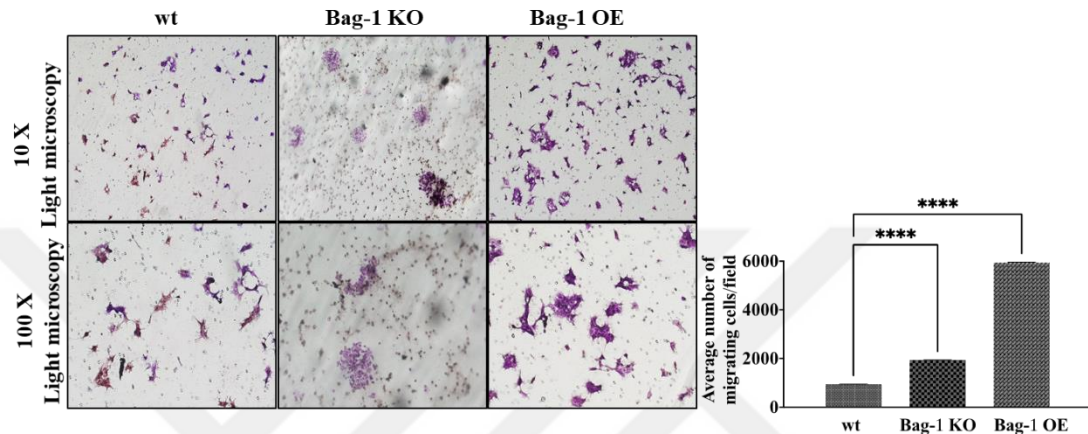


Figure 2.49 : The invasion capacities of wt, Bag-1 KO, and Bag-1 OE cells were determined by transwell migration assay. Left panel, invading cells (magnification x10 and x100); right panel, quantification on the average number of migrating wt, Bag-1 KO and Bag-1 OE cells per well (**** $p < 0.0001$), counted by Image J and analyzed by One-way ANOVA, Dunnet's multiple comparison test.

2.4.1.17 Confirmation of the Bag-1 KO-mediated Akt hyperactivation and α -actinin downregulation profile in different clones of Bag-1 KO and MDA-MB-231 Bag-1 KO cells

To confirm our suggestion that Bag-1 KO caused actin disorganization via Akt activation-dependent manner, we investigated the expression levels of Akt phosphorylation and α -actinin in two different MCF-7 Bag-1 KO clones and Bag-1 KO, Control KO, Bag-1 shRNA, and Bag-1 shRNA plasmid transfected MDA-MB-231 cells, respectively. According to these results, two different MCF-7 Bag-1 KO clones also showed higher phospho-Akt Ser473 and lower α -actinin and FAK expression profile compared to wt MCF-7 cells (Figure 2.50).

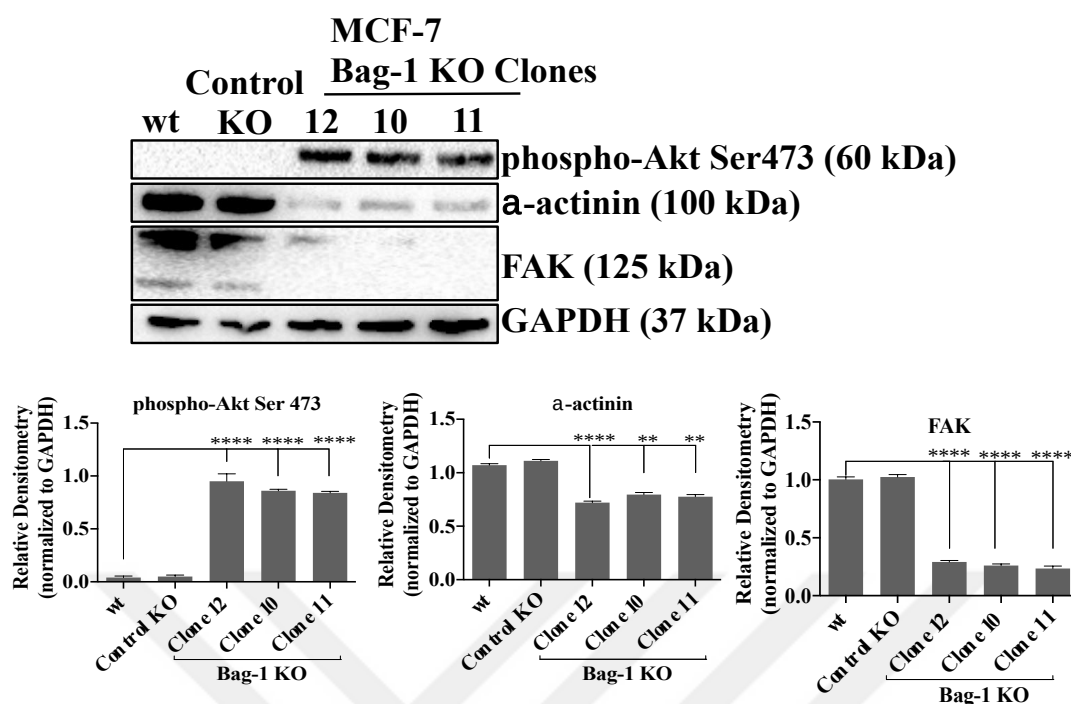


Figure 2.50 : Hyperactivation of Akt and α -actinin downregulation was investigated in different MCF-7 Bag-1 KO clones. The expression profiles of phospho-Akt Ser473, α -actinin, and FAK were determined by an immunoblotting assay in MCF-7 wt, Control KO, MCF-7 Bag-1 Clone 12, Clone 10, and Clone 11 cells. (**p=0.0021, ****p<0.0001 by Two-way ANOVA, Tukey's multiple comparison test).

Besides, we also checked the Bag-1 KO effect in triple-negative MDA-MB-231 cells and observed that knockout of Bag-1 caused the ablation and knockdown of Bag-1 caused significant downregulation on Bag-1 expression level. phospho-Akt Ser473 expression was similar in MDA-MB-231 wt, Control KO, Bag-1 KO, and Control shRNA cells, however, Bag-1 shRNA caused significant downregulation. This result confirmed our previous results suggesting that stable Bag-1 shRNA transfection enhanced the apoptotic parameters of breast cancer as well as in this study complete knockout of Bag-1 allowed the active Akt signaling. Concomitant downregulation of α -actinin was remarkable in Bag-1 KO MDA-MB-231 cells. We suggested that Bag-1 deficiency in the cells led to activation of Akt and altered actin organization in a hormone-receptor-independent manner (Figure 2.51).

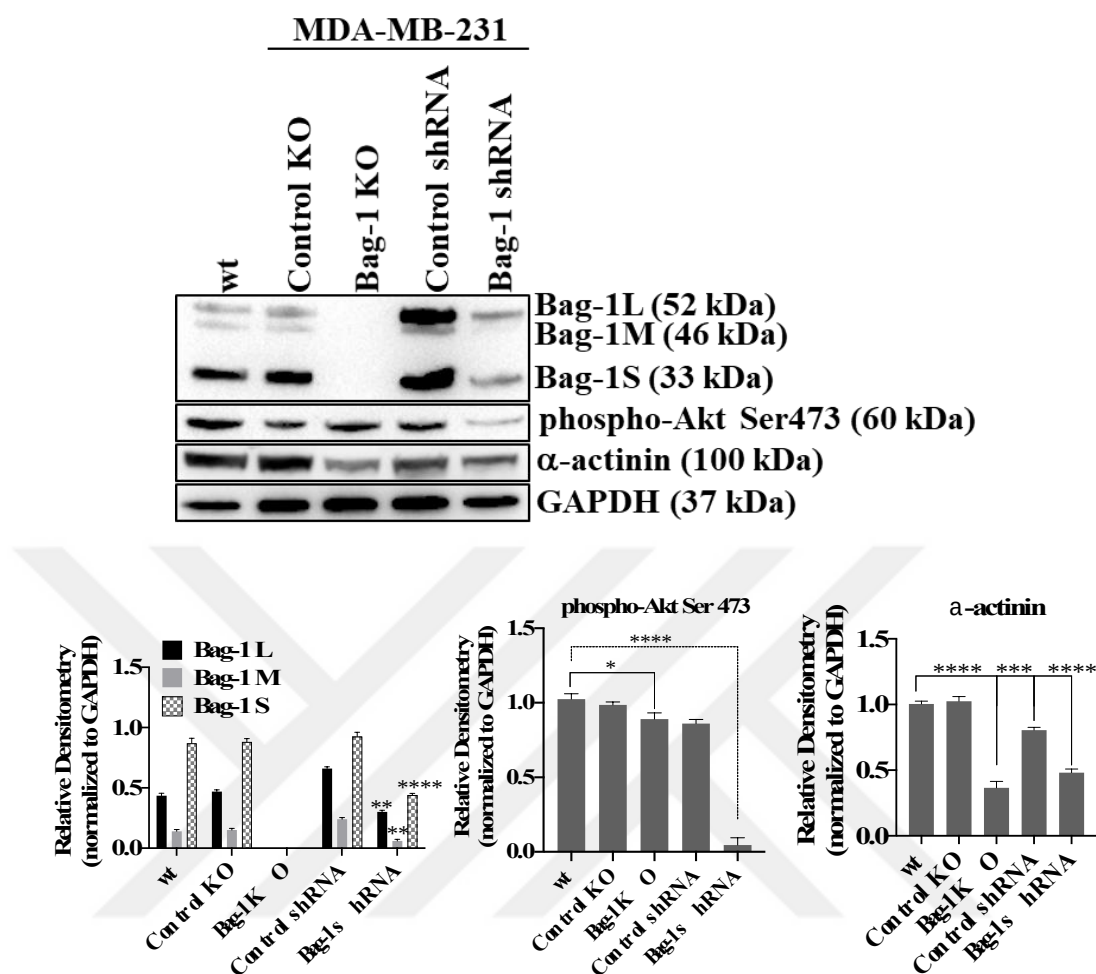


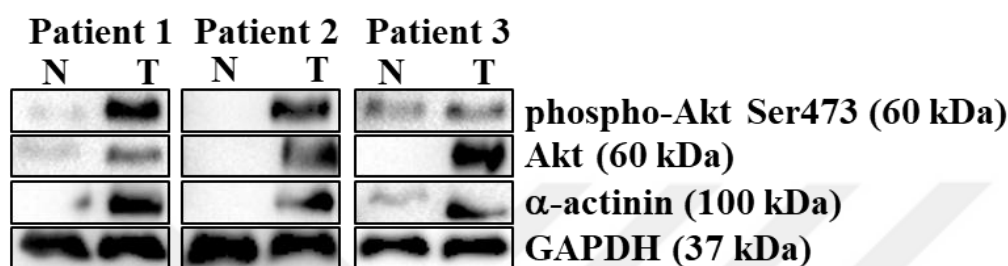
Figure 2.51 : The expression profiles of Bag-1, phospho-Akt Ser473, and α -actinin were investigated in MDA-MB-231 wt, Control KO, Bag-1 KO, and Control shRNA cells. (**p=0.0073, ***p= 0.0002, ****p<0.0001 by Two-way ANOVA, Tukey's multiple comparison test). The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control.

2.4.1.18 Distinct subtypes of breast cancer with Bag-1 expression showed significant Akt and α -actinin level

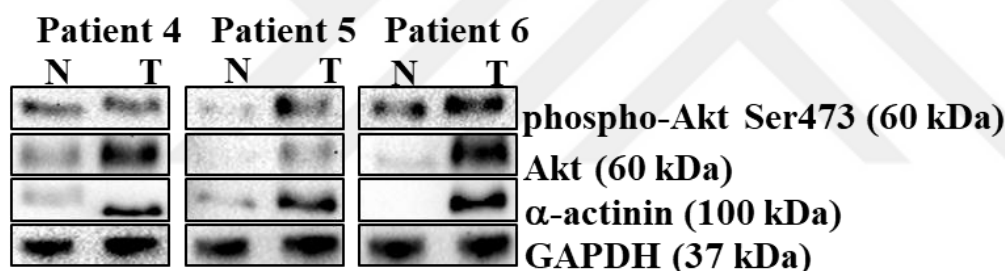
To determine the effect of endogenous Bag-1 expression on breast cancer cells, we performed immunoblotting experiments to investigate the phospho-Akt Ser473, total Akt, α -actinin, and N-cadherin expression in normal (N) and tumor (T) tissues from 8 breast cancer patients having three distinct subtypes. The patients were classified into ER/PR- Her2-, ER/PR+ Her2-, ER/PR/Her2+. Phospho-Akt Ser473, total Akt, and α -actinin levels were significantly upregulated in tumor samples compared to normal samples in different patients of each breast cancer subtype. However, none of the

normal or tumor tissues showed N-cadherin expression (Figure 2.52). Bag-1 expression observed from different patients of the same subtypes was elevated in tumor samples (not shown), which has already been shown in our previous study [182]. Based on this result, we suggest that Bag-1 expression enhanced the tumorigenic potential whereas knockout of Bag-1 caused a differential mesenchymal phenotype for breast cancer cells.

ER/PR-, Her2-



ER/PR+, Her2-



ER/PR/Her2+

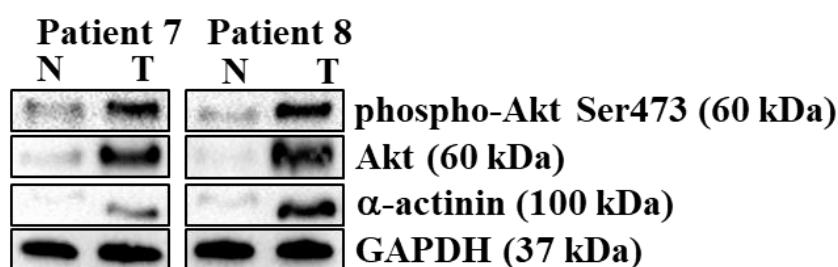


Figure 2.52 : The expression profiles of phospho-Akt Ser473, total Akt, and α -actinin were determined by an immunoblotting assay in normal and tumor tissues of ER/PR- Her2- (A), ER/PR+ Her2- (B), and ER/PR Her2+ (C) breast cancer cells. (* $p=0.0162$, *** $p=0.0007$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test). The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control.

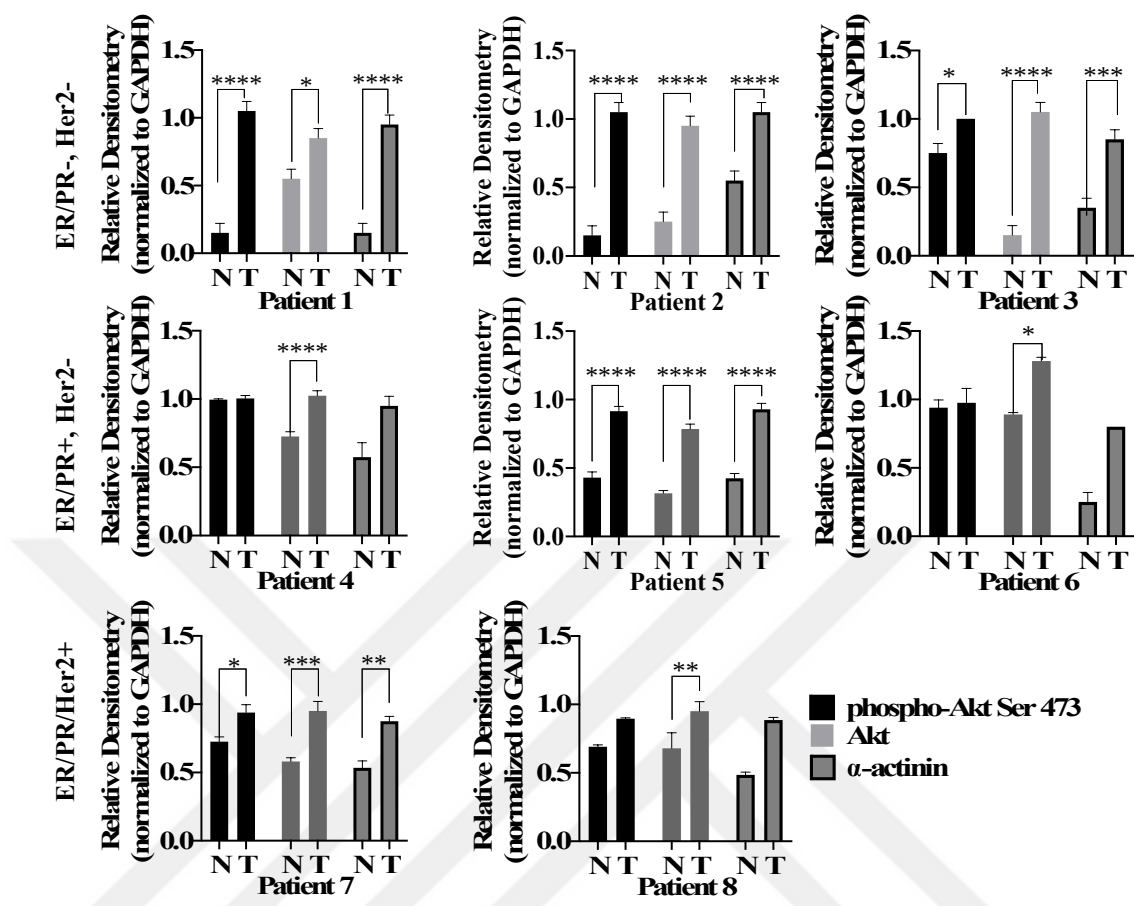


Figure 2.52 (continue): The expression profiles of phospho-Akt Ser473, total Akt, and α -actinin were determined by an immunoblotting assay in normal and tumor tissues of ER/PR- Her2- (A), ER/PR+ Her2- (B), and ER/PR Her2+ (C) breast cancer cells. (* $p=0.0162$, *** $p=0.0007$, **** $p<0.0001$ by Two-way ANOVA, Tukey's multiple comparison test). The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. GAPDH was used as a loading control.

2.4.1.19 Re-gain of each Bag-1 isoform prevented mesenchymal transition-related to Akt activation in Bag-1 KO cells

To confirm the potential role of Bag-1 on the cytoskeleton network via Akt-mediated actin dysregulation in our experimental MCF-7 cell models, we transiently transfected Bag-1 KO cells to isoform-specific expression constructs for 48 h, respectively. First, we compared the relative cell viability of Bag-1 KO cells following Bag-1 S, M, or L isoform transfection. We found that isoform-specific transfection caused by about a 12% cell viability decrease in each transfected cell line compared to that of parental Bag-1 KO cells (* $p=0.0121$, ** $p=0.0070$) (Figure 2.53).

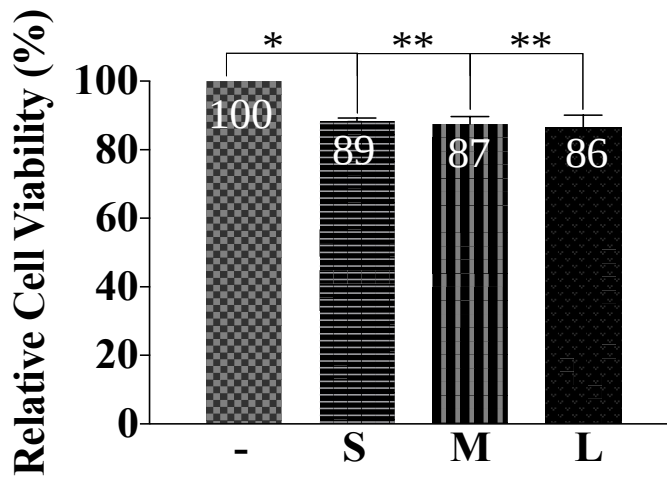


Figure 2.53 : The cell viability of Bag-1 KO cells and transfected with Bag-1S, M, and L isoforms, respectively were determined by MTT Cell Viability assay. (* $p=0.0121$, *** $p=0.0070$, by Two-way ANOVA, Dunnett's multiple comparison test).

According to the immunoblotting assay of Bag-1, the transfection of specific Bag-1 isoforms was successfully performed (**** $p<0.0001$). phospho-PI3K expression was higher in S isoform transfection (**** $p<0.0001$) but decreased in M isoform transfection. phospho-PTEN expression increased in an isoform-specific manner and the highest expression was shown in Bag-1L isoform transfected cells (* $p=0.0102$, ** $p=0.0018$). In contrast, phospho-Akt Ser473 levels were downregulated after Bag-1 S, M, or L isoform transfections (**** $p<0.0001$). A similar expression profile was observed in phospho-Akt Thr308 (**** $p<0.0001$). Total Akt was downregulated in Bag-1M isoform transfected cells (* $p=0.0165$) (Figure 2.54).

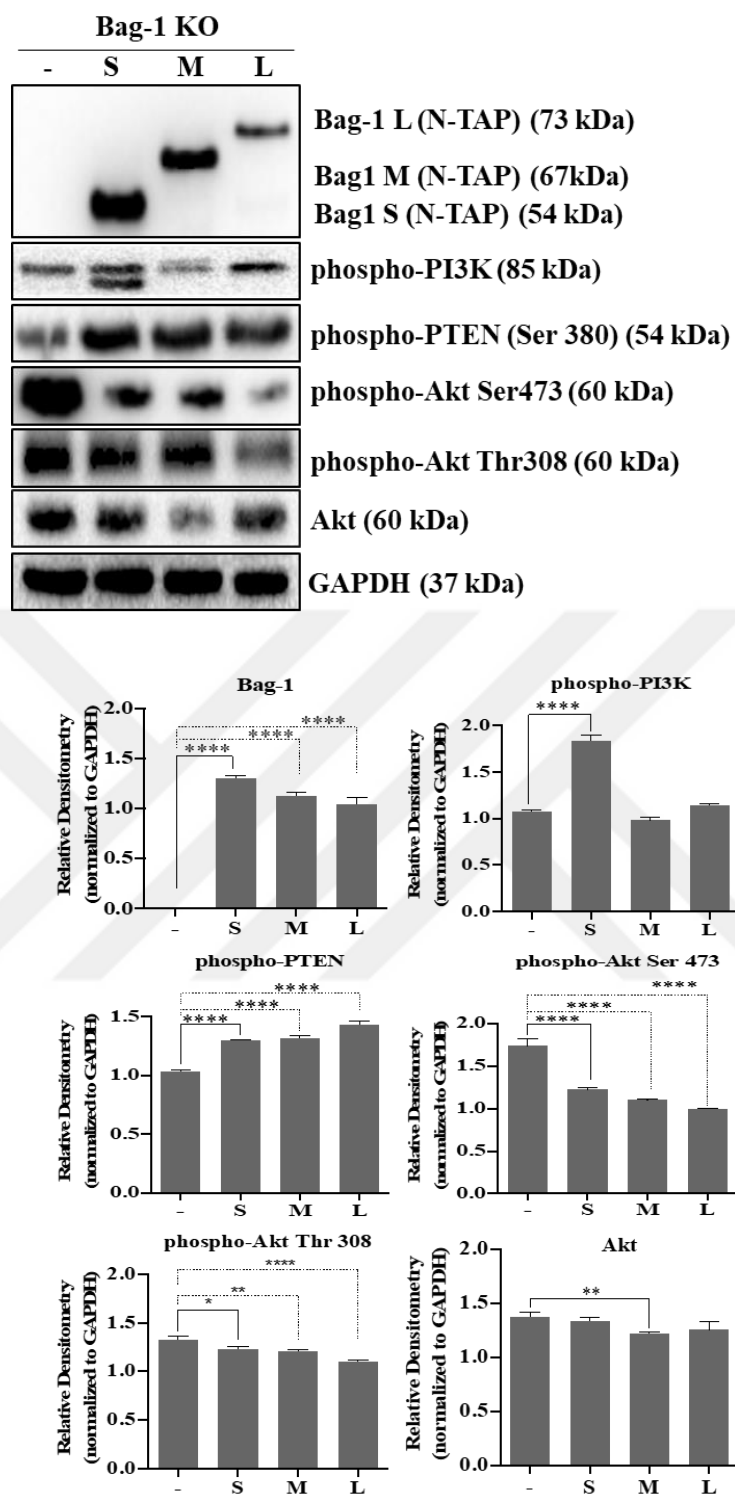


Figure 2.54 : Expression profiles of Bag-1, and Akt signaling pathway members after Bag-1 isoform-specific transfections were analyzed by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. **** $p < 0.0001$ by Two-way ANOVA, Tukey's multiple comparison test.

In addition, we investigated the expression profiles of EMT and actin-mediated cytoskeleton proteins. However, epithelial marker E-cadherin was significantly upregulated, mesenchymal marker N-cadherin and Vimentin were downregulated in Bag-1S, M, or L transfection (**** $p < 0.0001$). the β -catenin expression did not show a significant change after S and M isoform transfection but increased through Bag-1L transfection (* $p = 0.0206$). Transfection factors TWIST1 and ZEB1 were also downregulated after S, M, or L isoform transfection in Bag-1 KO cells (**** $p < 0.0001$). The tight junction protein ZO-1 expression did not show any significant change. Actin cytoskeleton-related focal adhesion proteins FAK, paxillin, and Vinculin expressions were upregulated (**** $p < 0.0001$) associated with α -actinin expression (**** $p < 0.0001$) but the β -actin expression did not show significant change. Actin-binding protein cofilin phosphorylation level was decreased (**** $p < 0.0001$), whereas total cofilin expression was upregulated following S, M, and L isoforms transfection (**** $p < 0.0001$) (Figure 2.55).

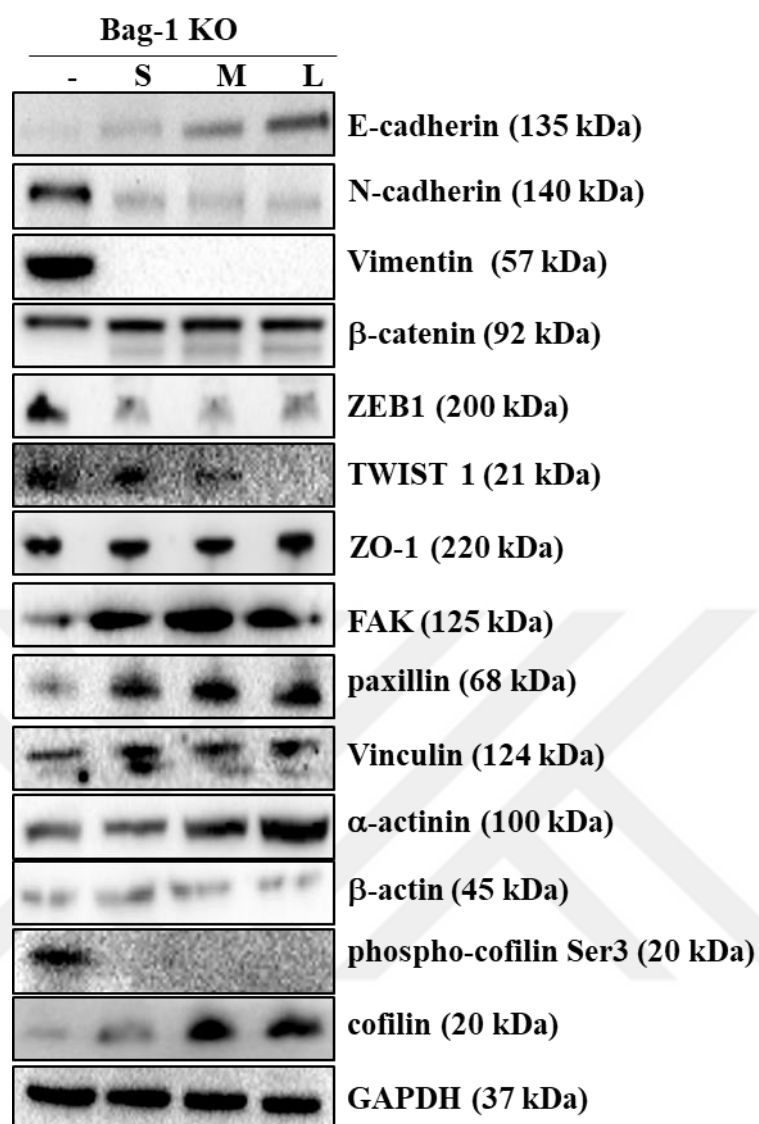


Figure 2.55 : Expression profiles of Akt signaling pathway members according to the Bag-1 isoform-specific transfection were determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. **** $p < 0.0001$ by Two-way ANOVA, Tukey's multiple comparison.

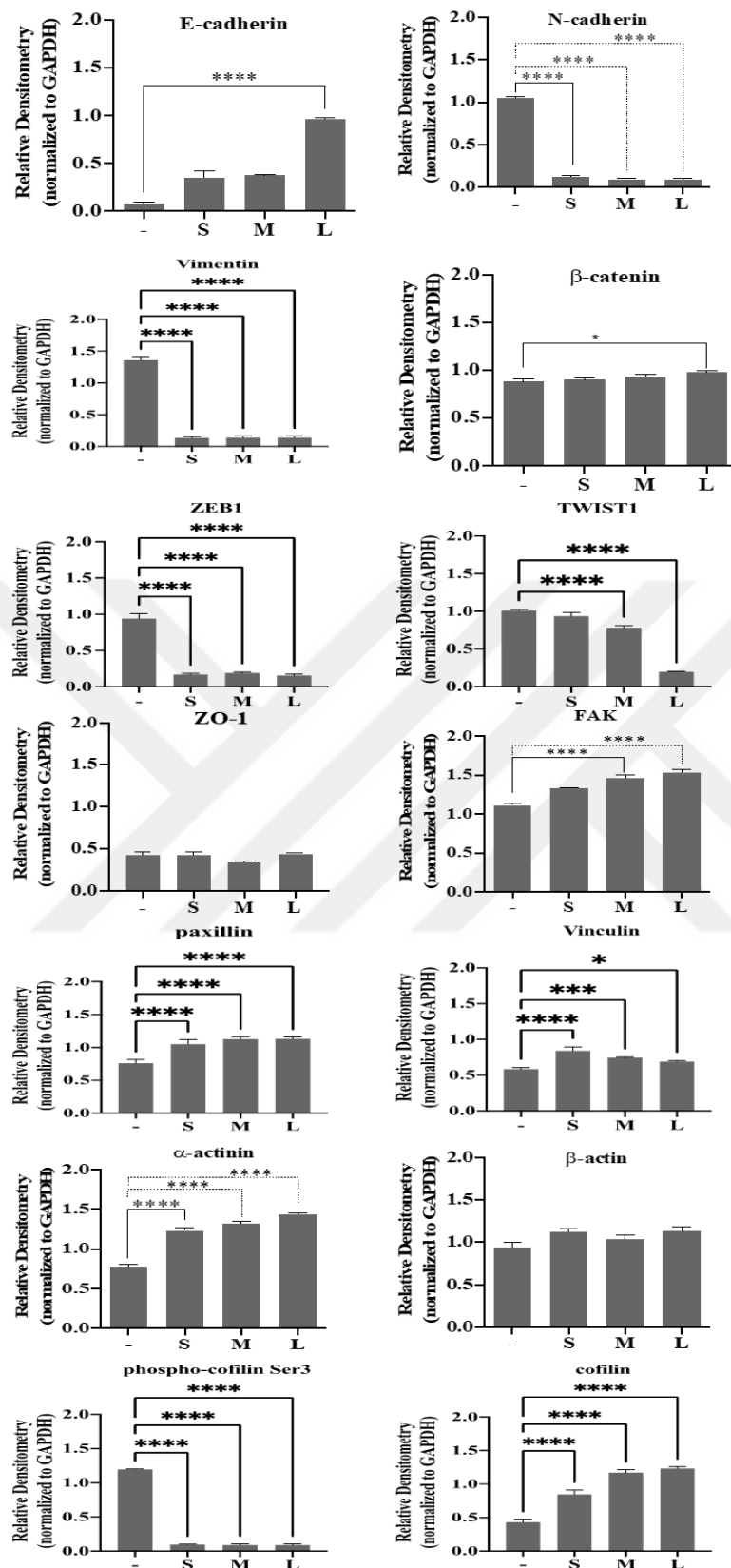


Figure 2.55 (continued): Expression profiles of Akt signaling pathway members according to the Bag-1 isoform-specific transfection were determined by immunoblotting assay. The relative densitometry analysis represented the mean $\pm$ SD of three independent experiments. **** $p < 00001$ by Two-way ANOVA, Tukey's multiple comparison test.

We also performed a qRT-PCR assay, which revealed that the relative mRNA expression levels of Akt, E-cadherin, Snail1, Snail2, TWIST1, and β -actin were increased following S, M, and L isoforms transfection (Figure 2.56).

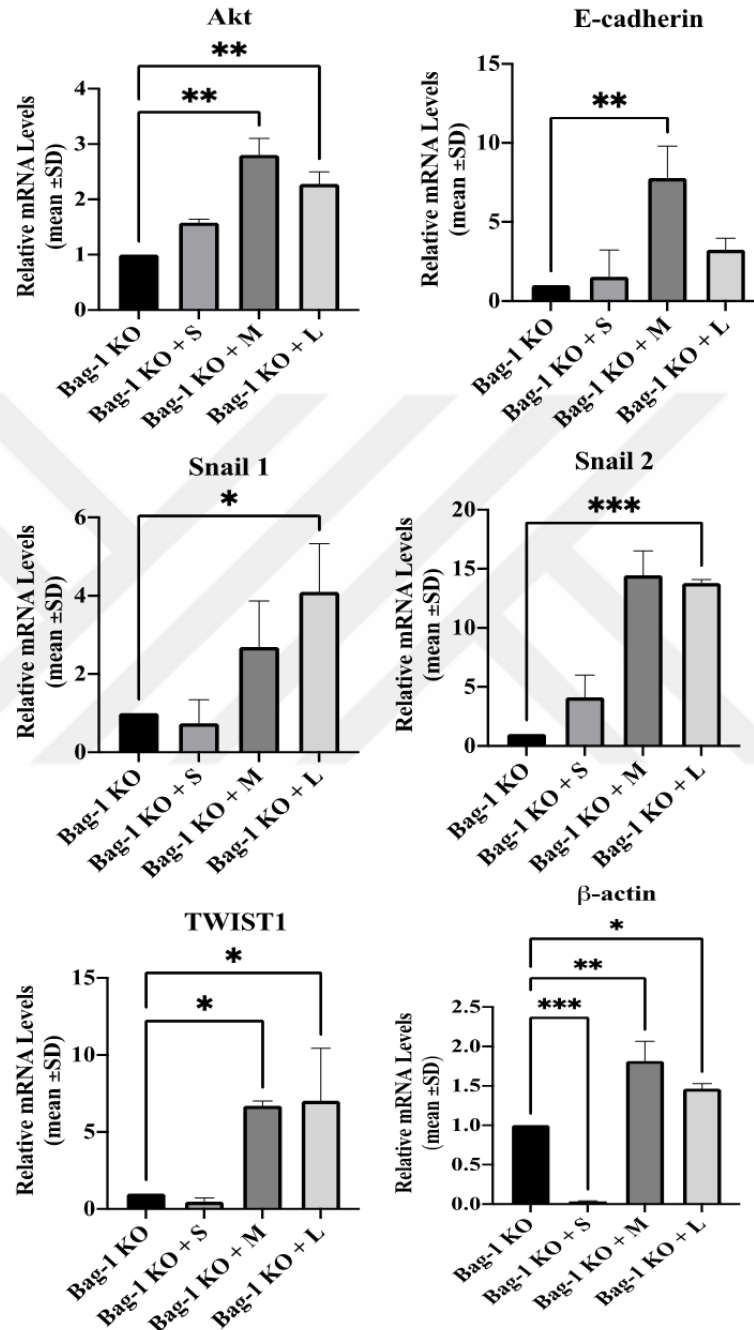


Figure 2.56 : The mRNA levels of Akt, E-cadherin, Snail1, Snail2, TWIST1 and β -actin were detected in qRT-PCR analysis. (Akt **=0.0015, E-cadherin **p=0.0031, Snail1 *p=0.0207, Snail2 ***p=0.0003, TWIST1 *p=0.0187, β -actin *p=0.0175, **p=0.0015, ***p=0.0007.

Considering the role of re-gaining of Bag-1 isoforms into Bag-1 KO cells decreased the mesenchymal properties of cells, we investigated the role of isoform transfection on migration in Bag-1 KO cells. The wound-healing assay showed that isoform-specific transfection of Bag-1 limited the migratory roles of Bag-1 KO cells (Figure 2.57). Time-dependent (0-24 and 48 h) wound healing images were captured under light microscopy.

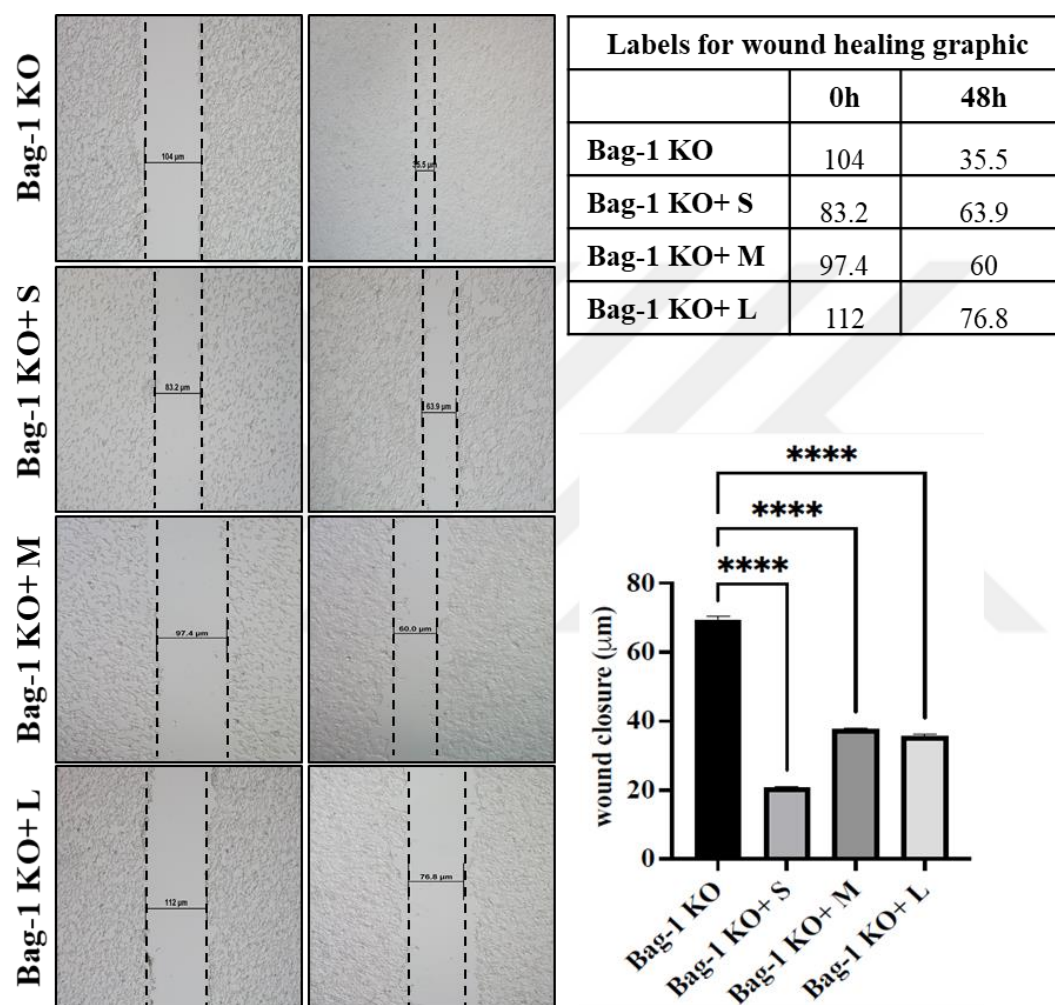


Figure 2.57 : The effect of Bag-1 isoform transfection into Bag-1 KO cells on cell migration of MCF-7 cells was assessed by wound healing assay, which was repeated by two-independent assays. Wound closure was imaged by light microscopy (magnification 4x, scale 10 μm), and analyzed by Two-way ANOVA, Tukey's multiple comparison test, **** p< 00001.

To examine the effect of different Bag-1 expression levels on actin cytoskeleton regulation, generated MCF-7 cell models were stained with FITC-Phalloidin to mark F-actin and were visualized by immunofluorescence imaging. Untransfected wt, isoform-specific Bag-1 transfected wt and Bag-1 OE cells showed increased F-actin

accumulation in the cell, whereas knockout of Bag-1 caused cytoplasmic condensation with the decrease in F-actin stress fiber. Bag-1 S, M and, L isoform transfected Bag-1 KO cells showed slightly more phalloidin stain compared to untransfected Bag-1 KO cells. The nuclear formation of cells was visualized by DAPI staining (Figure 2.58).

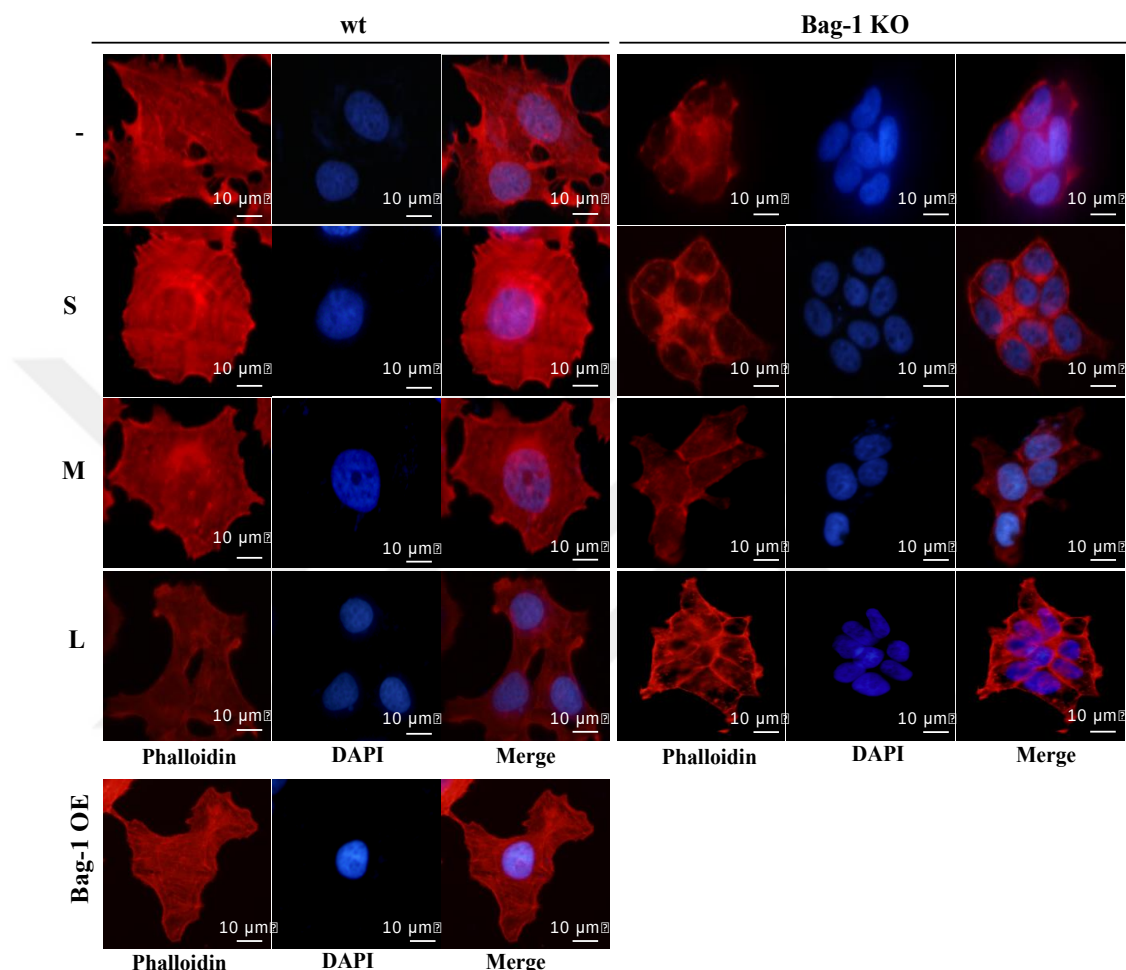


Figure 2.58 : Visualization of the actin cytoskeleton in Bag-1 isoform plasmid transfected MCF-7 wt and Bag-1 KO cells through fluorescent-labeled Phalloidin incubation by immunofluorescence experiment. Scale bar is 10 μ m and magnification 64x.

2.5 Discussion

Breast cancer is the most common type of cancer with the highest incidence and mortality rates among women worldwide [183]. Although there are improvements in adjuvant/neoadjuvant chemotherapy models, current treatment options have limitations in the treatment of metastatic breast cancer because of the heterogeneous nature of this disease. Genomic and proteomic differences of individuals cause resistance against current therapeutics and prevent successful therapies [184].

Therefore, current knowledge of gene-editing techniques promises to overcome the genomic problems in the diagnosis and treatment of breast cancer [185].

The multifunctional Bag-1 interacts with several molecular targets to regulate cell survival, proliferation, and apoptosis pathways. Early studies to understand the molecular function of Bag-1 were based on the relationship between the overexpression of Bag-1 and cell survival of cancer cells. One of these studies showed that the elevated level of Bag-1 in cancer cells was due to the transactivation of its promoter by mutant p53 [157]. Parental and stably Bag-1S isoform overexpressed MCF-7 breast cancer cells were exposed to several stress conditions such as γ -radiation, hypoxia, paclitaxel, doxorubicin, etoposide, and vincristine and heat shock. It was observed that the growth potential of parental cells decreased and apoptosis was induced by approximately 90% under these stress conditions, whereas in Bag-1S stably overexpression protected MCF-7 cells from heat shock-induced growth loss and apoptosis, as well as improved the long-term growth [166]. When performing functional studies of specific Bag-1 isoforms, low Bag-1-expressed HEK293 cells were used instead of MCF-7 cells. Because the endogenous level of Bag-1 in MCF-7 cells could affect the isoform-specific response [166].

Several studies explained the anti-apoptotic potential and survival-targeted role of Bag-1 overexpression in cancer, although the effect of complete knockout of Bag-1 in breast cancer has not been exploited in depth. In previous studies, complete loss of Bag-1 expression or ablation of its expression was found to be associated with the cell death of neuronal precursor cells [186]. Generated Bag-1^{-/-} mice through the deletion of the first two exons of Bag-1 had revealed normal body size but defects in the liver and nervous system cells with massive cell death [186]. Similarly, Bag-1 was found in the nucleus of neuronal precursor cells and overexpression of Bag-1 suppressed the cell death of neural cells [179]. However, in a recent study, CRISPR/Cas9-mediated double Bag-1 KO mouse embryonic stem cells (Bag-1KO-mES) showed high levels of alkaline phosphatase with ES-specific dome shape morphology and maintenance of their pluripotent stemness properties with expressing Oct4, Sox2, and Nanog stem cell markers [178].

In this study, we generated Bag-1 KO MCF-7 cells via editing of exon 2, 3, and 6 in the Bag-1 gene to elucidate the molecular consequences of the survival function of Bag-1. In addition, the generation of Control KO MCF-7 cells was used for the control

of the gene-editing system. The molecular alterations related to cell survival mechanism in these cells were compared to wt and Bag-1 OE MCF-7 breast cancer cells. The proliferation experiments showed that Bag-1 KO MCF-7 cells were not capable of adhering within 24 h and their known 29 h doubling time, which was delayed to 48 h. In contrast, Bag-1 OE cells were found to be highly capable of attachment due to their extended sizes. Although we observed in our previous study that transient Bag-1 silencing attenuates the survival-related PI3K/Akt/mTOR signaling axis, quite interestingly in this study, Bag-1 knockout induced phosphorylation of Akt on Ser473 and Thr308 and led to its activation in MCF-7 cells. This result suggested that Bag-1 was not required for the activation of Akt.

Akt has been indicated to be hyperactivated as an energy producer for the survival of cancer cells under increased ROS-mediated metabolic stress conditions [187]. Bag-1 was also found to prevent cellular ROS generation and stress-mediated growth inhibition in breast cancer cells [166]. In our study, it was observed that p38 phosphorylation and ROS generation were significantly increased in Bag-1 KO cells. Thus, we have suggested that the significant Akt activation in Bag-1 KO cells is due to ROS generation caused by Bag-1 deprivation and may be a cause of survival of Bag-1 KO cells. To overcome Akt-mediated survival of Bag-1 KO MCF-7 cells, wt, Control KO, Bag-1 KO, and Bag-1 OE cells were exposed to Akt inhibitor (MK-2206). Bag-1 has been shown to suppress elevated stress levels in different cell models, such as in neuronally differentiated rat pheochromocytoma PC12 cells. In addition, ectopic expression of Bag-1 was required for the suppression of caspase-activation in nerve-growth factor (NGF) deprivation-induced apoptosis and inhibition of the cellular ROS generation [188]. By these findings, Bag-1 prevented stress-mediated growth inhibition by interacting with chaperone-dependent pathways in MCF-7 breast cancer cells. Structural analysis showed that both NH₂-terminal and COOH-terminal parts of Bag-1 were required for the interaction between Bag-1 and Hsc70/Hsp70 to protect cells from cellular stress conditions [161]. Thus, in our experimental model, the increased cellular stress with excess ROS generation and significant alterations on MAPKs was meaningful in Bag-1 KO MCF-7 breast cancer cells. While a decrease in cellular ROS level was observed in wt and Bag-1 OE cells following MK-2206 treatment, continued stress in Bag-1 KO cells might be due to the induction of other signaling pathways instead of Akt. The mTOR might be a targeted downstream

mediator under elevated ROS conditions due to the inhibition of the PI3K/Akt/mTOR pathway leading to a reduction in stress-related survival of melanoma cells [189].

Considering the Akt activation, the expression levels of upstream and downstream signaling members of this pathway were investigated and found that Bag-1 KO caused a significant reduction in both total and phosphorylation of PTEN expression on Ser 380. Active PTEN co-localizes with β -catenin/E-cadherin at cell-cell junctions for the maintenance of tissue integrity, and the loss of E-cadherin expression reduces PTEN and caused the loss of growth control through inhibiting cell-cell contact [190]. In addition, inactivation or deletion of PTEN leads to activation of Akt. The phosphorylation of both Ser 473 and Thr 308 residues, which are required for the full activation of Akt was the consequence of upstream PI3K activation [191].

According to our results, activation of Akt was initiated from the upstream PI3K signaling and continued with the diminished PTEN expression in Bag-1 KO cells. Moreover, although a dramatic increase in PI3K expression levels was observed in Bag-1 OE MCF-7 cells, Akt phosphorylation was not significant compared to Bag-1 KO MCF-7 cells. In Bag-1^{-/-} mice neuronal stem cells, Akt was highly phosphorylated, but the phosphorylation of Bad at Ser136, which as a specific Akt target site was significantly reduced and therefore increased neuronal cell death, was observed [186]. Considering downstream signaling members of Akt members in Bag-1 KO MCF-7 cells, the deletion of Bag-1 removed the Akt-dependent cellular decision. On the contrary, Bag-1 OE cells showed Akt-dependent regulation of this signaling axis.

Previous studies showed that active PTEN served as a stabilizer of intercellular junctions through co-localizing with β -catenin/E-cadherin at cell-cell contact during acini formation of non-malignant Human Microvascular Endothelial Cells (HMEC). However, the blocking of E-cadherin in HMEC cells reduced PTEN expression and caused loss of growth control through disrupted organized acinar polarity [190].

In our study, we showed that significant E-cadherin loss was associated with a diminished PTEN expression profile in Bag-1 KO and Bag-1 OE MCF-7 cells. In the E-cadherin-mediated cell-cell adherence junctions, the cytoplasmic region of transmembrane E-cadherin binds β -catenin and p120-catenin and links to actin cytoskeleton with α -catenin to maintain cell-cell adhesion [192]. In the integrin-

mediated adhesion, the extracellular domain of cell-cell adhesion molecule integrin binds extracellular matrix proteins including laminin, fibronectin, or collagen. This binding change in the conformation of integrin leads to the exposure of the cytoplasmic tail to bind a multi-protein complex termed adhesome to link with the actin cytoskeleton. The integrin-actin linkage protein complex consists of actin-binding protein talin and vinculin, actin-crosslink protein α -actinin, Src, FAK, and Kindlins. Integrins bind and activate Src and FAK and lead the disorganization in the actin cytoskeleton [193].

Considering our result, we suggested Bag-1 KO cells gained mesenchymal characteristics due to their distinct morphological features from wt cells. As known from the literature, polarized epithelial cells are laterally connected and can interact with the basal surface but spindle-like mesenchymal cells lose their cell-cell junctions and have increased motility and migration capacity through decreased focal adhesions and remodeled cytoskeleton [140].

We suggested that decreased expression levels of epithelial marker E-cadherin and PTEN in Bag-1 KO and Bag-1 OE cells were associated with the motility potential of Bag-1 KO and Bag-1 OE MCF-7 cells. We further confirmed this mesenchymal potential of Bag-1 KO cells with the significant N-cadherin upregulation, which was reduced with MK-2206 treatment. To be functional, mature, and active cadherin proteins, the precursor cadherins are exogenously cleaved from conserved proteolytic cleavage sites within their pro-regions at the cell surface. The band pattern differences in N-cadherin between Bag-1 KO and Bag-1 OE were noticeable, which is related to the proteolytic processing of N-cadherin.

According to studies using a monoclonal antibody specific to the N-cadherin in HeLa cells, N-cadherin also needed to be processed to be functional and mature. The furin subgroup of subtilisin-like proprotein convertases was responsible for the cadherin processing and the furin protease sequences were the same in both E-cadherin and N-cadherin. N-cadherin was firstly synthesized in the endoplasmic reticulum (ER) due to the determination of proN-cadherin localization at ER or Golgi rather than at the plasma membrane. The p120-catenin binds to the cytoplasmic domain of proN-cadherin and is phosphorylated by casein kinase II at the cytoplasmic region. The phosphorylated proN-cadherin was associated with α -catenin, β -catenin, and p120-catenin, and the cadherin-catenin complex occurred while proN-cadherin in the

endoplasmic reticulum. Furin proteases triggered the cleavage of ~15 kDa pro-region fragment and converted the precursor N-cadherin to mature form, then the complex was transported to the plasma membrane to associate with actin cytoskeleton [181]. Due to these findings, our results indicated that Bag-1 OE increased N-cadherin expression levels independent of Akt. In Bag-1 KO cells, N-cadherin containing the pro-region was observed at a higher band level. On the contrary, Akt inhibition cleaved N-cadherin from its pro-region in Bag-1 KO cells. Therefore, Akt inhibition converted precursor N-cadherin to active N-cadherin form, the expression levels appeared to be considerably reduced. We conclude that hyperactivation of Akt led to an immature form of N-Cadherin in Bag-1 KO cells via inhibiting its pro-region cleavage, whereas Akt inhibition was associated with the cleavage of N-cadherin from its pro-region, leading to an active N-cadherin.

EMT is regulated by several transcription factors: Snail, Slug, TWIST, ZEB1 [194]. Increased ZEB-1 and TWIST expression confirmed the mesenchymal characteristics of Bag-1 KO cells. Bag-1 KO and Bag-1 OE cells differ in ZO-1 expression which is a tight junction protein in EMT. Loss of ZO-1 expression deregulates actin cytoskeleton remodeling and suppresses the E-cadherin-mediated cell-cell adhesion [195], [196]. Therefore, in the induction of EMT in Bag-1 KO and Bag-1 OE cells, cell adherence function differs from Bag-1 OE cells in that Bag-1 KO cells, which tend to lose cell adherence. Focal adhesion proteins have a role in the E-cadherin-mediated cell-cell adherence junctions through linking to the actin cytoskeleton [197].

In addition to the significant downregulation of focal adhesion protein FAK, β -actin, and α -actinin in Bag-1 KO cells compared to Bag-1 OE cells, the occurrence of FAK phosphorylation on Try 397 and paxillin expression in only Bag-1 OE cells confirmed our suggestion that Bag-1 KO cells lose their cell adherence characteristics. In Bag-1 KO cells, we also suggested that the E-cadherin/ β -catenin complex was disrupted and therefore the adhesome complex did not form and link to actin cytoskeleton due to the significant FAK, β -actin, and α -actinin ablation in Bag-1 KO cells [198]. Although inhibition of Akt caused an increase in FAK, β -actin, and α -actinin expression levels, the expression profiles of these proteins were still appeared to be low compared to that of wt MCF-7 breast cancer cells. Therefore, the mature form of N-cadherin in MK-2206 treated Bag-1 KO cells might not functionally be linked to the actin cytoskeleton to form cell-cell adhesion complexes. Reorganization of the cytoskeleton through Akt

inhibition in Bag-1 KO cells is also confirmed by the alternation of phosphorylation of cofilin at Ser3. It is indicated that phosphorylation of cofilin at Ser3 inactivates its function by severing F-actin and promotes actin polymerization [199]. Akt inhibitor decreased the phospho-cofilin level at Ser3 in Bag-1 KO cells which led to the polymerization of F-actin.

Recovery of Bag-1 isoforms (Bag-1S, M, and L) one by one led to upregulation in PTEN, E-cadherin, FAK, paxillin, Vinculin, α -actinin, and total cofilin expression in Bag-1 KO MCF-7 cells, allowed the suppression of EMT and induced the cell-cell adhesion potential of cells. Besides these results, downregulation of N-cadherin, TWIST1, and ZEB1 were remarkable signatures of the relation between Bag-1 expression and EMT suppression in rescued MCF-7 cells. We found that regain of each Bag-1 isoform reduced phospho-Akt Ser473 levels compared to Bag-1 KO MCF-7 cells. Although transfection of each Bag-1S or M isoform did not alter the expression of adhesion proteins, Bag-1L transfection upregulated FAK, β -actin, and α -actinin expression. Therefore, we suggested that the full-length form of the Bag-1 protein is required for the formation of cell adhesion complexes. Accordingly, we proposed that Bag-1 overexpression enhances the tumorigenic potential, whereas knockout of Bag-1 causes a mesenchymal phenotype for breast cancer cells.

Due to the morphological changes of Bag-1 KO MCF-7 cells compared to wt, Control KO, and Bag-1 KO cells, Bag-1 KO caused the redistribution of actin filaments. According to the previous studies that phosphorylation of F-actin binding protein palladin by Akt1 at Ser507 caused disruptions of F-actin bundling in breast cancer [200]. Although Akt hyperphosphorylation in Bag-1 KO cells disrupted F-actin formation and F-actin bundling, Bag-1 isoform-specific transfection upregulated FAK, β -actin, and α -actinin expression levels but not their localization. We found that F-actin localized to the edge of the cell in isoform-expression-positive MCF-7 cells. The F-actin staining in Bag-1 KO cells was around the edges of cells and might be the outcome of colocalization of phospho-Akt, which is triggered by PIP3 signaling to increase cytoskeleton construction of cells [201].

Loss of Bag-1 is substantial in Akt-mediated survival of the cells and causes remodeling of stress fibers and reducing of focal adhesions. Overexpression of Bag-1 was found to be associated with more metastatic character than knockout of Bag-1 with improved migration and wound healing properties. The bag-1 loss showed a

higher migration rate compared to wt MCF-7 with their smaller cell size and colony-forming capabilities for survival resilience. Regaining of each Bag-1 isoform limited the migration profile of Bag-1 KO cells. All of these results highlighted that Bag-1 expression-dependent alterations in MCF-7 breast cancer cells sharply changed the expression profile of cytoskeleton proteins and affected EMT signaling. Therefore, Akt-dependent cytoskeletal regulatory potential is a novel understanding of the molecular role of Bag-1 in cell physiology.



3. CHAPTER II-INVESTIGATION OF BAG-1 EXPRESSION DEPENDENT MIRNA REGULATION OF MCF-7 CELLS

3.1 Purpose

The multifunctional Bag-1 protein promotes breast cancer survival through direct or indirect interaction partners. The number of the interacting partners determines its cellular role in different conditions. As well as interaction partner variability, the amount of Bag-1 in the cells could cause dramatic alterations. According to previous studies, while the transient silencing of Bag-1 enhanced drug-induced apoptosis, deletion of Bag-1 could induce stemness properties and Akt-mediated actin remodeling in MCF-7 breast cancer cells. Considering the heterogeneity of breast cancer and the variability of Bag-1-mediated cell response, it has become essential to determine differential expression of miRNAs during breast cancer progression depending on Bag-1 expression level. Although numerous studies have shown that different expression levels of Bag-1 show different functions in breast cancer, miRNA regulation in Bag-1-deficient breast cancer development remain largely unknown. Therefore, this study aims to screen the differential expressed miRNAs and determine the potential targeted genes and pathways in Bag-1 deficient conditions in MCF-7 breast cancer cells compared to wt cells.

3.2 Literature Review

3.2.1 Biological functions of microRNAs

MicroRNAs (miRNAs) are 22 nucleotide length endogenous, single-stranded small non-coding RNAs. miRNAs negatively modulate the expression of genes and affect several cellular pathways. They regulate post-transcriptional mechanisms by degrading mRNA and inhibiting target protein translation through sequence-specific binding to the 3'-untranslated region (UTR) of target mRNA or coding sequences [202], [203].

According to the latest version of miRBase (v22), the genomes of 271 different organisms consist of 38589 hairpin precursors and 48860 mature miRNAs. The human genome consists of 2654 mature miRNA sequences and more than 3000 miRNA genes regulate half of the protein transcripts in humans [204]. In eukaryotes, miRNA genes are considered to make up nearly 1-2% of all known genes [205]. In addition, more than one protein-coding gene and multiple cellular pathways can be a one miRNA target and one miRNA can target one mRNA as well, therefore miRNAs have an important role in gene regulation [206].

miRNAs have important biological functions in cell proliferation, apoptosis and differentiation, drug resistance, and mostly in cancer. In addition, miRNAs have been found significantly dysregulated in cancer. Loss-of-function and gain-of-function investigations in human cancer cells or transgenic mouse models have demonstrated that miRNAs play important roles in the initiation of cancer, development, and metastasis [207]. The tumor-associated miRNAs are classified as tumor suppressor (TS)-miRNAs and onco-miRNAs [208]. Although miRNAs can be oncogenic or tumor suppressors, various studies showed that miRNA expression is broadly decreased in tumor cells compared to normal cells suggesting that miRNA biogenesis is dysregulated in cancers [209].

3.2.2 miRNA biogenesis and miRNA markers for breast cancer

The mature miRNA biogenesis is evolutionary conserved and miRNA genes are transcribed by RNA polymerase II (Pol II) in the nucleus as primary miRNAs (pri-miRNAs), then pri-miRNAs are spliced and polyadenylated. The cleavage of long pri-miRNAs is performed by a Microprocessor which consists of DROSHA, a double-stranded RNase III enzyme, its cofactor, and the double-stranded RNA binding protein DiGeorge syndrome critical region 8 (DGCR8) [210]. DROSHA cleaves the double-strand RNA to generate 60-70 nucleotide hairpin-shaped precursor miRNAs (pre-miRNAs). Then, the pre-miRNAs are exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, an RNase III enzyme that generates the mature miRNAs [211]. TRBP a transactivation-responsive RNA-binding protein that binds to dsRNA connects with DICER1, improves the accuracy of DICER1-mediated cleavage of pre-miRNAs, and also acts as a link between DICER1 and the Argonaute proteins (AGO1, AGO2, AGO3, or AGO4) to form the miRNA-

induced silencing complex (miRISC) [212]. miRISC complex which consists of DICER1 and Argonaute (AGO), leads to target mRNA by sequence complementary binding for post-transcriptional gene silencing. Gene silencing occurs through targeting mRNA degradation and translational repression in processing bodies (P-bodies) [213]. miRNA genes in humans are located in intergenic and intragenic regions in introns or within an exon of the gene.

Despite the fact that miRNA biogenesis is a tightly regulated process, dysregulation of miRNAs induced by changes in biogenesis pathway enzymes such as DROSHA, DICER, AGO2, TARBP2 has been observed in cancer cells. In addition, miRNA levels can be altered in cancer cells due to the tumor microenvironment which regulates miRNA biogenesis, methylation, and transcriptional changes [214].

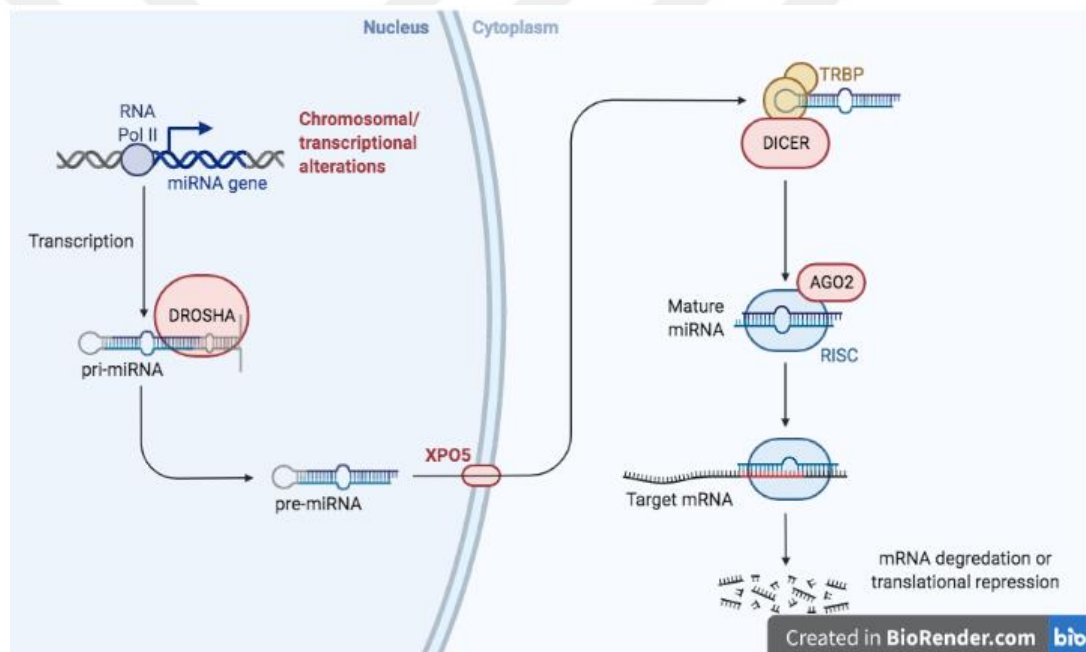


Figure 3.1 : The overview of miRNA biogenesis.

Moreover, the differential expression profiles of miRNAs are potential diagnostic markers and therapeutic targets in the progression of the various cancer types [215]. Defining miRNA expression profile in breast cancer is an improvement feature to understand the stage of tumor progression and metastasis [216]. Following the initial studies, miRNAs were referred to as a diagnostic and prognostic marker for breast cancer. These studies showed that miR-125b, miR-145, miR-21, miR-155, miR-205 were the most deregulated miRNAs in breast cancer related to the expression of hormone receptors, tumor subtype, and proliferation capacity [217]. miR-221 and

miR-222 were found in the association with ER and miR-145 decreased in tumors through participating with p53 and targetting ER [218], [219]. According to the studies based on the tumor subtyping related to miRNA profiling, 69 miRNA were found most differentially expressed among their receptor status. miR-135a, miR-135b, miR-365, and miR-7 were markers to classify breast cancer cells by ER status [220].

3.2.3 Next-generation sequencing

Next-generation sequencing (NGS) is a technique that allows the sequence of the whole genome, a small number of genes, or specific target regions of genes in parallel on the same bead or glass surface simultaneously. Small DNA fragments are sequenced in parallel and the sequenced fragments are assembled by mapping them to the human reference genome. This high throughput sequencing method leads millions of reads to be made in one single parallel sequencing [221].

While several NGS platforms are different in technologies, the basic steps of the NGS are the same. 1) Library preparation which consists of random fragmentation of genome and adaptor ligation, 2) amplification of library, and 3) sequencing [222].

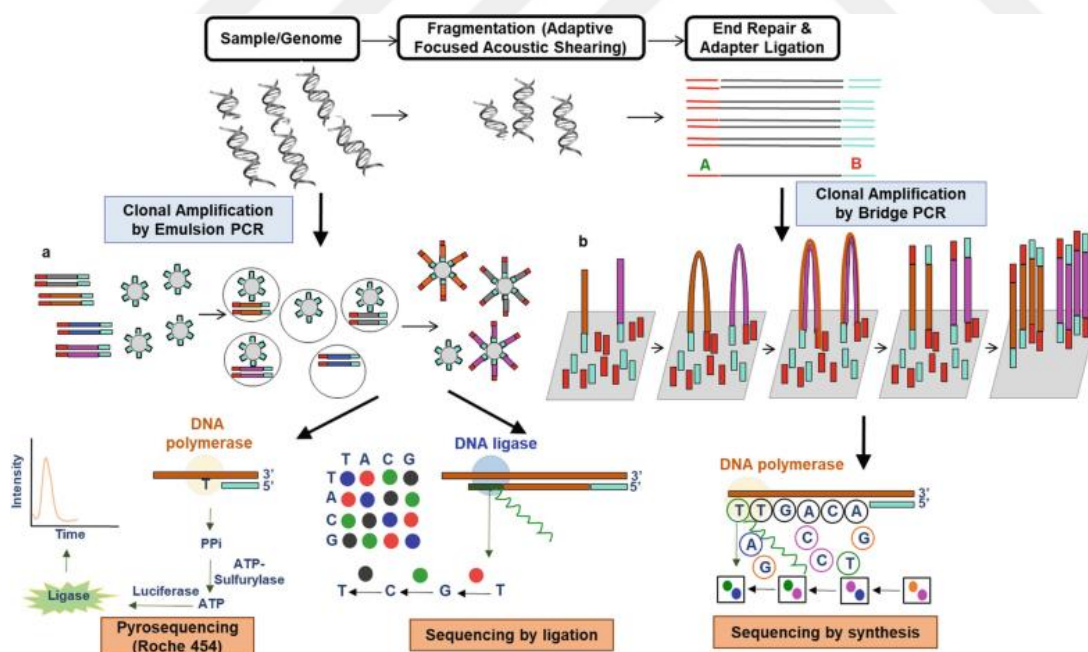


Figure 3.2 : Schematic diagram of the basic procedure for DNA sequencing using NGS platforms. The image was taken from [223].

Considering that NGS is a new technique, it has some limitations and advantages. DNA variations include substitutions (small base changes), small insertions or deletions, deleted large regions of genes, translocations, and inversions. Traditional

Sanger sequencing is limited to showing only minor insertions or deletions, while NGS provides a broader discovery of DNA variations and more sensitive investigations than Sanger sequencing, such as microdeletions or mosaic mutations in a single experiment. NGS is an effective and non-selective technique for discovering new mutations and novel genes that can be a potential cause of certain diseases or unexplained syndromes. NGS studies require large computer storage and capacity, and comprehensively bioinformatic analysis, prediction of obtained results, and extraction of clinically important information. However, as one of the significant limitations of this technique, NGS performs poor sequencing in guanine/cytosine-rich or repetitive regions [224].

The NGS technology is used in several fields of research areas such as; clinical genetics, microbiology, cancer genomics, metagenomics, agriculture, evolution studies. One of the common usages of NGS is interpreting differentially expressed miRNAs and profiling miRNAs. Considering the limited number of miRNAs compared to a higher number of mRNAs, it is known that one miRNA can regulate hundreds of mRNAs during gene regulation. Therefore, identifying miRNA expression profiles gains importance to determine their roles in cellular processes [225]. miRNA profiling is frequently performed in diverse research areas such as; tissue differentiation, stem cell studies, cancer development, investigations of human diseases molecular diagnostic applications.

3.2.3.1 Illumina next-generation sequencing technology

Illumina sequence technology is based on sequence-by-synthesis technology which consists of library preparation, generation/amplification of clusters, and parallel sequencing.

The templates are immobilized onto the flow cell surface and solid-phase amplification leads the 1,000 copies of each template. Parallel sequencing was performed based on the sequencing by synthesis (SBS) reaction which uses four fluorescently labeled nucleotides to sequence template on the flow cell surface. An individual labeled deoxynucleoside triphosphate (dNTP) incorporates to the nucleic acid chain in each sequencing cycle. The general workflow of Illumina Sequencing consists of 1) Genomic DNA preparation by ligation of adapters to both ends of the randomly fragmented DNA, 2) These fragments bind onto the surface of the flow cells. 3) The

solid-phase bridge amplification occurs by the addition of unlabelled nucleotides and enzymes. 4) The double-stranded fragments are formed by nucleotide incorporation with enzyme on the solid-phase substrate. 5) Denaturation of the double-stranded templates to single-stranded. 6) Amplification leads to the generation of double-stranded DNA clusters in each flow cell channel. 7) Incorporation of four labeled reversible terminators, primers, and DNA polymerase initiates the sequencing. 8) The fluorescence emission of each cluster represents the base identification. 9) The repetitive cycles determine the sequence of bases in template fragments, one base at a time. 10) The raw read of sequencing is aligned to a reference genome and the differences are identified [226].

MiSeq next-generation sequencing platform is one of the sequencing technology of Illumina (San Diego, CA), which was firstly used in 2011 by performing SBS technology. This system allows rapid library sequencing, production of large data, analysis, and monitoring of the data in a single run [226]. The quality of raw data obtained from the MiSeq system depends on the preparation and quantification of libraries, denaturation, and dilution of libraries, cluster densities, the concentration of loading, and sequencing parameters [227].

3.3 Hypothesis

The multifunctional Bag-1 (Bcl-2 athanogene-1) protein promotes breast cancer survival through direct or indirect interaction partners. Silencing of Bag-1 enhanced drug-induced apoptosis, whereas knockout of Bag-1 induced Akt-mediated actin remodeling in MCF-7 breast cancer cells. Considering the heterogeneous characteristics of breast cancer, it became essential to investigate the regulatory mechanism in breast cancer. Bag-1 expression-mediated alternations in cellular responses in breast cancer development, the differentially expressed miRNAs, and miRNA targets can be potential targets to elucidate the network between Akt and Bag-1 triggering specific survival-related pathways.

3.4 Materials and Methods

3.4.1 Materials

3.4.1.1 Equipment

Types of equipment that are used in this study are listed in APPENDIX A.

3.4.1.2 Chemicals, media components, antibodies, and kits

Chemicals, media components, antibodies, and molecular biology kits that were used in this study are shown in APPENDIX B.

3.4.1.3 Buffers and solutions

Buffers and solutions that were used in this study are shown in APPENDIX C.

3.4.2 Methods

3.4.2.1 Cell culture of wt and Bag-1 KO MCF-7 cells

The wt MCF-7 (HTB-22) human breast cancer cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, PanBiotech, Germany) augmented with 10% Fetal Bovine Serum (FBS, PanBiotech, Germany), 100-units/ml penicillin, and 100-µg/ml streptomycin (Pan Biotech, Germany). Cells were kept at 37°C in a humidified 5% CO₂ incubator. Bag-1 KO MCF-7 cells were generated through co-transfection of Bag-1 CRISPR/Cas9 Knockout (KO) plasmid (sc-417179) (Santa Cruz Biotechnology, USA) and Bag-1 Homology Directed Repair (HDR) plasmid (sc-417179-HDR) (Santa Cruz Biotechnology, USA) into MCF-7 cells. Control CRISPR/Cas9 Knockout plasmid (sc-418922) was transfected into MCF-7 cells for negative control. Cells were transfected with UltraCruz Transfection Reagent (Santa Cruz Biotechnology, USA) for 48 h according to the manufacturer's protocol. Following transfection cells were treated with puromycin (Santa Cruz Biotechnology, USA) for 48h, and selected by single-cell dilution process. Comparison of wt and Control KO cells showed similar survival, proliferation and, growth potentials. After validation and characterization of Bag-1 deficiency in MCF-7 cells, one colony showing the lack of Bag-1 expression was selected for further experiments. Bag-1 KO MCF-7 cells were also grown in the same conditions with wt MCF-7 cells.

3.4.2.2 RNA extraction and quality control

According to the manufacturer's protocol, the total RNA of wt and Bag-1 KO MCF-7 cells were isolated using SPLIT RNA Extraction Kit (Lexogen, Austria). According to the manufacturer's instructions, total RNA quality, concentration, and integrity were measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

3.4.2.3 Construction of sequencing libraries

The total RNA from two biological replicates for each group was used in sequencing experiments. Small RNA Libraries were constructed directly from total RNA using the Small RNA-Seq Library Prep Kit for Illumina Platforms (Lexogen, Austria) following the manufacturer's instructions. Small RNAs are ligated at 3' and 5' ends, respectively, and converted into cDNA.

3.4.2.4 RNA-Seq and data analysis of small RNA sequencing

High-throughput RNA-seq was performed by Illumina MiSeq Kit V3 sequencing platform (Illumina, USA). The data were uploaded to the Galaxy web platform, and all downstream analyses were done at usegalaxy.org. FastQC reported the quality check of raw reads with sequence quality scores, sequence-based quality scores, sequence GC and Kmer content, duplicate sequences, overrepresented sequences, and overall sequence length distribution read distribution plots. The Illumina small RNA-Seq Library kit adapter sequence 5'-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-3' was trimmed, leaving a minimum overlap length=5 and maximum error rate= 0.1 by Cutadapt. The minimum length was selected 18 to discard sequences fewer than 18 base pairs in size, and the total length was selected 35 to exclude more than 35 base pairs in length. The trimmed reads were aligned to the reference human genome GRCh37-hg19 by Bowtie2 with BAM format output. The aligned reads were annotated to mature human miRNA (<http://www.mirbase.org/ftp.shtml>) by featureCounts. Differential expression of miRNAs was determined using DESeq2, and the results have False Discovery Rates (FDR) ≤ 0.01 was accepted significantly. The fold change determined the expression ratio between wt and Bag-1 KO cells.

3.4.2.5 Identification of differentially expressed miRNAs in wt and Bag-1 KO cells

To analyze differential expressions of miRNAs obtained from featureCounts annotation, DESeq2 differential expression analysis was performed. The output of DESeq2 gives the fold changes (log2) of genes based on the factors (untreated/treated) and generates a tabular file. In our study, the fold changes of "Bag-1KO samples" against "wt" samples were compared. The False Discovery Rates (FDR) ≤ 0.01 was accepted significantly. The fold change determined the expression ratio between wt and Bag-1 KO cells.

3.4.2.6 The functional analysis of miRNAs using DIANA-miRPath v3.0 prediction algorithm

DIANA-miRPath v.3.0 web platform was used to investigate the molecular function of miRNAs and enrichment of pathways. The algorithm performs a statistical model mentioned in Vlachos et al. [228] (<http://snf-515788.vm.okeanos.grnet.gr/>). Functional enrichment of target genes for downregulated and upregulated miRNAs was also performed on DIANA-miRPath v.3.0. KEGG pathway analysis was performed to predict target genes and molecular pathways associated with differentially expressed miRNAs in Bag-1 KO and wt samples. GO analysis was performed to annotate the biological processes, cellular components, and molecular functions of miRNA-associated mRNA targets. Both functional enrichments analyzed FDR p-value < 0.05 were enriched using the Fisher's exact t-test hypergeometric distribution.

3.4.2.7 Validation of selected differentially expressed miRNAs by quantitative polymerase chain reaction (RT-qPCR)

Total RNA, including smaller than ~200 nucleotides was extracted from MCF-7 wt and Bag-1 KO cells using miRNeasy Mini Kit (Qiagen, USA), and the reverse transcription of total RNA containing miRNA was performed using miScript II RT Kit (Qiagen, USA) according to the manufacturer's protocol. The qRT-PCR experiment was performed using miScript SYBR Green PCR Kit (Qiagen, USA), and the cycling conditions were as follows: initial activation 95 °C for 15 min, followed by 40 cycles of denaturation 94 °C for 15 sec, annealing 55 °C for 30 sec, extension 70 °C for 30 sec. The reactions were performed in two replicates and the selected miRNA primer

sequences were listed in (Table 3.1). RNU6 small nuclear RNA was used as a standard internal control and to normalize expression profiles. Relative expression of miRNA samples was calculated using the $2^{-\Delta\Delta C_q}$ methods. Data analyses were performed via GraphPad Prism 8.

Table 3.1 : Primer sequences for selected miRNA targets for qRT-PCR experiments.

miRNA ID	Forward Primer	Reverse Primer
RNU6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
hsa-miR-21-5p	GCTAGCTTATCAGACTGATGTTGAAA	GTGCAGGGTCCGAGGT
hsa-miR-429	GCTAATACTGTCTGGTAAAACCGTAA	GTGCAGGGTCCGAGGT
hsa-miR-200a-5p	CGGACAGTGCTGGAAAAA	GTGCAGGGTCCGAGGT
hsa-miR-27a-3p	CAGTGGCTAAGTTCCGCAAA	GTGCAGGGTCCGAGGT
hsa-miR-342-3p	CACAGAAATCGCACCCGTAA	GTGCAGGGTCCGAGGT
hsa-miR-345-5p	GACTCCTAGTCCAGGGCTCAAA	GTGCAGGGTCCGAGGT
hsa-miR-151a-3p	GACTGAAGCTCCTTGAGGAAAA	GTGCAGGGTCCGAGGT
hsa-miR-146b-5p	TGAGAACTGAATTCCATAGGCTAA	GTGCAGGGTCCGAGGT
hsa-miR-31-5p	CAGGCAAGATGCTGGCATAG	GTGCAGGGTCCGAGGT
hsa-miR-887-3p	GCCATCCCGAGGAAAAA	GTGCAGGGTCCGAGGT
hsa-miR-767-5p	CACCATGGTTGTCTGAGCAT	GTGCAGGGTCCGAGGT
hsa-miR-4521	CTAAGGAAGTCCTGTGCTCAGAAA	GTGCAGGGTCCGAGGT
hsa-miR-130b-3p	TGCAATGATGAAAGGGCATA	GTGCAGGGTCCGAGGT
hsa-miR-10a-5p	ACCCTGTAGATCCGAATTTGTG	GTGCAGGGTCCGAGGT

3.4.2.8 Protein-protein interaction (PPI) network analysis

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to predict protein-protein interactions derived from genomic context, high-throughput lab experiments, co-expression, text mining database knowledge [229]. The active interaction sources were selected from experiments and databases for reliable results. Moreover, the minimum required interaction score was set at high confidence (0.700). The mRNA regulatory network was visualized by Cytoscape open-source platform version 3. 8. 2 [230].

3.4.2.9 Statistical analysis

The differential expression of miRNAs was performed using the Galaxy web platform, and the $FDR \leq 0.01$ expression results were considered significant. Then, the functional KEGG and GO enrichment analysis was performed using $FDR < 0.05$. The qRT-PCR results were calculated using $2^{-\Delta\Delta C_q}$ and RNU6 was used as a normalization control. For the analyses of results “Two-way ANOVA followed by Sidak’s multiple

comparisons tests was performed using GraphPad Prism 8.0.0, Graphpad Software, San Diego, California USA, www.graphpad.com". To predict protein-protein interaction STRING was used at 0.7 high confidence ratio, and interaction network was visualized by Cytoscape web-platform using CytoHubba application in the Cytoscape database using the "the degree" calculation method. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics tools (<https://david.ncifcrf.gov/>) was performed for the functional characterization of mRNA targets.

3.5 Results

3.5.1 Analysis workflow/pipeline

High-throughput next-generation sequencing was performed from two biological replicates of wt and Bag-1 KO MCF-7 cells. The overall workflow is composed of five main steps: The sequencing experiment, raw data processing obtained from sequencing, adapter trimming, alignment to the human genome, and differential expression analysis of miRNAs (Figure 3.3).

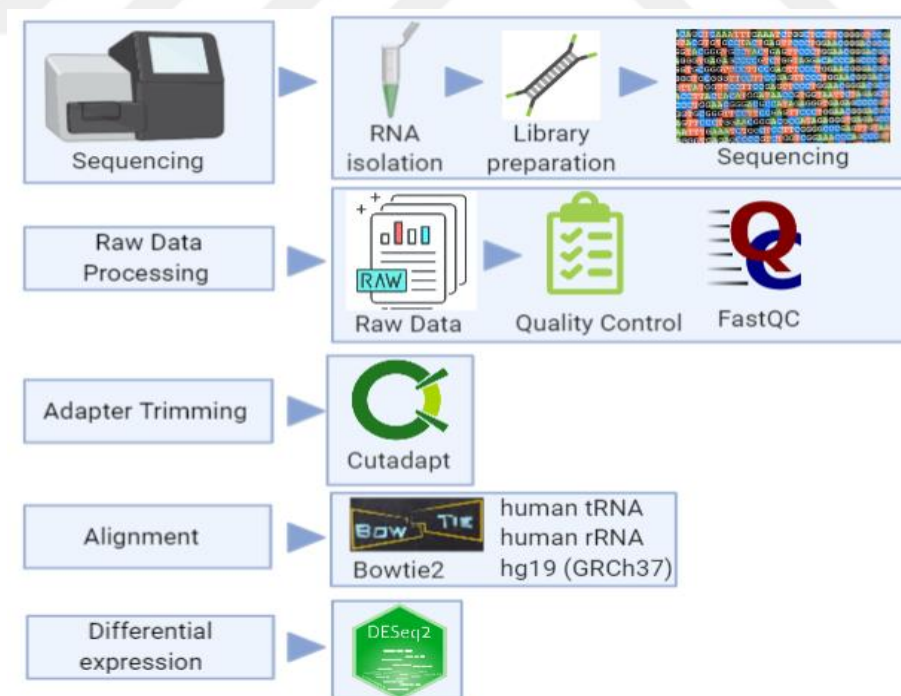


Figure 3.3 : The pipeline of small RNA sequencing of wt and Bag-1 KO MCF7 samples. The scheme illustrated the five steps of RNA sequencing analysis: the quality control of raw reads, the adapter trimming, alignment, annotation, and differential expression of miRNAs in Bag-1 KO samples compared to wt cells.

3.5.1.1 Quality control of raw reads of two replicated wt and Bag-1 KO samples by FASTQC

The data were uploaded to the Galaxy open web platform and analyzed from usegalaxy.org. Following sequencing, it is required to perform quality control of millions of raw sequences before processing further analysis. FastQC is a tool that provides a quality control report per sequence quality scores, sequence-based quality scores, sequence GC and Kmer content, duplicate sequences, overrepresented sequences, and overall sequence length distribution read distribution plots. According to the basic statistical report, in the first replicate of sequencing results, a total number of sequences in wt and 2387018 sequences in Bag-1 KO were processed. In the second replicate of the sequencing results total of 3021275 sequences in wt and 3713839 sequences in Bag-1 KO were processed. The shortest sequence length was 136 and the longest sequence length was 151 in all processed sequences in MCF-7 wt cells. Similarly, in Bag-1 KO sequences the shortest was 137 and the longest was 151 sequences length. The overall GC% content of all bases was 50% (replicate 1) and 53% (replicate 2) in all wt and 54% (replicate 1) and 56% (replicate 2) in Bag-1 KO sequences (Figure 3.4).

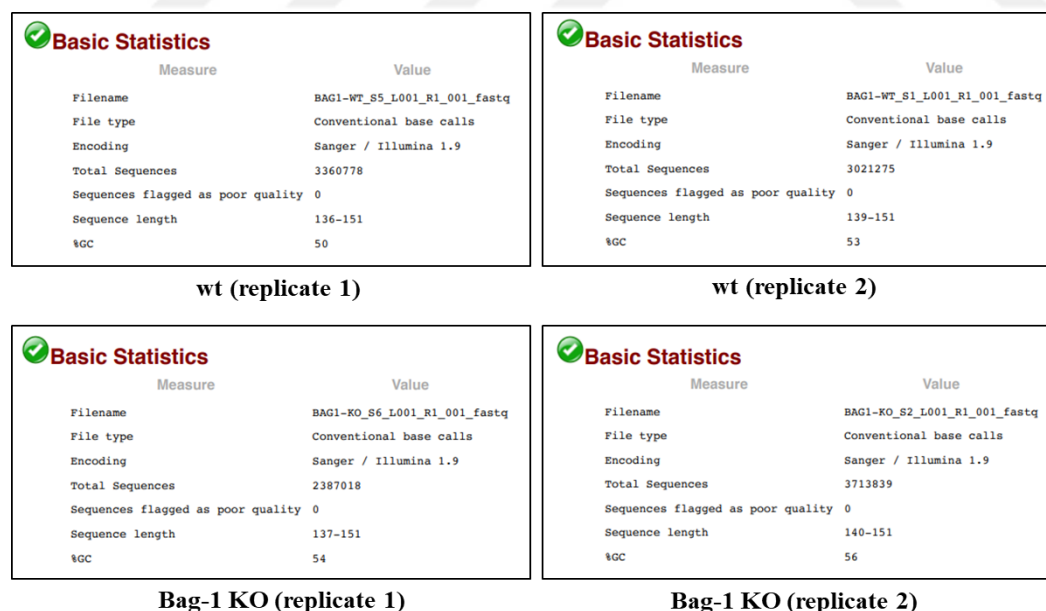


Figure 3.4 : The basic statistics reports of small RNA sequencing of two replicates of wt and Bag-1 KO MCF-7 cells. The total sequenced reads, length, and GC% rate is the main output of the FASTQC report.

The per base sequence quality report showed the overview of the quality values across all bases. The y-axis showed the quality scores and the higher score represented the

better base calls. The good-quality bases are represented in the green area, the reasonable calls are shown in the orange area and poor-quality calls are depicted in the red area. The quality of calls is decreased towards the end of the reads due to the degradation of bases. The per-base quality reports of two replicated wt and Bag-1 KO samples showed that the quality score of all bases in wt at 28-38% quality score and in bases Bag-1 KO at 32-38% quality score and both appeared in the very good quality scores green area. The per sequence quality scores show the low-quality values and the error rate is 0.2% if the mean quality is below 27 and the error rate is 1% if the mean quality is below 20. Our results showed that the mean quality per reading in both wt and Bag-1 KO sequences was above 27 which means the error rate was below 0.2% (Figure 3.5).

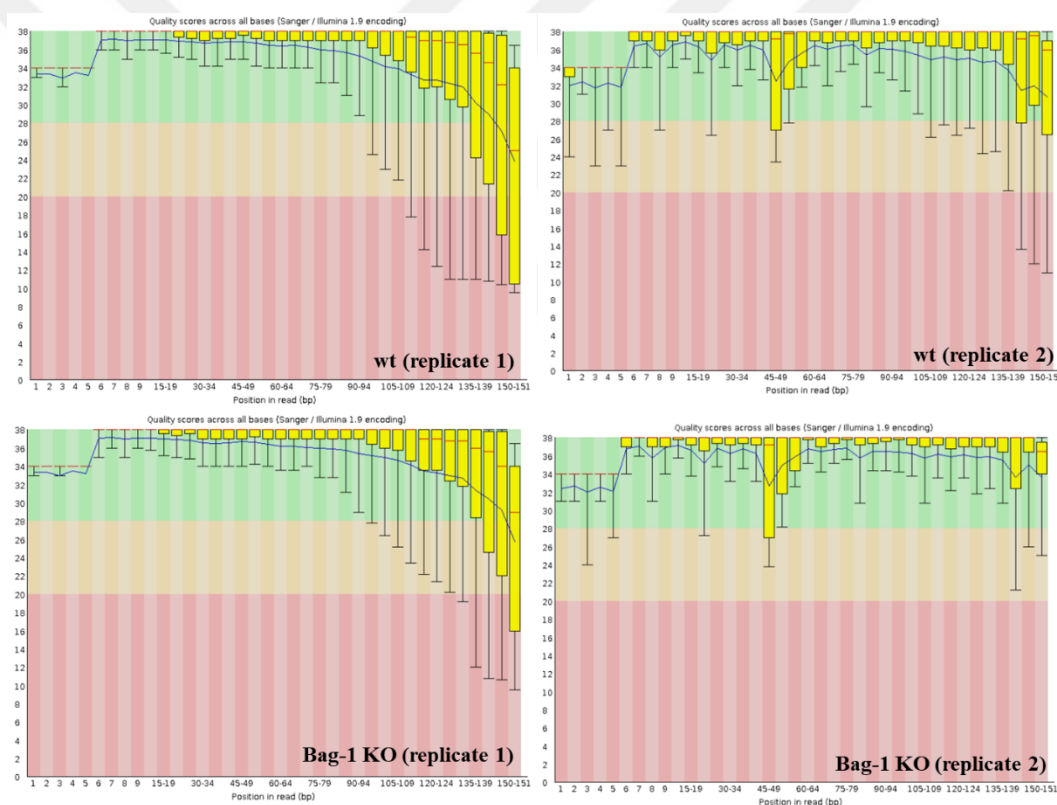


Figure 3.5 : The per base sequence quality report of small RNA sequencing of two replicates of wt and Bag-1 KO MCF-7 cells. The bar graphs illustrated the quality scores across all bases.

3.5.1.2 Trimming adapter sequences from all reads by Cutadapt

The sequenced reads start with the 5' adapter sequencing and contain the 3' adapter sequence. Normally, mature miRNAs are 25 bp in length therefore trimming process is required to remove adapter sequences. Cutadapt tool removes adapter sequences

from high-throughput sequences by searching the adapter sequences in all reads. The trimmed reads should be at a minimum of 18 bp for reliable analysis.

In our results, the adapter sequence 5'-TGGAATTCTCGGGTGCCAAGGAACCTCCAGTCAC-3' was removed by Cutadapt selecting the sequence length 18-35 bp. The quality of trimmed reads was controlled by FastQC and according to the basic statistical analysis 198187 in replicate 1 and 95336 reads in replicate 2 in wt samples, and 52703 reads in replicate 1 and 33134 reads in replicate 2 in Bag-1 KO samples were sequenced between an 18-35 bp with a miRNA length.

3.5.1.3 Alignment of trimmed reads to hg19 human genome by Bowtie2

The trimmed and processed reads of wt and Bag-1 KO bases were sequentially aligned to other types of RNAs such as; tRNA and rRNA with the Bowtie2 aligner program. Following Cutadapt trimming processes, the alignment of 198187 reads of wt bases with human tRNA showed a 0.0% overall alignment rate and showed 0.02% overall alignment rate with human rRNA. 52703 Bag-1 KO reads showed a 0.00% alignment ratio with tRNA and a 0.03% alignment score with rRNA.

The unaligned reads of 198187 wt bases and also unaligned reads of 52703 Bag-1 KO bases with tRNA and rRNA were aligned with the reference genome GRCh37-hg19. Overall 95.94% alignment ratios were found in wt reads and 93.97% alignment ratios were found in Bag-1 KO reads with the alignment of the hg19 genome.

3.5.1.4 Annotation of sequence counts by featureCounts

Following mapping the reads using Bowtie2, the read counts are required for each sample. Therefore, featureCounts were used to count the number of reads mapped to human miRNA sequences using the gff file. The output of the featureCounts read counting program is a table that contains table reads, per gene and row. In our results, we aligned the reads that were obtained from Bowtie2 with human mature miRNA sequences provided by miRBase online web tool. Aligned reads of two replicated wt Bag-1 KO samples were annotated to mature human miRNAs using the featureCounts tool. The sequence counts for 2576 miRNAs of two biological replicates of wt and Bag-1 KO samples annotated in the hg19. Considering our samples, The 144 miRNAs were found in either in two replicated wt or Bag-1 KO samples (Appendix F), 88 miRNAs were only present in two replicated wt samples (Appendix G), and 71

miRNAs were only present in two replicated Bag-1 KO samples (Appendix H) with at least 1 TPM (transcripts per million).

3.5.1.5 Identification of differentially expressed miRNAs in wt and Bag-1 KO samples by DESeq2 analysis

For the determination of differential expression profiles of miRNA reads that were obtained from alignment results, DESeq2 is used to perform normalized counts. DESeq2 is a test for analyzing the differential expression of count data from high-throughput sequencing assays based on variance-mean dependence using the negative binomial distribution. The output gives the fold changes (log2) of genes based on the factors (untreated/treated) and generates a tabular file. In our study, “Bag-1 KO” and “wt” factor names were given to compare the fold changes of “Bag-1KO samples” against “wt” samples and according to these comparisons total of 2576 known miRNA was found differentially expressed. To analyze differential expressions of 2576 miRNA counts obtained from featureCounts annotation, DESeq2 differential expression analysis was performed. The mean average (MA) plot of differentially expressed miRNAs between Bag-1 KO and wt MCF-7 samples were shown in Figure 3.6.

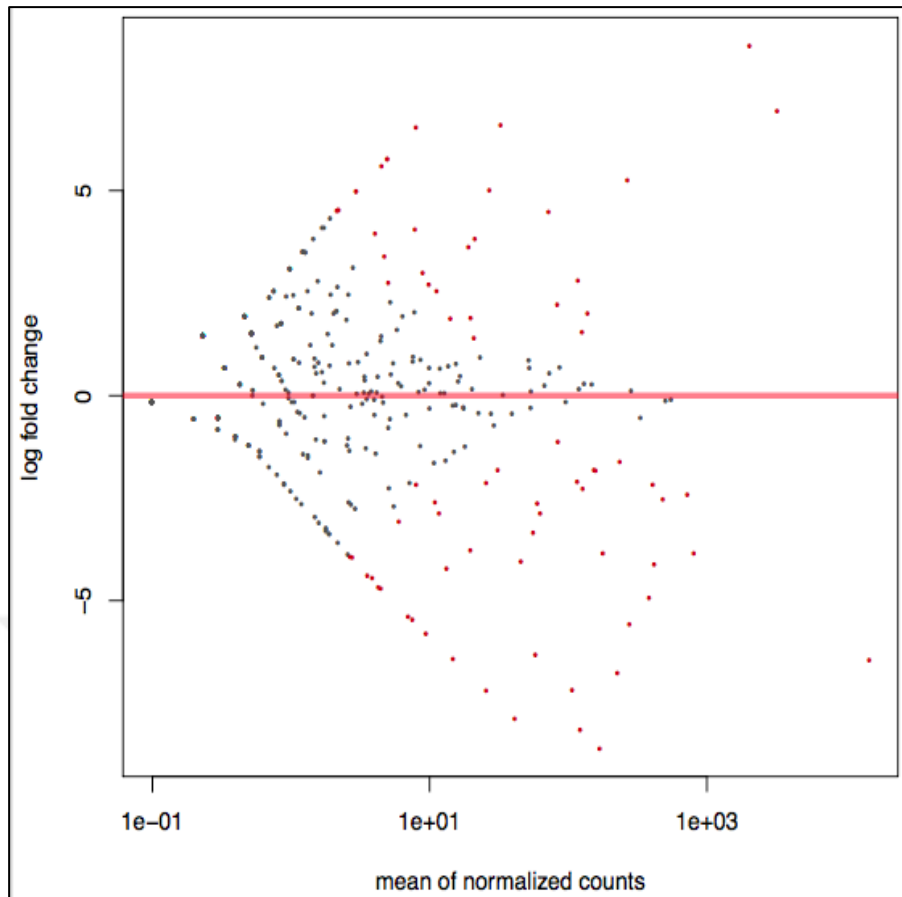


Figure 3.6 : MA plot of differentially expressed miRNAs between Bag-1 KO and wt MCF-7 samples. Y-axis showed the log₂ fold change, and the x-axis showed the average expression. Red dots represented the differentially expressed miRNAs, and gray dots were shown in no change. Negative values of fold changes represented downregulated, positive values represented upregulated miRNAs.

Following the Benjamini-Hochberg correction method, False Discovery Rates (FDR) ≤ 0.01 was set to obtain significantly differentially expressed miRNAs, and with this correction method, 25 known miRNAs were found significantly differentially expressed in Bag-1 KO reads comparing wt reads. According to the comparison results in Bag-1 KO and wt MCF-7 cells, while 14 different miRNA targets were downregulated in Bag-1 KO cells, 11 of the significantly upregulated miRNA targets were determined (Table 3.2).

Table 3.2 : Significantly differentially expressed miRNAs in Bag-1 KO samples compared to wt samples.

Downregulated miRNAs			Upregulated miRNAs		
miRNA ID	Log ₂ fold change	p-value	miRNA ID	Log ₂ fold change	p-value
hsa-miR-21-3p	-6,767	4,97E-11	hsa-miR-92a-3p	1,895	0.013
hsa-miR-21-5p	-6,447	6,37E-248	hsa-miR-30a-3p	2,559	0.008
hsa-miR-429	-5,805	0.000	hsa-miR-130b-3p	2,709	0.008
hsa-miR-296-3p	-5,466	0.001	hsa-miR-148a-5p	2,99	0.006
hsa-miR-200a-5p	-5,388	0.002	hsa-miR-4521	4,055	0.001
hsa-miR-27a-3p	-4,218	0.001	hsa-miR-873-5p	5,602	0.004
hsa-let-7b-5p	-4,115	1,25E-21	hsa-miR-767-5p	5,771	0.002
hsa-let-7i-5p	-3,838	5,99E-28	hsa-miR-887-3p	5,771	0.002
hsa-miR-342-5p	-3,772	0.000	hsa-miR-31-5p	6,539	0.000
hsa-miR-342-3p	-2,875	0.008	hsa-miR-10a-5p	6,939	6,09E-133
hsa-miR-345-5p	-2,131	0.003	hsa-miR-10b-5p	8,538	1,83E-111
hsa-miR-151a-3p	-1,811	0.006			
hsa-miR-98-5p	-4,938	3,55E-26			
hsa-miR-146b-5p	-1,123	0.010			

3.5.1.6 Determination of predicted target genes of differentially expressed miRNAs in wt and Bag-1 KO samples

To determine the potential functions of differentially expressed miRNAs and further explore miRNAs associated with target genes controlling molecular pathways, in silico DIANA-miRPath v3.0 miRNA target prediction algorithm was performed incorporating KEGG and GO functional enrichment analysis.

3.5.1.7 KEGG pathway enrichment analysis

We explored the potential mRNA targets and significantly enriched pathways of all downregulated 14 miRNAs and 11 upregulated miRNAs in Bag-1KO cells compared with wt MCF-7 cells.

According to KEGG pathway analysis of 14 downregulated miRNA targets, we found that signaling pathways regulating the metabolism of xenobiotics by cytochrome P450, glycosphingolipid biosynthesis, ErbB signaling, lysine degradation, Mucin type O-Glycan biosynthesis, proteoglycans in cancer, the pluripotency of stem cells, TGF- β signaling, glycosaminoglycan biosynthesis, PI3K/Akt signaling mechanism, ECM-receptor interaction, and amoebiasis were targeted in Bag-1KO MCF-7 samples

compared to wt samples. The pathway enrichment ratio increased from yellow to red, and the red color represented the higher ratio of enrichment (Figure 3.7).

In table 2.7. the detailed KEGG pathway enrichment analysis was shown with the name of targeted miRNAs and, the name and number of miRNA-associated mRNA targets. Considering the detailed KEGG analysis, downregulated miRNA-associated 95 target genes as the highest number were involved in PI3K/Akt signaling pathway. Then, proteoglycans in cancer were found as the second enriched pathway with 57 target gene involvement. 44 genes were involved in the pluripotency of stem cell signaling pathways, 30 genes were involved in ErbB signaling, and 26 genes were involved in ECM-receptor interaction in Bag-1 KO MCF-7 samples compared to wt samples (Figure 3.8).



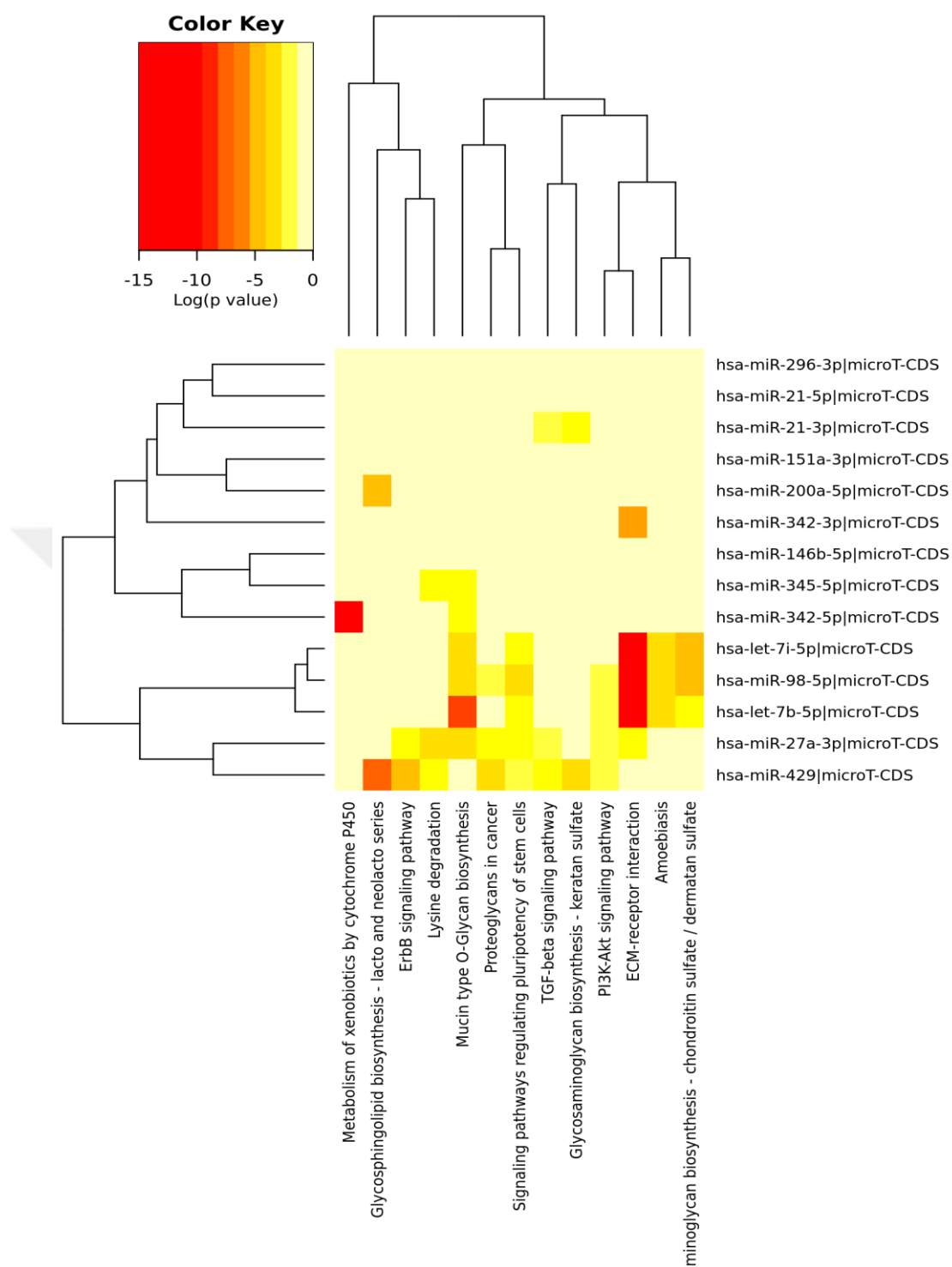


Figure 3.7 : KEGG Pathway enrichment analysis results were extracted from DIANA-miRPath v.3.0 for downregulated 14 miRNA targets in Bag-1 KO MCF-7 samples compared to wt MCF-7 samples based on the microT-CDS (v.5.0) miRNA input. Red colors represented the higher level of pathway enrichment of the miRNA target genes.

Table 3.3 : Enriched KEGG pathways of miRNAs and target genes of 14 downregulated miRNAs.

Downregulated miRNAs				
KEGG pathway	p-value	Gene number	target genes	miRNA number miRNA ID
ECM-receptor interaction	0,00	26	ITGB8, LAMB1, SV2B, THBS1, ITGA8, COL24A1, COL27A1, ITGB6, AGRN, ITGA5, ITGA1, COL3A1, SV2A, RELN, COL1A1, ITGA2, COL11A2, COL1A2, ITGA7, THBS3, COL4A6, SV2C, FN1, TNF, COL5A2, COL4A1	5 hsa-miR-27a-3p hsa-let-7b-5p hsa-let-7i-5p hsa-miR-342-3p hsa-miR-98-5p
Mucin type O-Glycan biosynthesis	4,8E-05	11	GALNT7, GALNTL6, GCNT4, GALNT1, GALNT3, GALNT14, GALNT2, C1GALT1, GALNT12, GALNT5, GALNT16	6 hsa-miR-27a-3p hsa-let-7b-5p hsa-let-7i-5p hsa-miR-342-5p hsa-miR-345-5p hsa-miR-98-5p
Signaling Pathways regulating pluripotency of stem cells	2,1E+01	44	BMI1, FZD7, GSK3B, DVL3, WNT16, PAX6, SMAD2, NRAS, SMAD9, APC, HOXB1, ACVR1B, MAPK14, HAND1, SMARCA4, INHBA, IGF1R, ZFXH3, KRAS, FZD3, ACVR2B, FZD4, RIF1, SMAD4, LIFR, SKIL, SMAD5, ACVR2A, ACVR1C, IGF1, AKT3, BMPR1A, PIK3CA, IL6ST, ISL1, ID3, DUSP9, SOX2, PCGF3, GRB2, DVL2, KLF4, WNT9A, BMPR2	5 hsa-miR-429 hsa-miR-27a-3p hsa-let-7b-5p hsa-let-7i-5p hsa-miR-98-5p
Metabolism of xenobiotics by cytochrome P450	0,00	2	GSTM3, GSTM2	1 hsa-miR-342-5p
Glycosphingolipid biosynthesis Lacto and neolacto series	0,00	4	FUT4, B3GNT1, FUT9, B3GNT2	2 hsa-miR-429 hsa-miR-200a-5p
Proteoglycans in cancer	0,00	57	FZD7, CAMK2D, BRAF, ACTB, HBEGF, WNT16, MET, SMAD2, CBL, NRAS, THBS1, PTCH1, PPP1CC, MAPK14, PXN, ROCK2, FRS2, RDX, ITGA5, IGF1R, EGFR, KRAS, FZD3, RRAS2, MSN, RPS6KB2, COL21A1, VAV2, PTK2, CBLB, ANK3, FZD4, PPP1R12A, PLCG1, CASP3, FLNA, ITGA2, SOS1, PTPN11, PRKX, RAC1, PRKCB, FAS, IGF1, GAB1, AKT3, PIK3CA, SDC2, FN1, CDKN1A, PDPK1, VEGFA, KDR, GRB2, WNT9A, ERBB4, RPS6KB1	3 hsa-miR-429 hsa-miR-27a-3p hsa-miR-98-5p
Glycosaminoglycan biosynthesis chondroitin sulfate dermatan sulfate	0,00	4	UST, CHST3, CHSY3, XYLT2	3 hsa-let-7b-5p hsa-let-7i-5p hsa-miR-98-5p
Amoebiasis	0,01	11	ARG2, COL27A1, COL3A1, CASP3, COL1A, COL1A2, COL4A6, IL10, COL5A, PLCB4, COL4A1	3 hsa-let-7b-5p hsa-let-7i-5p hsa-miR-98-5p

Table 3.3 (continued): Enriched KEGG pathways of miRNAs and target genes of 14 downregulated miRNAs.

ErbB signaling pathway	0,01	30	CAMK2D, BRAF, GSK3B, HBEGF, CBL, CRKL, PAK2, MAP2K7, PAK7, CDKN1B, EGFR, KRAS, PTK2, CBLB, PLCG1, JUN, SOS1, PAK6, NRG1, PRKCB, GAB1, SHC4, AKT3, PIK3CA, MAP2K4, ABL2, GRB2, ERBB4, MAPK10, RPS6KB1	2	hsa-miR-429 hsa-miR-27a-3p
Lysine degradation	0,02	15	CAMKMT, WHSC1L1, SETD7, ALDH3A2, SETD2, NSD1, ASH1L, COLGALT2, SUV420H1, DOT1L, WHSC1, SUV420H2, EHHADH, KMT2E, KMT2C	3	hsa-miR-429 hsa-miR-27a-3p hsa-miR-345-5p
Glycosaminoglycan biosynthesis keratan sulfate	0,03	6	ST3GAL1, B4GALT1, B3GNT1, ST3GAL2, CHST2, B3GNT2	2	hsa-miR-21-3p hsa-miR-429
PI3K-Akt signaling pathway	0,04	95	PHLPP2, PRLR, GSK3B, TSC1, PDGFRA, GNG13, PPP2R5E, MET, MYB, ITGB8, LAMB1, FGF14, NRAS, PRKAA2, THBS1, PPP2R2C, ITGA8, CREB5, SYK, COL24A1, YWHAG, CDK2, CCND2, COL27A1, ITGB6, IL7, ITGA5, CDKN1B, YWHAB, MTCP1, EFNA3, IGF1R, EGFR, ITGA1, KRAS, CDK6, COL3A1, LPAR6, YWHAQ, RPS6KB2, GHR, CREB1, IKBKB, FGF11, PTK2, BRCA1, THEM4, CCNE2, RELN, COL1A1, FLT1, VEGFC, COL4A3, ITGA2, SOS1, FGF9, KITLG, COL11A2, IRS1, RAC1, INSR, CHRM2, FGF18, NGF, COL1A2, LAMC1, IGF1, ITGA7, BCL2L1, AKT3, EIF4E2, CREB3L2, PIK3CA, COL4A6, FN1, PKN2, CDKN1A, TNF, PDK1, VEGFA, OSMR, PPP2R1B, KDR, CSF1, GNG5, GRB2, COL5A2, GNB4, NGFR, BCL2L11, RPS6KB1, COL4A1, CHRM1, IL6R, EFNA1	4	hsa-miR-429 hsa-miR-27a-3p hsa-let-7b-5p hsa-miR-98-5p
TGF- β signaling pathway	0,04	20	SMAD2, SMAD9, SMAD3, INHBA, ACVR2B, SMAD4, SMAD5, ACVR2A, GDF6, SP1, ACVR1C, EP300, BMPR1A, IFNG, LTBP1, ID3, NOG, PPP2R1B, BMPR2, RPS6KB1	3	hsa-miR-21-3p hsa-miR-429 hsa-miR-27a-3p

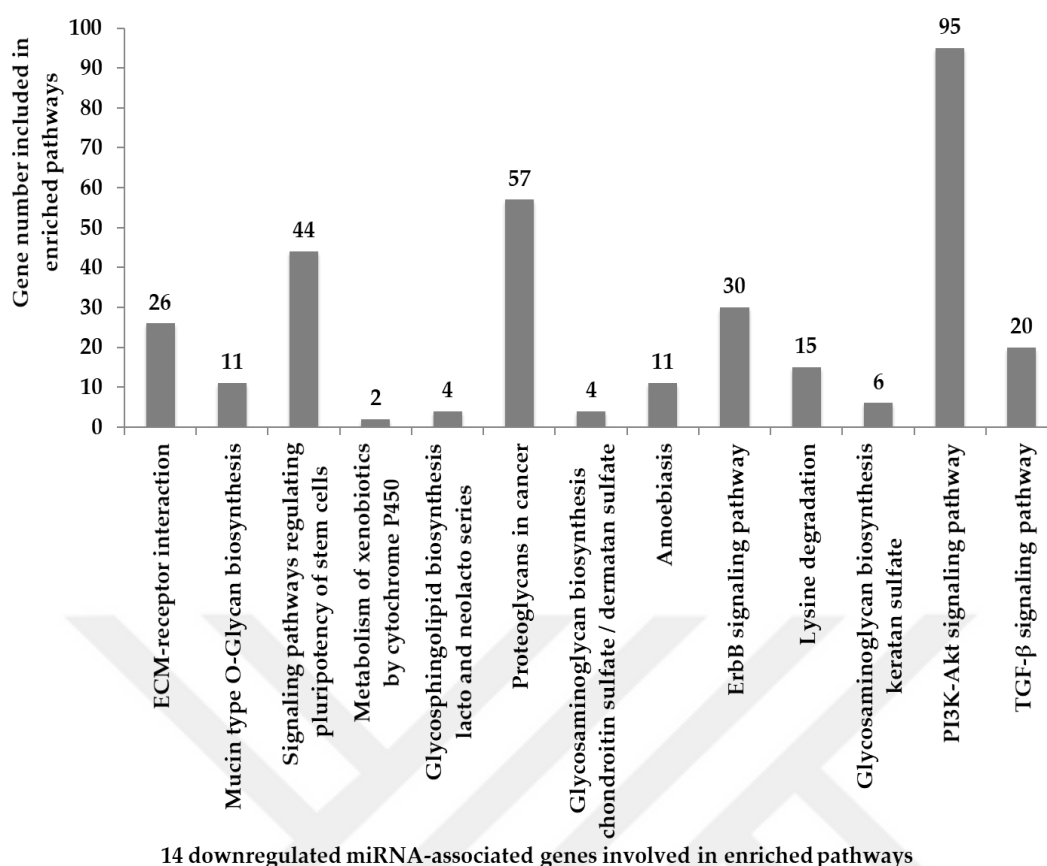


Figure 3.8 : Downregulated miRNA target gene numbers involved in enriched pathways were extracted from DIANA-miRPath v.3.0 for downregulated 14 miRNA targets in Bag-1 KO MCF-7 samples compared to wt MCF-7 samples based on the microT-CDS (v.5.0) miRNA input.

According to the KEGG pathway enrichment results of upregulated 11 miRNAs, morphine addiction, ECM-receptor interaction, mucin-type O-Glycan biosynthesis, and metabolism of xenobiotics by cytochrome p450, TGF-β signaling pathway, and prion diseases pathways were found highly enriched (Figure 3.9). Enriched KEGG pathways, differentially expressed miRNAs, and miRNA-associated target genes of 11 upregulated miRNAs were shown in Table 2.8. However, it was observed that the number of enriched pathways of the upregulated miRNA-targeted genes was small compared to the downregulated miRNA-targeted genes enriched pathways. ECM-receptor interaction was highly enriched with 18 genes, then, TGF-β signaling pathways were the second highly enriched pathway with 16 genes associated with upregulated miRNAs (Figure 3.10).

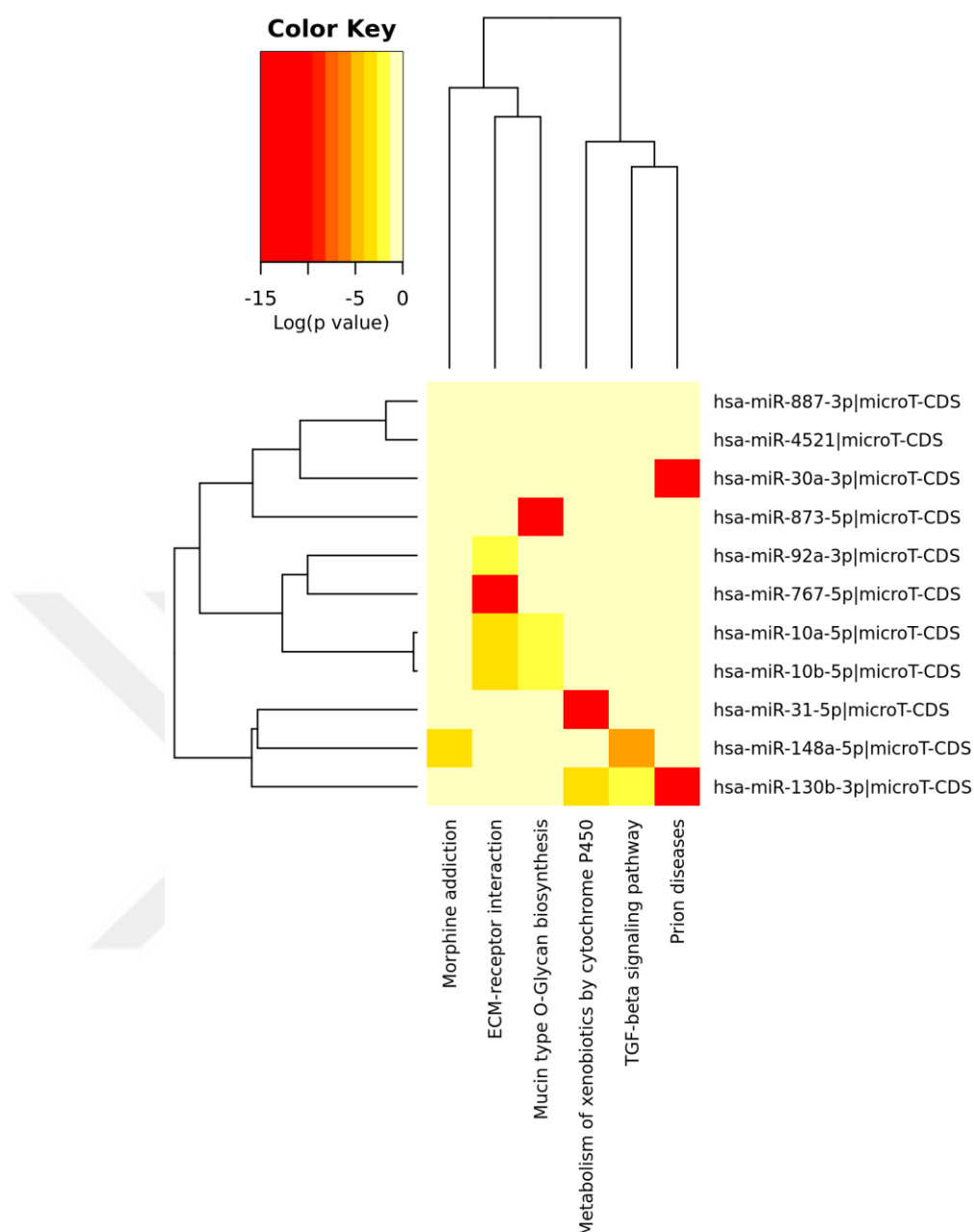


Figure 3.9 : KEGG Pathway enrichment KEGG analysis results were extracted from DIANA-miRPath v.3.0 for upregulated 11 miRNA targets in Bag-1 KO MCF-7 samples compared to wt MCF-7 samples based on the microT-CDS (v.5.0) miRNA input. Red colors represented the higher level of pathway enrichment of the miRNA target genes.

Table 3.4 : Enriched KEGG pathways, differentially expressed miRNAs, and miRNA-associated target genes of 11 upregulated miRNAs.

Upregulated miRNAs					
KEGG pathway	p-value	gene number	target genes	miRNA number	miRNA ID
Prion diseases	<1e-325	1	PRNP	2	hsa-miR-30a-3p hsa-miR-130b-3p
Mucin type O-Glycan biosynthesis	<1e-325	7	POC1B-GALNT4, GALNTL6, GALNT13, GALNT4, GALNT8, GALNT1, ST6GALNAC1	3	hsa-miR-873-5p hsa-miR-10a-5p hsa-miR-10b-5p
ECM-receptor interaction	<1e-325	18	SDC1, ITGA8, COL4A5, COL24A1, COL27A1, ITGA5, COL3A1, ITGAV, COL2A1, COL5A1, COL4A4, COL1A2, COL11A1, COL6A3, ITGA6, COL5A3, COL4A1, ITGB3	4	hsa-miR-92a-3p hsa-miR-767-5p hsa-miR-10a-5p hsa-miR-10b-5p
Metabolism of xenobiotics by cytochrome P450	3,4E+00	6	GSTM5, MGST1, GSTM4, ADH4, GSTK1, CYP1B1	2	hsa-miR-130b-3p hsa-miR-31-5p
Morphine addiction	0.01	7	ADRBK2, GABRG2, PDE10A, GNAI3, GNB1, PDE4D, KCNJ6	1	hsa-miR-148b-5p
TGF-beta signaling pathway	0.03	16	FST, SMAD2, INHBB, SMURF2, SMAD3, INHBA, ID4, ACVR1, SKP1, MYC, ZFYVE9, SMAD5, ACVR1C, TGFB2, TGFB2R2	2	hsa-miR-130b-3p hsa-miR-148b-5p

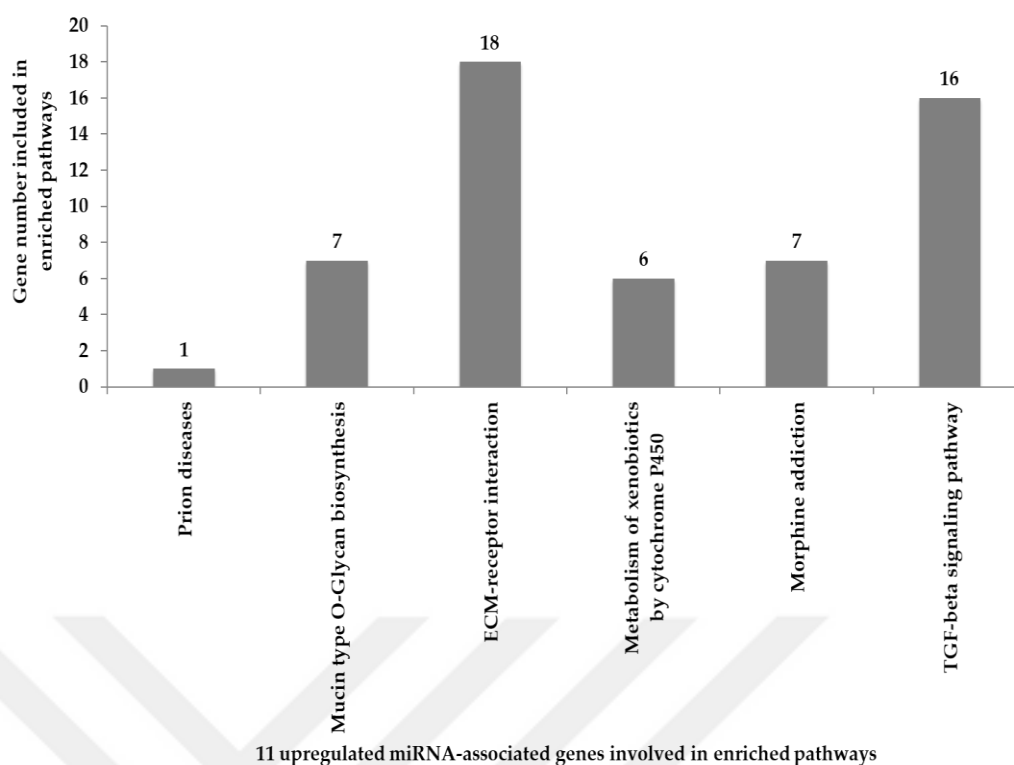


Figure 3.10 : Upregulated miRNA target gene numbers involved in enriched pathways were extracted from DIANA-miRPath v.3.0 for upregulated 11 miRNA targets in Bag-1 KO MCF-7 samples compared to wt MCF-7 samples based on the microT-CDS (v.5.0) miRNA input.

3.5.1.8 GO functional annotation analysis

The significance of downregulated and upregulated miRNAs was analyzed using GO analysis in DIANA-miRPath v.3.0 by examining biological processes, molecular functions, and cellular components. The downregulated 14 miRNAs were enriched in biological processes involving nucleic acid binding transcription factor activity, cellular component, protein binding transcription factor activity, gene expression, Fc-epsilon receptor signaling pathway, neurotrophin TRK receptor signaling pathway, cellular protein modification process, biosynthetic process, epidermal growth factor receptor signaling pathway, response to stress, cytoskeletal protein binding, extracellular matrix disassembly and organization and cell-cell signaling (Figure 3.11).

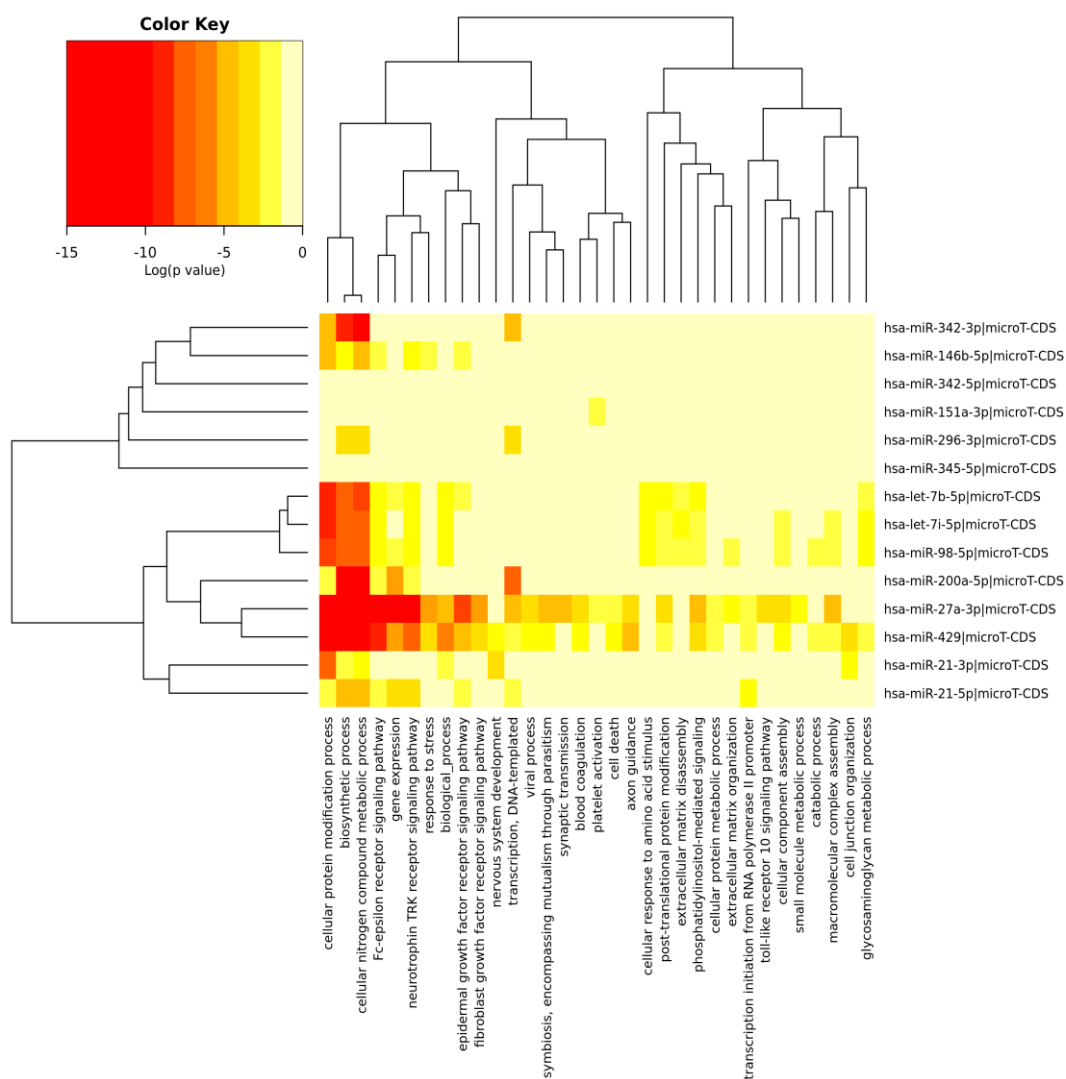


Figure 3.11 : GO enrichment analysis of 14 downregulated miRNAs at biological process category.

The detailed GO functional annotation analysis table showed the biological process of enriched pathways based on p-values and the number of miRNA and miRNA-associated gene targets (Table 2.9).

Table 3.5 : The detailed GO functional enrichment analysis at biological process category.

Downregulated miRNAs			
GO Category	p-value	genes	miRNAs
nucleic acid binding transcription factor activity	<1e-325	218	5
cellular_component	<1e-325	2591	6
protein binding transcription factor activity	<1e-325	133	7
gene expression	<1e-325	133	7
Fc-epsilon receptor signaling pathway	<1e-325	62	8
neurotrophin TRK receptor signaling pathway	<1e-325	81	8
molecular_function	<1e-325	3335	10
The cellular protein modification process	<1e-325	622	10
biosynthetic process	<1e-325	956	11
cellular nitrogen compound metabolic process	<1e-325	1096	11
ion binding	<1e-325	1471	12
organelle	<1e-325	2233	13
transcription DNA-templated	1,38E-13	447	6
biological_process	1,34E-12	2494	6
enzyme binding	1,45E-12	212	3
epidermal growth factor receptor signaling pathway	2,27E-11	60	7
protein complex	1,41E-07	487	3
response to stress	3,20E-07	320	3
nucleoplasm	1,06E-05	188	4
fibroblast growth factor receptor signaling pathway	1,07E-05	39	2
cytosol	1,60E-05	346	2
post-translational protein modification	1,79E-05	38	5
cellular component assembly	2,70E-05	195	5
cytoskeletal protein binding	0.0001531536	110	2
axon guidance	0.0002622114	84	2
symbiosis encompassing mutualism through parasitism	0.0002916611	72	2
blood coagulation	0.0004116226	64	2
enzyme regulator activity	0.000470201	69	1
macromolecular complex assembly	0.0008923938	138	4
glycosaminoglycan metabolic process	0.002947115	18	4
viral process	0.004114641	63	2
synaptic transmission	0.005407267	43	1
nervous system development	0.007019097	56	2
RNA binding	0.007276568	127	1
cellular response to amino acid stimulus	0.007545761	11	3
cell death	0.008313612	121	2
cell junction organization	0.01001545	24	2
extracellular matrix disassembly	0.01119196	10	3
cellular protein metabolic process	0.01460577	57	2
catabolic process	0.01706224	168	2
extracellular matrix organization	0.02170607	55	2
Small-molecule metabolic process	0.0230613	151	1
transcription initiation from RNA polymerase II promoter	0.02326048	44	3
miRNA binding	0.02552213	7	3
toll-like receptor 10 signaling pathway	0.02979234	10	1
protein complex assembly	0.03265043	118	3
platelet activation	0.03764083	19	1
cell-cell signaling	0.04982579	81	2

The molecular functions of downregulated miRNAs were enriched in nucleic acid and protein binding transcription factor activity, ion binding, enzyme binding, cytoskeletal protein binding, enzyme regulator activity, RNA, and mRNA binding (Figure 3.12).

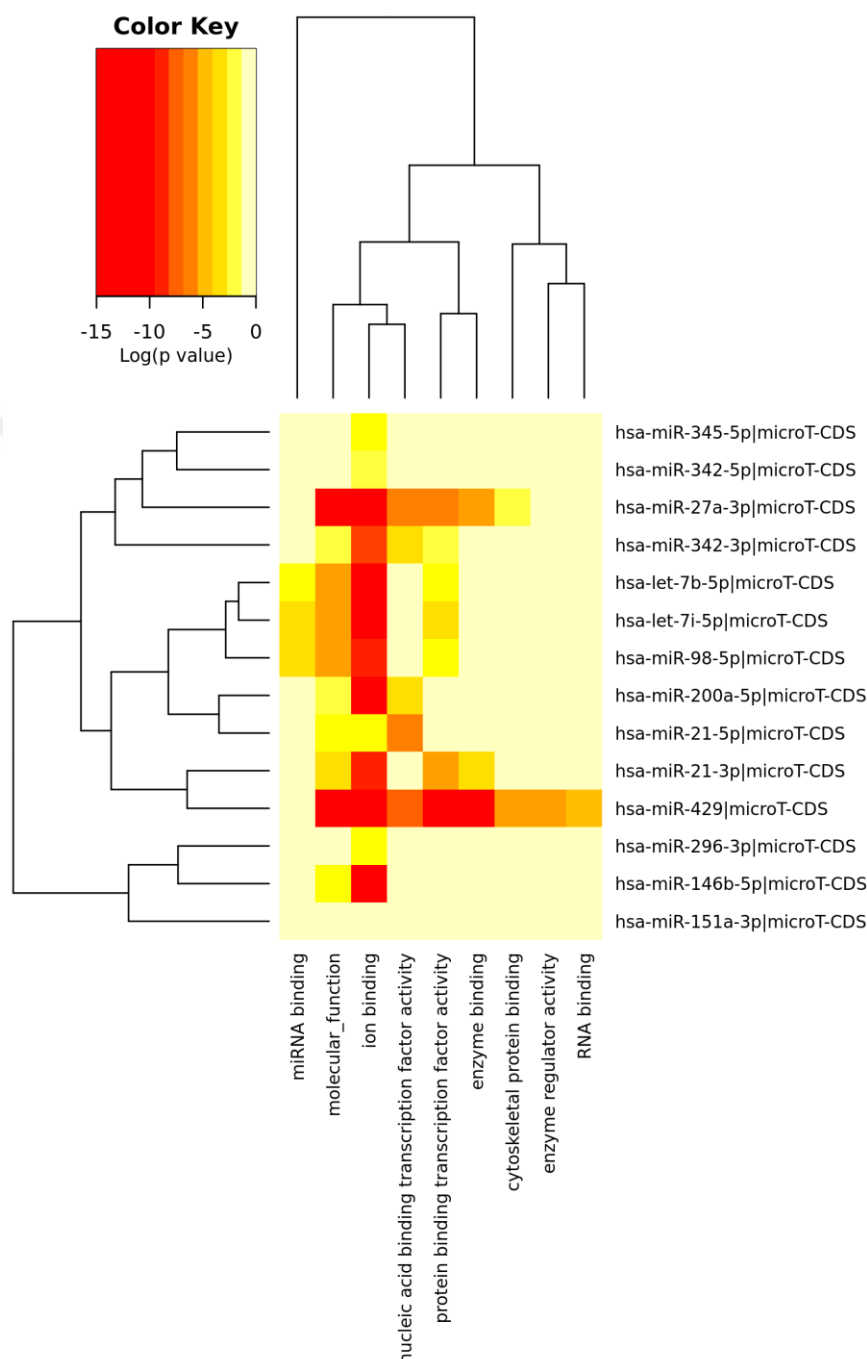


Figure 3.12 : GO enrichment analysis of 14 downregulated miRNAs at molecular function category.

The detailed molecular function category of GO annotation of 14 downregulated miRNA-associated targeted genes showed that nucleic acid and protein binding

transcription factor activity, ion and enzyme binding categories were highly enriched (Table 3.6).

Table 3.6 : The detailed GO functional enrichment analysis at molecular function category.

Downregulated miRNAs			
GO Category	p-value	genes	miRNAs
nucleic acid binding transcription factor activity	<1e-325	218	5
protein binding transcription factor activity	<1e-325	133	7
molecular_function	<1e-325	3335	10
ion binding	<1e-325	1501	13
enzyme binding	7,25E-13	212	3
cytoskeletal protein binding	0.0001950282	110	2
enzyme regulator activity	0.0005244524	69	1
RNA binding	0.009176097	127	1
miRNA binding	0.01865131	7	3

The enriched cellular components category of GO analysis of downregulated miRNAs include organelle, protein complex, nucleoplasm, and cytosol (Figure 3.13).

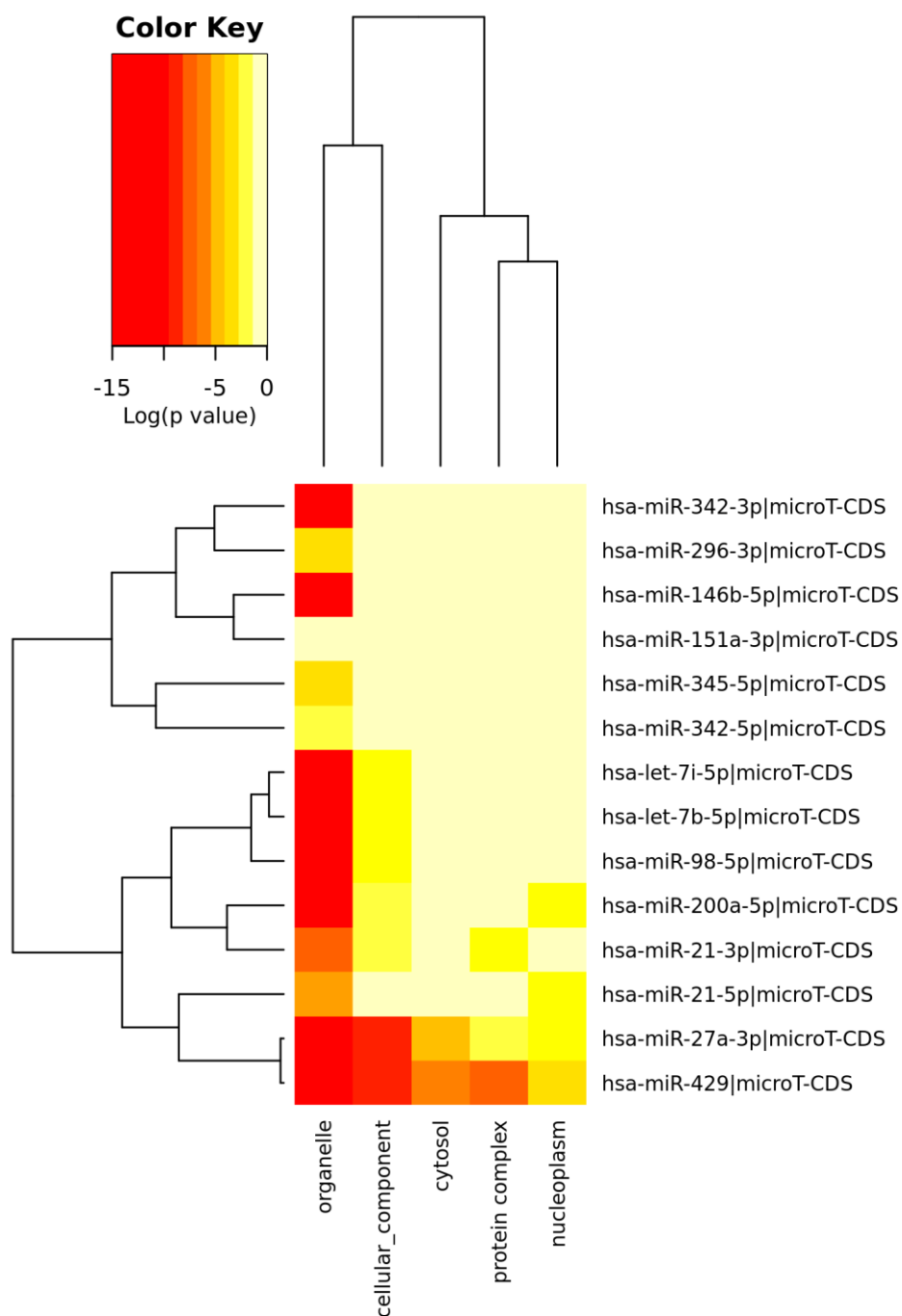


Figure 3.13 : GO enrichment analysis of 14 downregulated miRNAs at cellular component category.

The detailed cellular organization of GO analysis of downregulated miRNAs was shown in Table 3.7 based on the p-value, target genes, and miRNA numbers.

Table 3.7 : The detailed GO functional enrichment analysis at cellular organization category.

GO Category	Downregulated miRNAs		
	p-value	genes	miRNAs
cellular_component	<1e-325	2704	7
organelle	<1e-325	2233	13
protein complex	1,62E-06	487	3
nucleoplasm	2,76E-05	188	4
cytosol	0.0001237179	346	2

The biological process of upregulated 11 miRNAs was enriched in the biosynthetic process, cellular protein modification process, cellular nitrogen compound metabolic process, gene expression, phosphatidylinositol-mediated signaling, mitotic cell cycle, and cell junction organization (Figure 3.14)

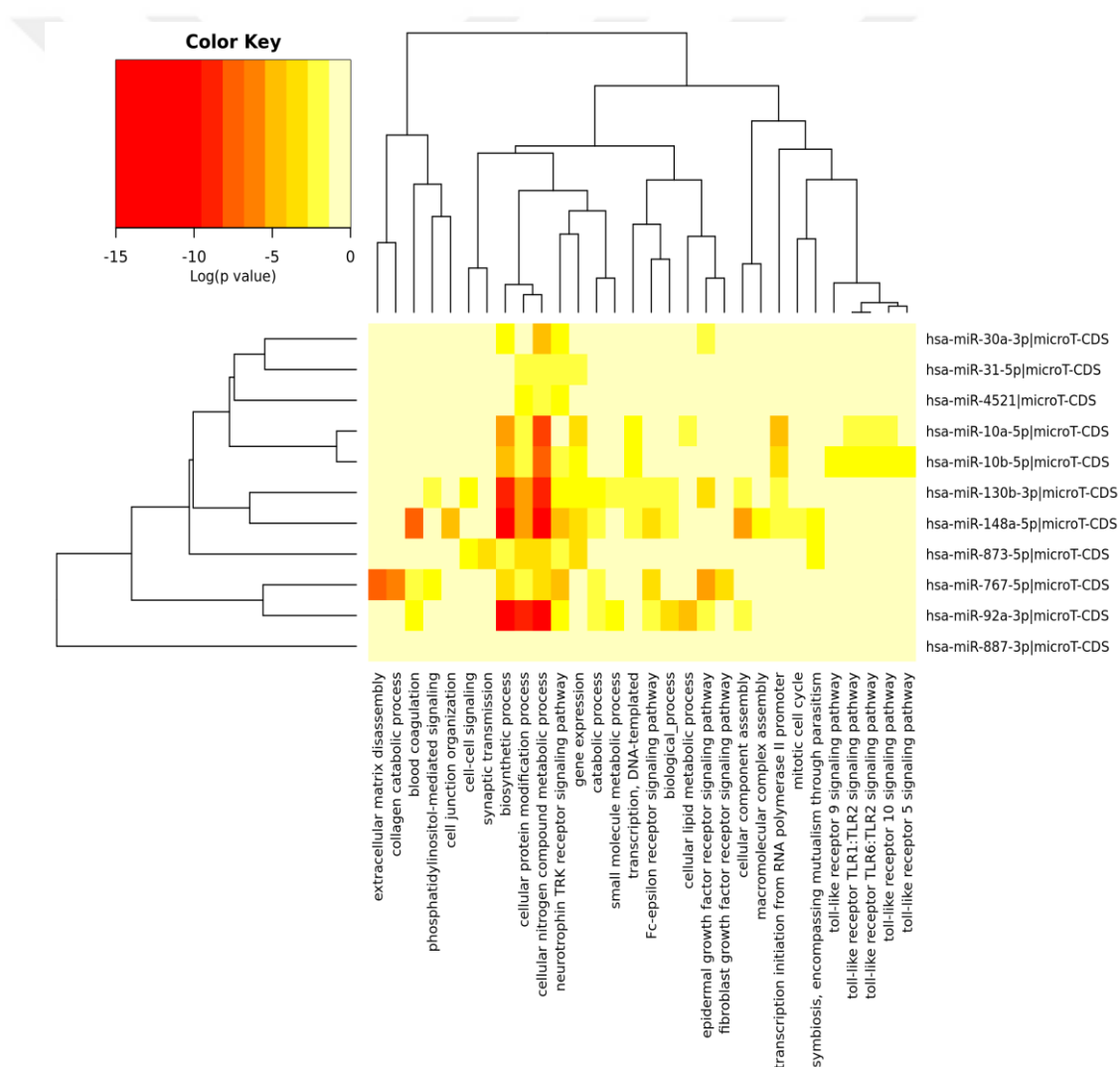


Figure 3.14 : GO enrichment analysis of 14 downregulated miRNAs at biological process category.

Table 3.8 represented the biological function of GO annotation of upregulated miRNA and miRNA-target gene numbers with p-values.

Table 3.8 : The detailed GO annotation of upregulated miRNA-target genes at the biological function level.

Upregulated miRNAs			
GO Category	p-value	genes	miRNAs
biosynthetic process	<1e-325	662	8
The cellular protein modification process	<1e-325	439	9
cellular nitrogen compound metabolic process	<1e-325	855	10
neurotrophin TRK receptor signaling pathway	1,91E-14	81	9
gene expression	4,35E-09	87	6
epidermal growth factor receptor signaling pathway	1,35E-06	43	4
Fc-epsilon receptor signaling pathway	1,42E-06	33	4
blood coagulation	6,66E-06	62	3
transcription initiation from RNA polymerase II promoter	2,31E-05	36	4
cellular component assembly	3,59E-05	149	3
catabolic process	0.0003791966	225	4
transcription DNA-templated	0.0005248616	201	4
biological_process	0.001100289	1502	3
cellular lipid metabolic process	0.001212784	20	2
extracellular matrix disassembly	0.002697809	13	1
Small-molecule metabolic process	0.003444109	165	2
cell-cell signaling	0.005410739	58	2
synaptic transmission	0.006088044	23	1
fibroblast growth factor receptor signaling pathway	0.007360359	12	1
collagen catabolic process	0.01246843	11	1
macromolecular complex assembly	0.01524561	40	1
phosphatidylinositol-mediated signaling	0.01807528	14	2
toll-like receptor TLR1:TLR2 signaling pathway	0.01911617	5	2
toll-like receptor TLR6:TLR2 signaling pathway	0.01911617	5	2
symbiosis encompassing mutualism through parasitism	0.01937155	42	2
toll-like receptor 10 signaling pathway	0.02065922	5	2
toll-like receptor 5 signaling pathway	0.02741777	5	1
toll-like receptor 9 signaling pathway	0.02833552	5	1
mitotic cell cycle	0.0373686	18	1
cell junction organization	0.04967624	15	1

The molecular function category of GO terms showed nucleic acid binding transcription factor activity, ion binding, protein binding transcription factor activity, and enzyme binding pathways of 11 upregulated miRNAs (Figure 3.15).

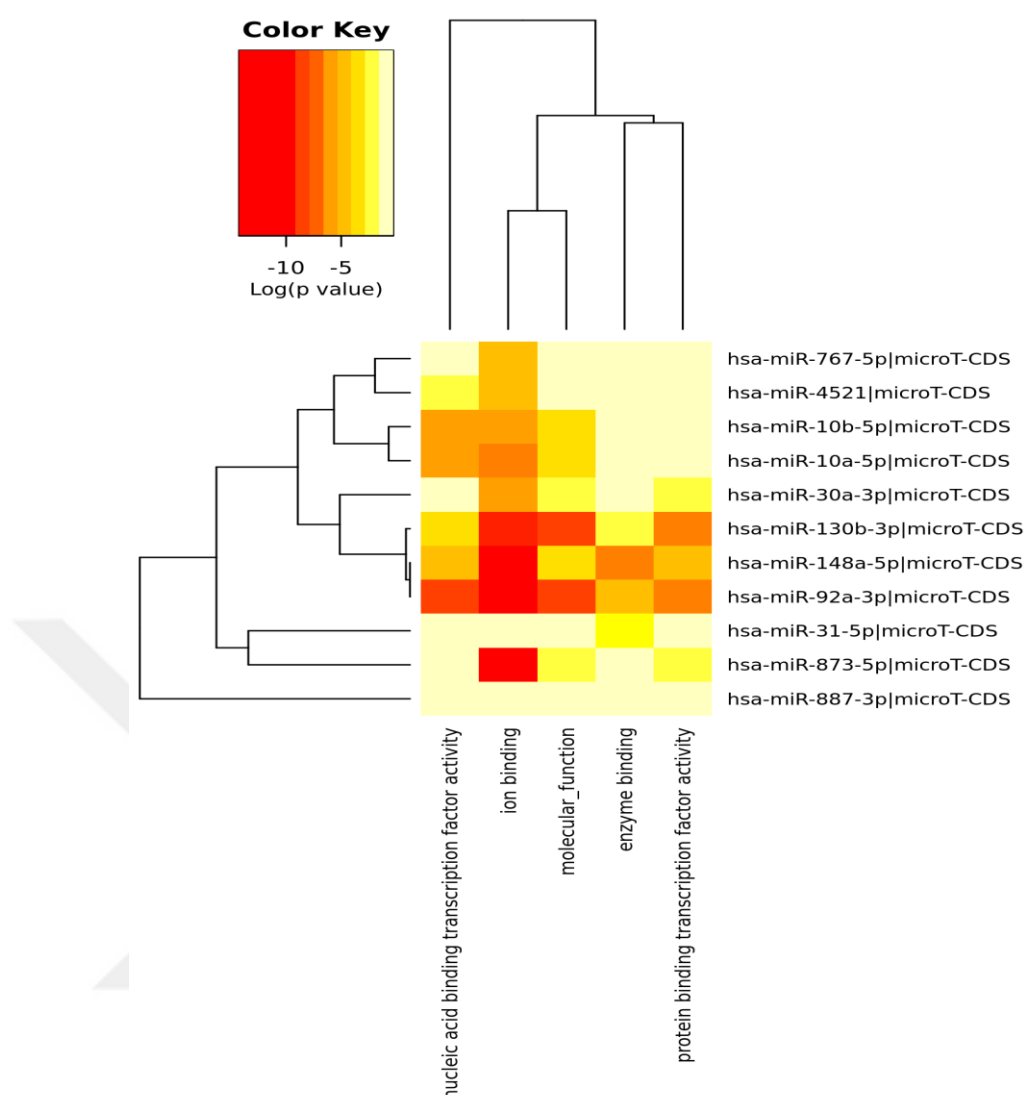


Figure 3.15 : GO enrichment analysis of 11 upregulated miRNAs at molecular function category.

The detailed molecular function category of GO annotation of 11 upregulated miRNA-associated target genes were shown in Table 3.9 The table represented the molecular function category of GO analysis with p-values, target miRNAs, and genes numbers.

Table 3.9 : The detailed GO functional enrichment analysis at molecular function category of 11 downregulated miRNA-associated genes.

Upregulated miRNAs			
GO Category	p-value	genes	miRNAs
nucleic acid binding transcription factor activity	<1e-325	170	6
molecular_function	<1e-325	2240	7
ion binding	<1e-325	1025	9
protein binding transcription factor activity	5,27E-14	100	5
enzyme binding	5,66E-11	184	4

The enriched cellular components of 11 downregulated miRNA-associated target genes include organelle, cytosol, protein complex, and nucleoplasm (Figure 3.16).

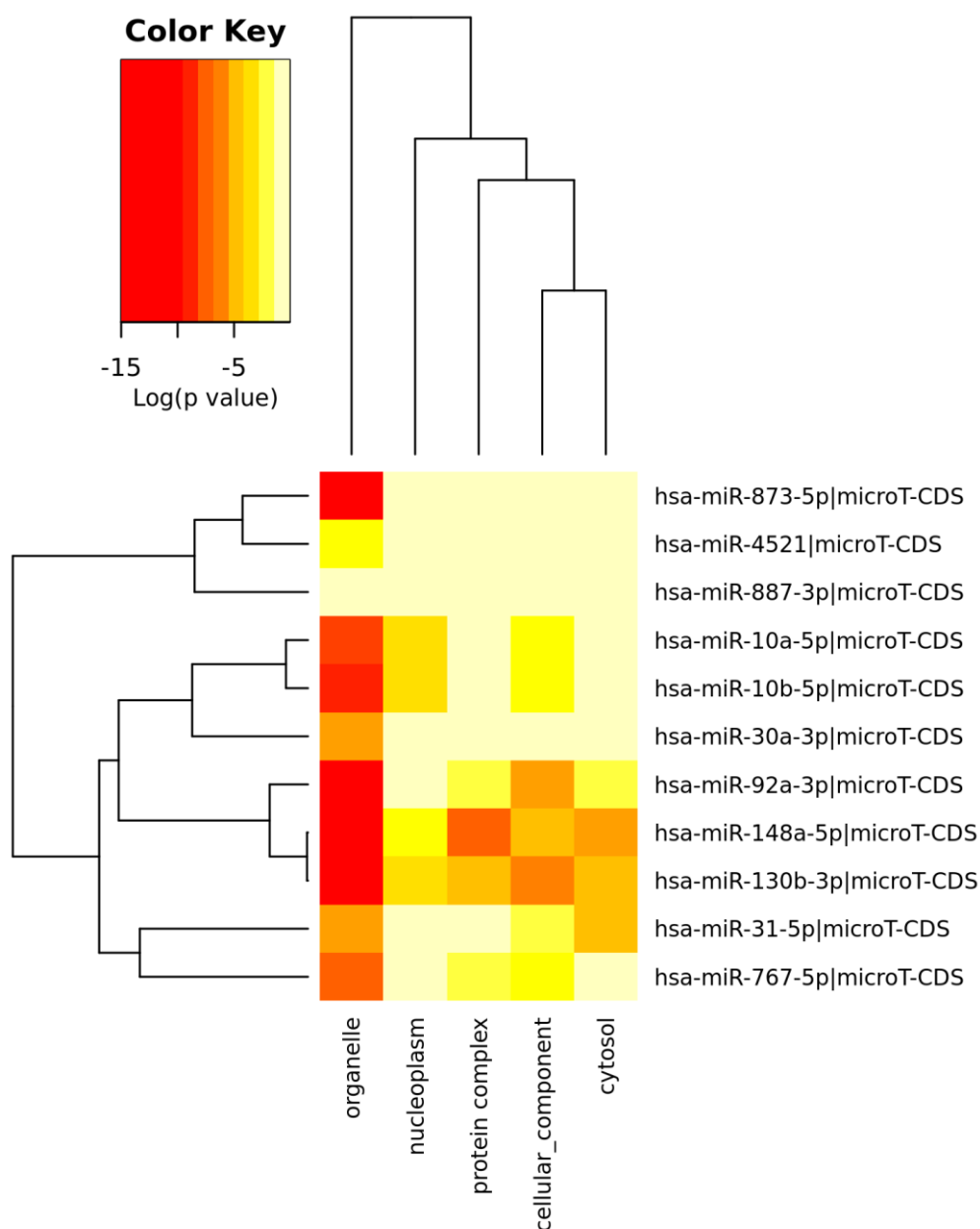


Figure 3.16 : GO enrichment analysis of 11 upregulated miRNAs at cellular component category.

Table 3.10 : The detailed GO functional enrichment analysis at cellular organization category of 11 downregulated miRNA-associated genes.

GO Category	Upregulated miRNAs		
	p-value	genes	miRNAs
organelle	<1e-325	1721	10
cellular_component	7,77E-16	2125	7
cytosol	2,01E-09	330	4
protein complex	8,28E-08	435	4
nucleoplasm	3,71E-07	111	4

3.5.2 Experimental qRT-PCR validation of 14 key miRNAs

The predicted expression profiles of significantly differentially expressed 14 miRNAs of 25 known miRNAs were validated using qRT-PCR analysis. The relative expression profiles of hsa-miR-27a-3p, hsa-miR-151a-3p, and hsa-miR-342-3p showed ~10-fold; hsa-miR-429, and hsa-miR-146b-5p showed ~4-fold; hsa-miR-21-5p, hsa-miR-200a-5p, and hsa-miR-345-5p showed ~2.5-fold downregulation in Bag-1 KO samples compared to wt samples. Besides, hsa-miR-31-5p showed 2.9-fold, and hsa-miR-10a-5p showed 4-fold upregulation in Bag-1 KO cells compared to wt cells (Figure 3.17).



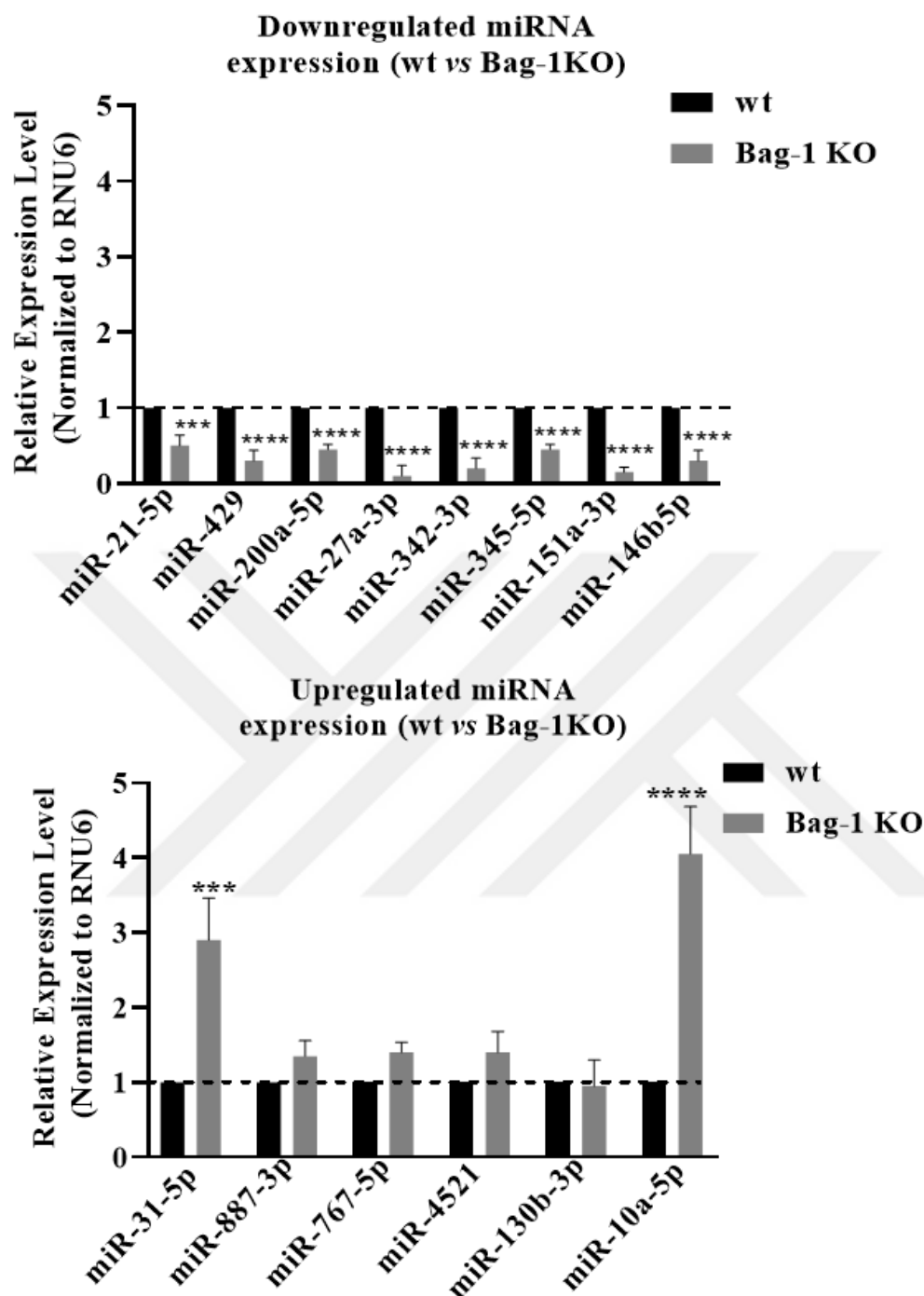


Figure 3.17 : qRT-PCR validation of differentially expressed selected miRNAs in wt and Bag-1 KO samples. The expression patterns of significantly changed selected 14 miRNAs were validated by qRT-PCR analysis and calculated using the $2^{-\Delta\Delta C_q}$ methods. RNU6 was used as a reference gene for qRT-PCR normalization. All data collected from three independent experiments and the designed primer sequences were shown in Table 3.1 (** $p=0.0002$, **** $p<0.0001$).

3.5.3 Selection of hsa-miR-429 as a potential target and analyzing the PPI network of genes targeted by hsa-miR-429

According to the results of differentially expressed 25 miRNAs in Bag-1 KO samples, hsa-miR-429 was found the third most downregulated expression profile of the 14-downregulated miRNAs after hsa-miR-21-3p and hsa-miR-21-5p. It was also involved in 8 KEGG terms out of 13 enriched KEGG pathways of downregulated miRNA-associated target genes and experimentally validated with the confirmation of its downregulated expression profile. The results of hsa-miR-429 were found to be consistent in both sequencing and experimental PCR analysis as they showed a similar downregulation profile. Therefore, hsa-miR-429 was selected as a potential target for Bag-1 KO samples in further analyses.

The target genes of hsa-miR-429 from DIANA-miRPath v.3.0 were compared in the miRDB online database used for miRNA target prediction. Potential gene targets of hsa-miR-429 were identified from common predicted target genes in both DIANA-miRPath v.3.0 (Table 3.11).

Table 3.11 : The predicted target genes and target scores of hsa-miR-429 obtained from DIANA-miRPath v.3.0 and miRDB.

Target Score	Gene Symbol	Gene Description
98	RPS6KB1	ribosomal protein S6 kinase B1
98	MSN	moesin
97	CRKL	CRK like proto-oncogene, adaptor protein
97	PPP2R5E	protein phosphatase 2 regulatory subunits B'epsilon
96	PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
95	CBL	Cbl proto-oncogene
95	WNT16	Wnt family member 16
95	KDR	kinase insert domain receptor
94	FN1	fibronectin 1
94	JUN	Jun proto-oncogene, AP-1 transcription factor subunit
93	B3GNT2	UDP-GlcNAc:betaGal
93	VEGFA	beta-1,3-N-acetylglucosaminyltransferase 2
91	YWHAG	vascular endothelial growth factor A tyrosine 3-monooxygenase/tryptophan 5-monooxygenase
91	CHRM2	activation protein gamma
90	ITGA1	cholinergic receptor muscarinic 2
89	NOG	integrin subunit alpha 1
87	ROCK2	noggin
85	KLF4	Rho-associated coiled-coil containing protein kinase 2
84	PPP2R1B	Kruppel like factor 4
		protein phosphatase 2 scaffold subunit Abeta

Table 3.11 (continued): The predicted target genes and target scores of hsa-miR-429 obtained from DIANA-miRPath v.3.0 and miRDB.

83	ANK3	ankyrin 3
83	SRY-box 2	SRY-box 2
83	SDC2	syndecan 2
83	RDX	radixin
82	EIF4E2	eukaryotic translation initiation factor 4E family member 2
81	MYB	MYB proto-oncogene, transcription factor
81	CCNE2	cyclin E2
80	ACVR1C	activin A receptor type 1C
80	NRG1	neuregulin 1
79	LAMC1	laminin subunit gamma 1
77	IKBKB	inhibitor of nuclear factor-kappa B kinase subunit beta
77	EFNA1	ephrin A1
76	ASH1L	ASH1 like histone lysine methyltransferase
76	GAB1	GRB2 associated binding protein 1
76	PAK6	p21 (RAC1) activated kinase 6
76	KMT2C	lysine methyltransferase 2C
74	FUT9	fucosyltransferase 9
74	ACVR2B	activin A receptor type 2B
74	RRAS2	RAS related 2
73	EP300	E1A binding protein p300
69	RAC1	Rac family small GTPase 1
69	FUT4	fucosyltransferase 4
69	CHST2	carbohydrate sulfotransferase 2
67	SHC4	SHC adaptor protein 4
66	PPP2R2C	protein phosphatase 2 regulatory subunits Bgamma
64	PLCG1	phospholipase C gamma 1
64	RELN	reelin
63	PAX6	paired box 6
62	MTCP1	mature T cell proliferation 1
61	RIF1	replication timing regulatory factor 1
61	SMAD5	SMAD family member 5
60	FRS2	fibroblast growth factor receptor substrate 2
56	CREB5	cAMP-responsive element-binding protein 5
55	COL4A3	collagen type IV alpha 3 chain
54	SMARCAD1	SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1
54	PDPK1	3-phosphoinositide dependent protein kinase 1
50	PRKAA2	protein kinase AMP-activated catalytic subunit alpha 2
50	FGF9	fibroblast growth factor 9

The PPI network of the predicted gene targets for downregulated hsa-miR-429 was developed using the STRING database, and the visualization of the PPI network was established through Cytoscape software. The network was composed of 57 nodes and 97 edges (Figure 3.18).

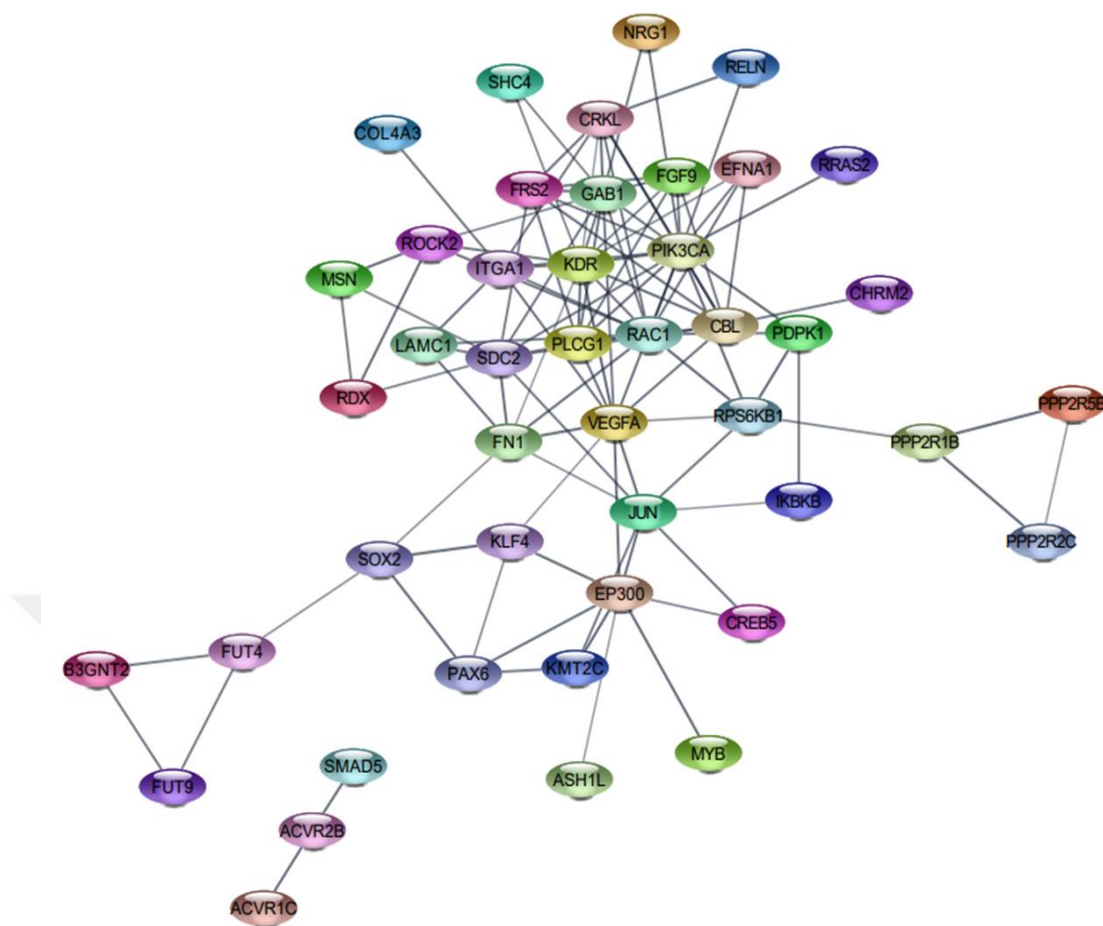


Figure 3.18 : PPI network of predicted targets for hsa-miR-429 using Cytoscape visualization database. The figure illustrates the putative interaction of genes targets of hsa-miR-429 and consists of 57 nodes and 97 edges.

The top ten hub proteins for the target of hsa-miR-429, which are the primary nodes of several interaction partners, were identified as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), kinase insert domain receptor (KDR), GRB2 associated binding protein 1 (GAB1), Rac family small GTPase1 (RAC1), vascular endothelial growth factor A (VEGFA), Cbl proto-oncogene (CBL), syndecan 2 (SDC2), phospholipase C gamma 1 (PLCG1), E1A binding protein p300 (EP300), and CRK like proto-oncogene, adaptor protein (CRKL). The PPI network of the top ten hub genes was shown in Figure 3.19 and Table 3.12.

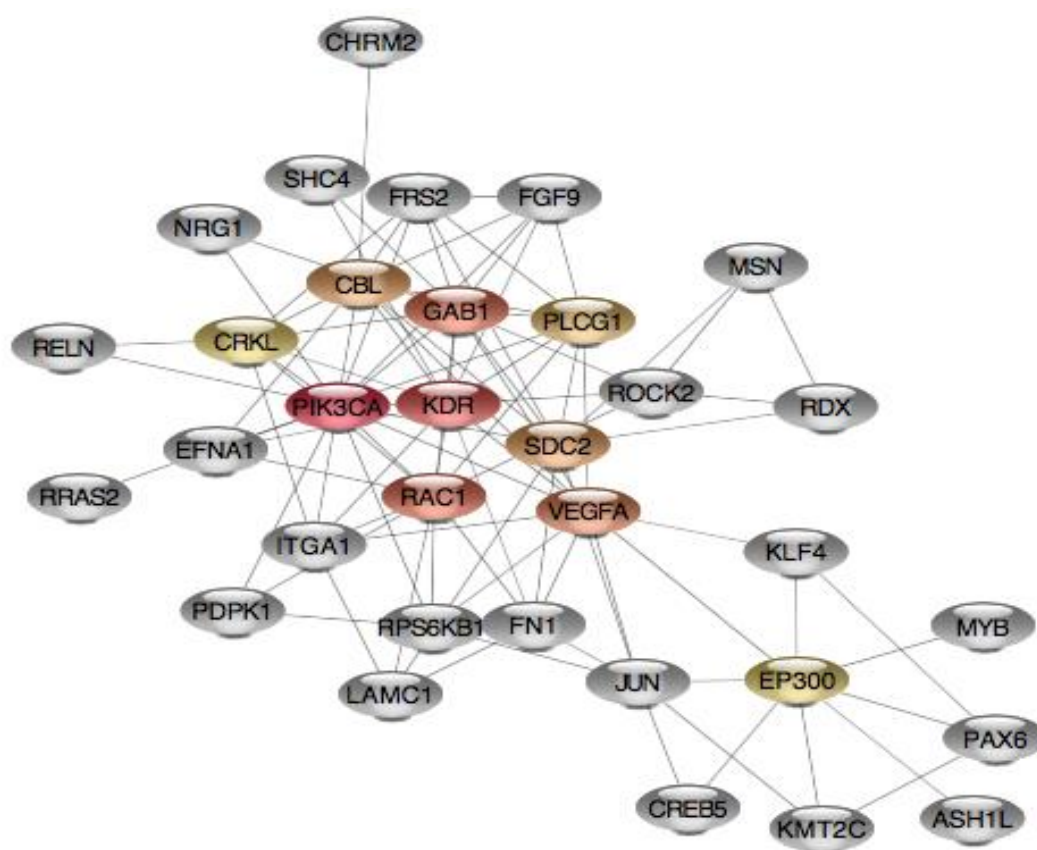


Figure 3.19 : PPI-network for the critical targets of hsa-miR-429. Circular yellow nodes represented the top 10 hub genes of predicted target genes for hsa-miR-429, grey nodes represented the significant targets of hsa-miR-429.

Table 3.12 : The top 10 proteins evaluated in the PPI network using the “degree” parameter

Gene name	Gene description	Degree
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase 110 kDa catalytic subunit alpha	17
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase	13
GAB1	Growth factor receptor-bound protein 2-associated protein 1	12
RAC1	Ras-related C3 botulinum toxin substrate 1	12
VEGFA	Vascular endothelial growth factor-A	12
CBL	Cbl proto-oncogene, E3 ubiquitin-protein ligase	11
SDC2	Heparan sulfate proteoglycan core protein	10
PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	10
EP300	Protein propionyltransferase p300	9
CRKL	V-crk avian sarcoma virus CT10 oncogene homolog-like	9

3.5.4 Functional annotation and pathway enrichment analyses for the target gene of hsa-miR-429

The GO functional annotation and KEGG pathway enrichment analyses of the top ten hub genes including essential proteins of predicted target genes for hsa-miR-429 were performed using the DAVID database. The most significant GO terms were listed in Table 3.13, including positive regulation on gene expression, phosphatidylinositol-mediated signaling, angiogenesis, cell migration, and cell adhesion in the biological process category. Moreover, plasma membrane, cytoplasm, intracellular membrane, and focal adhesion were found in the cellular compartment category, protein binding, phosphatidylinositol-4,5-biphosphate3-kinase activity, protein kinase, receptor tyrosine kinase, and integrin binding were observed in the molecular function category.

Table 3.13 : GO Functional Annotation analysis of the top ten hub genes included essential proteins of predicted target genes for hsa-miR-429.

GO Functional Annotation				
Biological Process	Description	P-value	Count	Genes
GO:0010628	<u>positive regulation of gene expression</u>	5.0E-7	8	KLF4, ROCK2, FGF9, FN1, MSN, PAX6, RDX, VEGFA
GO:0048015	<u>phosphatidylinositol-mediated signaling</u>	4.4E-8	7	PDPK1, GAB1, FGF9, FRS2, NRG1, PIK3CA, RPS6KB1
GO:0001525	<u>angiogenesis</u>	3.5E-6	7	JUN, EFNA1, FGF9, FN, KDR, PIK3CA, VEGFA
GO:0016477	cell migration	1.7E-5	6	PDPK1, EFNA1, LAMC1, PLCG1, PLCG, SDC2
GO:0007155	cell adhesion	5.6E-2	4	FN1, LAMC1, RAC1, RELN
Cellular Compartment	Description	P-value	Count	Genes
GO:0005886	<u>plasma membrane</u>	6.2E-4	17	PDPK1, CBL, ROCK2, SHC4, CHRM2, EFNA1, FRS2, ITGA1, KDR, MSN, PIK3CA, PLCG1, RDX, RAC1, RELN, RRAS2, SDC2
GO:0005737	<u>cytoplasm</u>	2.2E-2	16	PDPK1, ASH1L, CBL, EP300, KLF4, FGF9, FRS2, MSN, NRG1, PAX6, PLCG1, RDX, RAC1, RELN, RPS6KB1, VEGFA
GO:0005622	intracellular	7.3E-3	8	PDPK1, ROCK2, SHC4, EFNA1, PIK3CA, PLCG1, RAC1, RRAS2
GO:0016020	membrane	8.3E-2	8	PDPK1, EFNA1, FRS2, ITGA1, NRG1, RAC1, RRAS2, VEGFA
GO:0005925	focal adhesion	5.5E-4	6	PDPK1, ITGA1, MSN, RDX, RAC1, RRAS2

Table 3.13 (continued): GO Functional Annotation analysis of the top ten hub genes included essential proteins of predicted target genes for hsa-miR-429.

Molecular Function	Description	P-value	Count	Genes
GO:0005515	protein binding	6.0E-4	27	PDPK1, CRKL, CBL, EP300, GAB1, JUN, KLF4, MYB, ROCK2, SHC4, CREB5, EFNA1, FRS2, FN1, ITGA1, KDR, KMT2C, MSN, PAX6, PIK3CA, PLCG1, RDX, RAC1, RRAS2, RPS6KB1, SDC2, VEGFA
GO:0046934	<u>phosphatidylinositol-4,5-bisphosphate 3-kinase activity</u>	3.9E-6	5	GAB1, FGF9, FRS2, NRG1, PIK3CA
GO:0019901	<u>protein kinase binding</u>	5.3E-3	5	PDPK1, MSN, PAX6, PLCG1, RAC1
GO:0030971	<u>receptor tyrosine kinase binding</u>	7.3E-5	4	EFNA1, ITGA1, MSN, NRG1
GO:0005178	<u>integrin binding</u>	1.7E-2	3	FN1, KDR, NRG1

The KEGG pathway enrichment analysis revealed that the most significant genes were enriched in proteoglycans, PI3K-Akt, focal adhesion, cancer pathways, actin cytoskeleton regulation pathway, and Ras signaling pathway, which was listed in Table 3.14.

Table 3.14 : KEGG Pathway enrichment analysis of the top ten essential gene targets for hsa-miR-429.

KEGG Pathway Enrichment				
Description	p-value	Count	Genes	
<u>Proteoglycans in cancer</u>	2.0E-15	16	PDPK1, CBL, GAB1, ROCK2, FRS2, FN1, KDR, MSN, PIK3CA, PLCG1, RDX, RAC1, RRAS2, RPS6KB, SDC2, VEGFA	
<u>PI3K-Akt signaling pathway</u>	1.0E-10	15	PDPK1, MYB, CREB5, CHRM2, EFNA1, FGF9, FN1, ITGA1, KDR, LAMC1, PIK3CA, RAC1, RELN, RPS6KB1, VEGFA	
<u>Focal adhesion</u>	5.0E-11	13	PDPK1, CRKL, JUN, ROCK2, SHC4, FN1, ITGA1, KDR, LAMC1, PIK3CA, RAC1, RELN, VEGFA	
<u>Pathways in cancer</u>	8.0E-7	12	CRKL, CBL, EP300, JUN, ROCK2, FGF9, FN1, LAMC1, PIK3CA, PLCG1, RAC1, VEGFA	
<u>Regulation of actin cytoskeleton</u>	2.0E-8	11	CRKL, ROCK2, CHRM2, FGF9, FN1, ITGA1, MSN, PIK3CA, RDX, RAC1, RRAS2	
<u>Ras signaling pathway</u>	5.5E-7	10	GAB1, SHC4, EFNA1, FGF9, KDR, PIK3CA, PLCG1, RAC1, RRAS2, VEGFA	

3.6 Discussion

Bag-1 protein is one of the critical targets in breast cancer cells, which functions as a co-chaperone for Hsp70/Hsc70 proteins, triggers cellular survival, and results in a high proliferation rate compared to normal cells [161], [180]. Elevated levels of Bag-1 determined in metastatic cancer patients and associated with decreased drug-induced apoptosis in breast cancer [182]. On the contrary, elimination of Bag-1 expression through silencing increased the efficiency of therapeutics [7]. Following this, Bag-1 expression levels were shown as a limiting factor in the efficiency of breast cancer therapies.

miRNAs, the post-transcriptional regulators, are 18-23 nucleotide long noncoding RNAs [231]. They have regulatory roles controlling the development, progression, and metastasis of breast cancer at the post-transcriptional phase; however, these miRNAs' functions and expression profile differs according to the subtype of breast cancer [232]. Different miRNAs function independently to repress a single target, or combinatorially cause more dramatic effects in repressing a target [233]. Although previous studies have demonstrated miRNA-mediated breast cancer development, the miRNA expression profile in Bag-1 deficient-breast cancer cells is unknown [234]. One of the previous studies showed that the tumor suppressor hsa-miR-138 expression was decreased in the tumor tissues, and regain of miR-138 expression enhanced apoptosis of gallbladder cancer through directly targeting the 3'-UTR sites of Bag-1. The loss and gain-of-function studies confirmed that the Bag-1 is a direct target for miR-138 [235]. Moreover, miR-28-5p was found as a tumor suppressor directly targeting and deregulating Bag-1 in B cells [236].

Here, we discuss the differential expression profiles of miRNAs in Bag-1 deficiency leading to breast cancer progression in MCF-7 cells. Following small RNA sequencing analyses, 25 significant differentially expressed miRNAs were observed, 14 miRNAs were found downregulated, and 11 miRNAs were upregulated in Bag-1 KO samples compared to wt MCF-7 samples. It was noteworthy that the enriched pathways associated with upregulated miRNAs were less in number compared with downregulated miRNAs enriched pathways. To eliminate the false-positive rates of prediction algorithms, the relative expression of 14 miRNAs selected out of 25

differentially expressed miRNAs to sample different log2 fold change numbers was experimentally validated by qRT-PCR analyses.

According to the results of downregulated miRNA profiles in Bag-1 KO samples, previous studies showed that hsa-miR-21 is required for cell proliferation, PI3K/Akt activation, and EMT in MDA-MB-231 and SKM-1 cells [237], [238]. In addition, hsa-miR-27a-3p has been identified as an onco-miR and promotes proliferation and migration of colorectal and triple-negative breast cancer cells through targeting BTG-1 and GSK3 β , respectively [239]. hsa-miR-342 and hsa-miR-146 are associated with estrogen levels and have a role in the negative regulation of BRCA1 in breast cancer [240]. hsa-miR-345 has been known for its inhibitory role on tumor metastasis and induction of apoptosis in hepatocellular and pancreatic carcinoma [241]. hsa-miR-151 was found to repress migration in breast cancer through targeting TWIST1[242]. In addition, hsa-miR-98-5p has been known for its anti-oncogenic function in breast cancer [22]. The miR-200 family is one of the most studied miRNAs associated with EMT and metastasis. Significant downregulation of the miR-200 family, which consists of miR-141, miR-200a, miR-200b, miR-200c, and miR-429, regulates the expressions of mesenchymal markers ZEB1, ZEB2, TWIST1, TWIST2, Snai1, and Snai2 and promotes EMT in breast cancer [243]. Previous studies demonstrated that miR-200a showed decreased expression profile in EMT-induced cancer cells, but overexpression was associated with downregulated N-cadherin, ZEB1, vimentin, and upregulated E-cadherin profile [244], [245]. miR-429, a member of the miR-200 family, has been reported by its inhibitory role on EMT, tumor migration, and metastasis in hepatocellular, nasopharyngeal, and breast cancer [246]. Loss of function studies showed a decreased expression profile in breast cancer, inducing EMT and promoting bone metastasis directly targeting the ZEB1 transcription factor [192].

The downregulation of key miRNA biogenesis enzymes DROSHA and DICER have been recognized to deregulate miRNA expression in many cancers [247], [248]. The importance of downregulated DROSHA and DICER enzymes is associated with poor survival [249]. Previous studies showed that DROSHA enzyme is transcriptionally activated by c-Myc and downregulated by ADARB1 proteins [250]. The downregulation of DROSHA enzyme by ADARB1 decreased the specific miRNA expressions which allows the triggering of oncogenic stimulation [251]. Moreover, it was observed that DICER downregulation is associated with the loss of the TAp63

transcription factor which binds the promoter region of the DICER [252]. The downregulation of miR-200 was found associated with the low levels of DICER which allows the promotion of metastasis [253].

Considering the c-Myc and Bag-1 protein correlation, the expression profiles of miRNAs in Bag-1 KO cells compared to wt cell can be associated with the c-Myc-associated deregulation of the miRNAs. Future studies can be performed to provide the roles of c-Myc levels on the expression profiles of these miRNAs.

In the current study, hsa-miR-429 was consistently downregulated in Bag-1 KO MCF-7 cells in both bioinformatics and qRT-PCR validation assays. Moreover, hsa-miR-429 was involved in downregulated miRNAs-associated KEGG enrichments in 8 of a total 13 pathways; therefore, we suggested that hsa-miR-429 and its gene targets can be a potential purpose to explain the survival properties of Bag-1 KO cells.

It was observed from our results that the predicted gene targets of hsa-miR-429 were PIK3CA, KDR, GAB1, RAC1, VEGFA, CBL, SDC2, PLCG1, EP300, and CRKL, which are significantly enriched in PI3K/Akt signaling, cell migration, cell adhesion, focal adhesion, integrin binding, and actin cytoskeleton regulation.

Although there is no direct evidence that miR-429 targets PIK3CA, previous studies showed that it inhibits cell proliferation and migration through targeting AKT serine/threonine kinase 1 (AKT1) involved in PI3K/Akt pathway in renal cell carcinoma [254]. Similarly, although direct interaction of KDR with hsa-miR-429 has not been demonstrated, it was observed that hsa-miR-429 suppresses breast cancer invasion by inhibiting the Wnt/ β -catenin pathway by potentially targeting SOX2, CBL, FN1, KDR, KLF4, NR3C1, QK1, RBFOX3, SYNJ1, and VEGFA detected by CytoHubba algorithm in Cytoscape [255]. GAB1 was found to be directly targeted by hsa-miR-200a, a miRNA family including hsa-miR-429, to suppress cell invasion and migration of hepatocellular carcinoma [256]. hsa-miR-429 decreased the expression of VEGFA in human endothelial cells by directly inhibiting Hypoxia-inducible factor 1 (HIF1) [257]. In breast cancer, it was determined that hsa-miR-200bc/429 cluster directly targets PLCG1 and regulates EGF-driven invasion [258]. Moreover, hsa-miR-429 also directly targets and negatively regulates (v-crk sarcoma virus CT10 oncogene homolog (avian)-like) CRKL adaptor protein in breast cancer to promote cell migration and invasion [246]. Although several studies determined the direct or

indirect network between PIK3CA, KDR, GAB1, VEGFA, PLCG, and CRKL and hsa-miR-429 in various cancers, the functional regulation of these genes in Bag-1 deficiency conditions is still needed to elucidate. However, there is no direct or indirect interaction network between hsa-miR-429 and RAC1, CBL, SDC2, and EP300. Therefore, these preliminary screening data can be used to investigate the regulation of survival and proliferation conditions of Bag-1 knockout breast cancer cells.



4. CONCLUSIONS

Multifunctional Bag-1 possesses a central role in the survival-related signaling axis to maintain normal cell physiology. The enhanced stress conditions due to deletion of Bag-1 gene hyperactivated Akt with reduced PTEN expression to maintain cell survival with dysregulated mesenchymal cellular characteristics. The findings that have arisen in the first part of the thesis highlighted the isoform-specific functional role of Bag-1 on the PI3K/Akt signaling axis, which orchestrates cell cytoskeleton targets in MCF-7 breast cancer cells. Further studies are required to understand the molecular mediators of Akt hyperactivation in Bag-1 deficiency conditions, which prevent pro-region processing of N-Cadherin. In addition, these studies may provide preliminary results to investigate the regulatory role of mTOR like other strong candidates, which can activate Akt in Bag-1 KO multiple clones of MCF-7 breast cancer cells. It might be critical to study different Akt phosphorylation sites and their subcellular localization to understand the regulatory role of Bag-1 with Akt in cellular dynamics related to cytoskeleton organization. Re-gain of each isoform as the functional parts of Bag-1 enlighten the biological importance of each isoform in stress-mediated cellular responses. The findings arising from this study may help us to evaluate patient meta-analysis results, which showed Bag-1 deficiency may lead to a poor prognosis of breast cancer outcome.

The studies of the first part of the thesis that the deletion of the Bag-1 gene led to significant Akt activation with increased mesenchymal properties and resulted in actin cytoskeleton polymerization highlighted that Bag-1 expression is critical in cellular homeostasis. To clarify all aspects of this dramatic change in the MCF-7 breast cancer cells following Bag-1 deficiency and also considering the heterogeneous characteristics of breast cancer we aimed to investigate the other regulatory mechanisms in MCF-7 breast cancer cells. Therefore, in the second part of the thesis, we investigated the differential expression profiles of miRNAs in wt and Bag-1 KO cells. The results obtained from the miRNA screening studies in both samples, genes that are involved in PI3K/Akt and ECM signaling, and pluripotency markers were

highly enriched in Bag-1 KO cells compared to wt samples. According to the bioinformatic analysis of the results, hsa-miR-429 was suggested as the potential targeted miRNA in Bag-1 KO cells to associate with the induction of PI3K/Akt and EMT pathways due to targeting mRNAs that have roles in these pathways. Taken together, this study suggested that the PI3K/Akt and EMT induction in Bag-1 KO MCF-7 cells might be associated with the significantly downregulated miR-429 expression profile that was validated by bioinformatics analysis. However, further gain or loss of function studies would be necessary for further studies elucidating the molecular function of Bag-1 KO-mediated miR-429 in MCF-7 breast cancer cells.



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APPENDICES

APPENDIX A: Equipments used in this study.

APPENDIX B: Chemicals, media component, and antibodies used in this study.

APPENDIX C: Buffers and solutions that were used in this study.

APPENDIX D: The map of Bag-1 OE plasmid vector.

APPENDIX E: The list of the most possible 3 different off-targets determined by the CRISPOR web tool.

APPENDIX F: The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19.

APPENDIX G: The sequence counts for 88 miRNAs of two biological replicates of wt samples annotated in the hg19.

APPENDIX H: The sequence counts for 77 miRNAs of two biological replicates of wt samples annotated in the hg19.

APPENDIX I: Uncropped western blotting images of the results.

APPENDIX A: Equipments used in this study

Table A.1 : Equipments used in this study.

Name of equipment	Catalog No	Supplier company
Autoclave	OT 032	Nüve
Autoclave	OT 090	Nüve
Balance	LE6202S	Sartorius
Cell culture plate- 6 wells	92006	TPP
Cell culture plate- 96 wells	92096	TPP
Cell culture flask- 25 cm ²	90026	TPP
Cell culture flask- 75 cm ²	90076	TPP
Cell culture flask- 60 mm	93060	TPP
Cell culture flask- 100 mm	93100	TPP
Cell scraper	280 mm	Greiner Bio-One
Centrifuge	5430R	Eppendorf
Centrifuge	5810R	Eppendorf
Centrifuge tubes	91015	TPP
Centrifuge tubes	91050	TPP
CO ₂ incubator	HeraCell 150i 51026280	Thermo Scientific
Chemiluminescence Imaging System ChemiDoc	MP 1708280	Bio-Rad
Cryotubes	5042000C	CAPP
DNA electrophoresis system	Wide Mini-Sub Cell GT	Bio-Rad
Deepfreeze	2041D	Arçelik
Distilled water machine	TANKPE030	Millipore
Dry air sterilizer	FN120	Nüve
Electrophoresis blot system	Tetra-Cell 1658004	Bio-Rad
Electrophoresis transfer system	Trans-Blot Turbo 10016505C	Bio-Rad
Electrophoresis power supply	PowerPac/Basic	Bio-Rad
Falcon tubes 15 ml	90015	TPP
Falcon tubes 50 ml	90050	TPP
Filter 0.22 µm	90022	TPP
Flow cytometer	Accuri C6	BD
Freezer	2041 D	Arcelik
Haemocytometer	40441	Assistant Germany
Ice machine	AF80	Scotsman
Incubating shakers	Excella E25	New Brunswick Scientific
Individual PCR Tubes 8-Tube Strip	TLS0801	Bio-Rad
Invert microscope	BDS-500	SOIF
Invert fluorescence microscope	1X71	Olympus
Laminar flow	HeraSafe KS 12	Thermo Scientific
Liquid nitrogen	Arpege 40	Air Liquid
Magnetic stirrer	SB162	Stuart/Prolab
Mini spin	Mini-6k	MiuLab
Micropipette (0.5µl-10µl)	Bravo B10-1	Capp
Micropipette (10µl-100µl)	Bravo B100-1	Capp

Micropipette(100µl-1000µl)	Bravo B1000-1	Capp
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Table A.1 (continued): Equipments used in this study.

Microplate reader	iMark	Bio-Rad
Microwave oven	MD595	Arçelik
Next-generation sequencing platform	MiSeq System	SY4101003
Optical Flat 8 Cap Strip for 0.2 ml	TCS0803	Bio-Rad
Orbital shaker	GyroTwister	Labnet
Polymerase Chain Reaction Machine	MyCycler	Bio-Rad
Polypropylene Eppendorf tube	25632	CAPP
pH meter	A111	Thermo Scientific
PVDF Membrane	88518	Thermo Scientific
Real-Time PCR Detection System	CFX96	Bio-Rad
Refrigrator	4263 TMB	Arçelik
Serological pipette 5 ml	94005	TPP
Serological pipette 10 ml	94010	TPP
Shaker	SRT6D	Stuart
Ultra-low-temperature freezer	ULUF	Arctico
Ultra-low-temperature freezer	U725	New Brunswick Scientific
Ultrapure water machine	Direct Q-5UV	Millipore
UV/Vis Spectrophotometer	NP80	Implen
UV Transilluminator	Imaging GelDoc XR	Bio-Rad
Machine		
Refrigrator	4260TMB	Arçelik
Rotator	SRT9D	Stuart/Prolab/Prolab
Water bath	BM 302	Nüve
Vacuum machine	5305BH020683	Eppendorf
Vortex	SA8	Stuart/ProLab

APPENDIX B: Chemicals, media component, and antibodies used in this study**Table B.1** : Chemicals, media component, and antibodies used in this study.

Name of chemicals	Catalog No	Supplier company
Absolute Etanol	100983.2511	Merck
Acetic acid	27225	Sigma Aldrich
Acrylamide/bis-acrylamide (30%)	A3699	Sigma Aldrich
Agarose	35-1010	PeqLab Biotech
Ammonium acetate	101115.1000	Merck
Ammonium persulfate (APS)	A2941	Applichem
Bag-1 CRISPR/Cas9 KO Plasmid (h)	sc-417179	SCBT
Bag-1 HDR Plasmid (h)	sc-417179-HDR	SCBT
β-merkaptoetanol	M3148	Sigma Aldrich
Bromophenol blue	A2331,0025	AppliChem
Bradford reagent	23200	Thermo Scientific
BSA	A2153	Sigma Aldrich
Control CRISPR/Cas9 Plasmid	sc-418922	SCBT
Chloroform	A2279	AppliChem
CM-H2DCF-DA	C6827	Invitrogen
Complete mini EDTA-free Lysis Reagent	047199640001	Roche
Coomassie brilliant blue G250	A3480	Applichem
Crystal violet	C0775	Sigma
DAPI	1322MG025	Biofroxx
DiOC6	318426	Sigma Aldrich
DMEM	P04-03590	PAN BioTech
DMSO	D8418	Sigma Aldrich
DNA Marker 100 bp	GeneRulerSM0241	Thermo Scientific
DNA Marker 1 kb	GeneRulerSM0311	Thermo Scientific
EDTA	A2937	Applichem
Ethanol	CAS 64-17-5	Alko Med
FBS	10500-064	Gibco
Fugene HD Transfection kit	04709705001	Roche
Glacial acetic acid	91190	Sigma Aldrich
Glycine	3570	Calbiochem
Hydrochloric acid (HCl)	100317	Merck
Hydrogen peroxide	107209	Merck
Isopropanol	34863	Sigma Aldrich
iScript cDNA Synthesis Kit	1708891	Bio-Rad
iQ SYBR Green Supermix	170-8880	Bio-Rad
Luminol	A2185	Applichem
2-Mercaptoethanol	S4805940517	Merck
MCF-7 cells	HTB-22	ATCC
Methanol	24229	Sigma Aldrich
miScript SYBR Green PCR Kit	218073	Qiagen
miScript II RT Kit	218160	Qiagen
miRNeasy Mini Kit	217004	Qiagen
M-PER Mammalian Protein Extraction Reagent	78501	Thermo Scientific
MTT	3580GR005	Biofroxx

Table B.1 (continued): Chemicals, media component, and antibodies used in this study.

p-Coumaric Acid	C9008		Sigma Aldrich
Phosphate buffer saline (PBS) (10X)	P04-53500		Pan Biotech
Phosphoric acid	100573.2500		Merck
PhosSTOP	04906837001		Roche
PI	P4170		Sigma Aldrich
Plasmid Transfection Medium	sc-108062		SCBT
Ponceau S	A1405		Applichem
Penicillin/Streptomycin	P06-07350		PAN BioTech
Propidium Iodide (PI)	A2261		AppliChem
Prestained Protein Ladder	PageRuler	Plus	Thermo Scientific
	26620		
ProteoJET Mammalian Cell Lysis Reagent	K0301		Fermentas
PureZol RNA Isolation Reagent	7326890		Bio-Rad
Puromycin dihydrochloride, 25 mg	sc-108071		SCBT
PVDF Membrane	88518		Thermo Scientific
RedSafe	21141		Intron Biotechnology
Skimmed Milk	1172KG001		Biofroxx
Sodium Chloride	S7653-5KG		Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	1177GR100		Neofroxx
Transfection Reagent	133215657		Qiagen
N,N,N',N'-Tetra methylethylenediamine (TEMED)	A1148		AppliChem
Triton-X-100	T8787-100ML		Sigma-Aldrich
Tris Base	648310		Calbiochem
Tris-HCl	648317		Calbiochem
Trypsin-EDTA	SH30042.01		Hyclone
Trypan Blue	EBT-001		NanoEnTek
Tween-20	822184		Merck
UltraCruz® Transfection Reagent	sc-395739		SCBT
Actin Cytoskeleton / Focal Adhesion Staining Kit	FAK100		Sigma-Aldrich
Adipogenesis Marker Antibody Sampler Kit	12589		Cell Signalling Technology
Bag-1 antibody	3920		Cell Signalling Technology
Epithelial-Mesenchymal Transition (EMT) Antibody Sampler Kit	9782		Cell Signalling Technology
Focal Adhesion Protein Antibody Sampler Kit	13430		Cell Signalling Technology
Loading control antibody sampler kit	5142		Cell Signalling Technology
MAPK antibody sampler kit	99265		Cell Signalling Technology
Page ruler prestained protein ladder	SM0671		Fermentas

APPENDIX C: Buffers and solutions that were used in this study

Ammonium persulfate

0.1 grams of ammonium persulfate was dissolved in 1 ml of distilled water, and stored at -20°C.

Bovine Serum Albumin (BSA, Protein Standard)

0.015 grams of BSA was dissolved in 10 ml of distilled water, passed through a 0.22 µm filter, and stored at +4°C.

Bradford Solution

0.05 grams of Coomassie Brilliant Blue (G-250) dye was dissolved in 50 ml of methanol. 100 ml of 85% phosphoric acid (H₃PO₄) was added to it. The prepared solution was added to 500 ml of distilled water, filtered with filter paper, and the final volume was completed to 1 liter. Stored at +4°C.

Coumaric acid

It was prepared by dissolving 0.15 grams of p-coumaric acid in 10 ml of DMSO and stored at +4°C.

Freezing Media

1 ml of DMSO was added to 9 ml of FBS and then passed through a 0.22 µm filter. Stored at -20°C.

5X Laemmli Loading Buffer

1 ml 1% bromophenol blue, 10 ml glycerol, 2 g SDS, 5 ml β-mercaptoethanol were added to 4 ml 1.5 M Tris-HCl pH 6.8. 5 ml total solution was aliquoted and stored in -20°C.

Luminol

0.44 grams of Luminol was prepared by dissolving in 10 ml of DMSO and stored at -20°C.

Mammalian Cell Lysis Buffer

1 tablet of Protease inhibitor was added to 10 ml of M-PER Mammalian Protein Extraction agent and stored at +4°C.

Membrane Stripping Buffer

15 grams of glycine, 1 gram of SDS, and 10 ml of Tween-20 were dissolved in approximately 800 ml of distilled water, the pH was adjusted to 2.2, and the final volume was completed to 1 liter. Stored at room temperature.

5% milk with TBS

2.5 grams of skimmed milk powder was dissolved up to 50 ml with TBS-T.

10X PBS (Phosphate buffered saline)

80 g NaCl, 2.25 g KCl, 23.27 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 2.05 g KH_2PO_4 were dissolved in 1000 ml dH_2O , pH adjusted to 7.4, and autoclaved.

10X Running Buffer

250 mM Tris base, 1.92 M glycine, and 1% (w/v) SDS were dissolved in 500 ml dH_2O , and pH was adjusted to 8.5.

10 X TBS (Tris-buffered saline)

86.6 g NaCl, 12, 1 g Tris, 4 ml HCl were dissolved in 1000 ml dH_2O , and pH was adjusted to 8.

10X TBE (Tris-Boric acid-EDTA)

108 g Tris and 55 g Boric acid were dissolved in 500 ml of distilled water, 40 ml 0.5 M EDTA (pH:8) was added and the final volume was made up to 1L.

10X Transfer Buffer

250 mM Tris base and 1.92 M glycine were dissolved in 500 ml dH_2O .

Table C.1 : SDS-PAGE gel electrophoresis.

	%12 Separating Gel (Bottom)	%4 Loading Gel (Upper)
dH_2O	1,7 ml	1,537 ml
Tris-HCl	1,25 ml (1,5 M, pH 8.8)	0,625 ml (0,5 M, pH 6.8)
% 10 SDS	50 μl	25 μl
Acrylamide/Bisacrylamide	2 ml	0,335 ml
%30 37,5:1		
%10 APS	40 μl	12,5 μl
TEMED	5 μl	2,5 μl

Table C.2 : Chemiluminescence imaging buffer.

	Buffer A	Buffer B
dH_2O	9 ml	9 ml
Tris Base pH: 8,5	1 ml	1 ml
Coumaric acid	75 μl	-
Luminol	150 μl	-
H_2O_2	-	15 μl

APPENDIX D: The map of Bag-1 OE plasmid vector

Plasmid Information

CS-L0198-M02 of OmicsLink Expression Clone was used as a plasmid. Bag-1 human cDNA ORF Clone was inserted in pEZ-M02 vector with the removal of the stop codon and addition of a C-terminal TAP (tandem affinity purification)-tag containing CBP (calmodulin-binding peptide)-TEV (tobacco etch virus protease)- Protein A from N to C terminus by Capital Biosciences. The whole plasmid size is 7285 bp with the 1038 bp ORF length. Ampicillin is the antibiotic for this plasmid, and neomycin is the stable selection marker. DH5 α is the suitable bacterial strain for transformation.

OmicsLink™ Expression Clone (CMV Promoter)

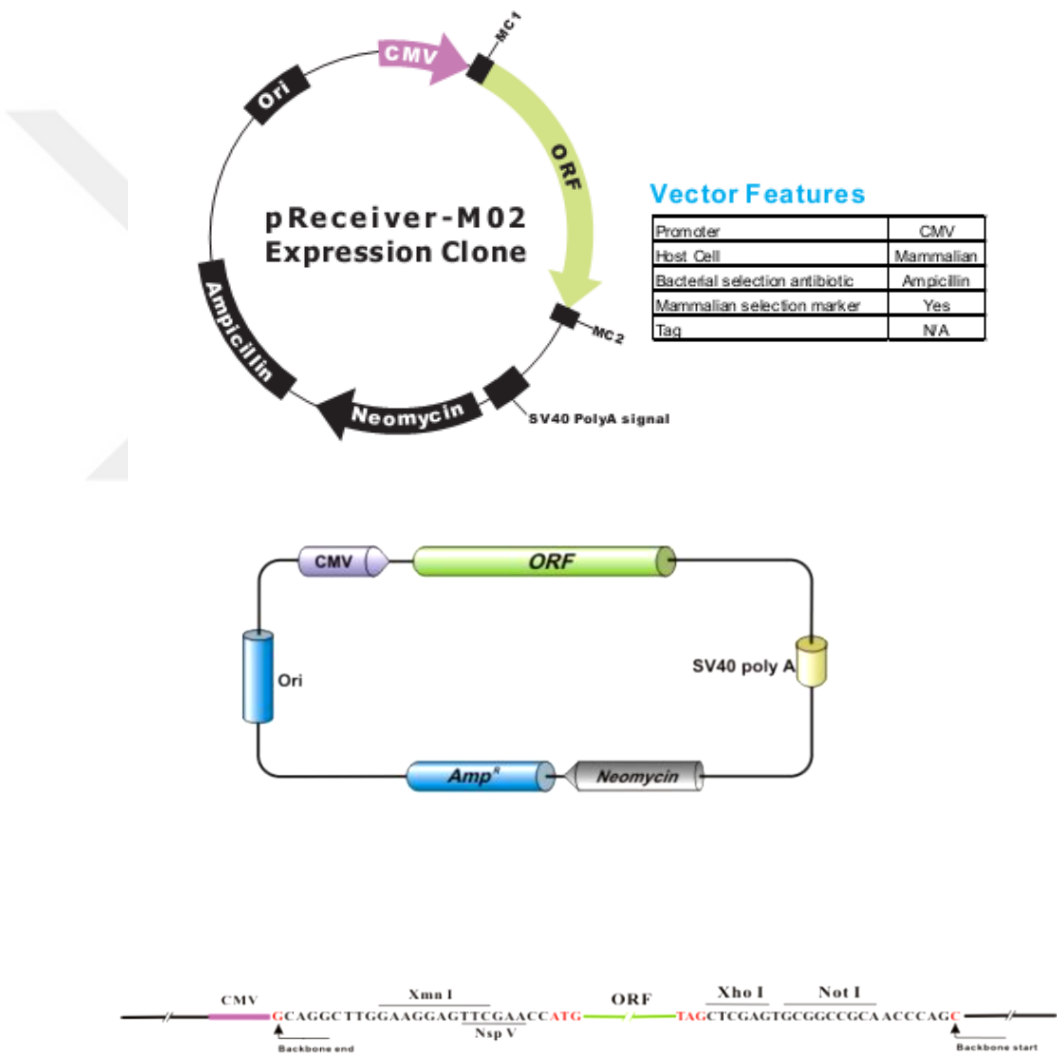


Figure D.1 : Vector information.

APPENDIX E: The list of the most possible 3 different off-targets determined by the CRISPOR web tool

Table E.1 : The list of the most possible 3 different off-targets determined by the CRISPOR web tool.

PCR primers for off-targets of TGAACCAGTTGTCCAAGACC TGG

In the table below, Illumina Nextera Handle sequences have been added and highlighted in bold. Primers for the on-target have been added for convenience. The table below is sorted by the CFD off-target score. Sites with very low CFD scores < 0.02 are unlikely to be cleaved, see Haussler et al. 2016, Figure 2.

In the protocol by Matthew Canver, Harvard, two PCRs are run: one PCR to amplify the potential off-target, then a second PCR to extend with Illumina barcodes. Please [click here](#) to download the protocol. Alternatively, you can have a look at [Fu et al. 2014](#).

Name	Primer Sequence	Tm	CFD S
ontarget_mm0_exon_BAG1_chr9_33262759_F	TCGTCGGCAGCGTC GTGTGGAACCCCTATGACCTCT	59.9	1.00
ontarget_mm0_exon_BAG1_chr9_33262759_R	GTCTCGTGGGCTCGG CAGGCAATGAGAAGCACGAC	59.5	1.00
mm4_intergenic_RP11-489M13.3 RP11-489M13.2_chr4_4104634_F	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_RP11-489M13.3 RP11-489M13.2_chr4_4104634_R	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_RBMX2 RP1-274L7.1_chrX_129552070_F	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_RBMX2 RP1-274L7.1_chrX_129552070_R	Primer3: not found at this Tm	N.d.	0.28
mm3_intergenic_AL451006.1 HIVEP3_chr1_42242681_F	Primer3: not found at this Tm	N.d.	0.26
mm3_intergenic_AL451006.1 HIVEP3_chr1_42242681_R	Primer3: not found at this Tm	N.d.	0.26
mm4_intron_FLNB_chr3_58087793_F	Primer3: not found at this Tm	N.d.	0.22
mm4_intron_FLNB_chr3_58087793_R	Primer3: not found at this Tm	N.d.	0.22
mm4_intergenic_CTD-2183H9.7 F8_chrX_154126402_F	TCGTCGGCAGCGTC AACACTGCAGCTGCGTATCT	60.0	0.21
mm4_intergenic_CTD-2183H9.7 F8_chrX_154126402_R	GTCTCGTGGGCTCGG AGAAGGCCTGAAGAGTGCAC	59.9	0.21
mm4_intergenic_AC007563.1 AC007557.1 AC007563.5_chr2_217839278_F	Primer3: not found at this Tm	N.d.	0.20
mm4_intergenic_AC007563.1 AC007557.1 AC007563.5_chr2_217839278_R	Primer3: not found at this Tm	N.d.	0.20
mm4_intergenic_AC007365.2 RN7SL341P_chr2_65032695_F	Primer3: not found at this Tm	N.d.	0.19
mm4_intergenic_AC007365.2 RN7SL341P_chr2_65032695_R	Primer3: not found at this Tm	N.d.	0.19
mm4_intergenic_RPL35P8 C22orf34_chr22_49743416_F	Primer3: not found at this Tm	N.d.	0.17
mm4_intergenic_RPL35P8 C22orf34_chr22_49743416_R	Primer3: not found at this Tm	N.d.	0.17
mm4_intron_CYP26B1_chr2_72368207_F	Primer3: not found at this Tm	N.d.	0.16
mm4_intron_CYP26B1_chr2_72368207_R	Primer3: not found at this Tm	N.d.	0.16
mm4_intron_SPIN1_chr9_91064245_F	Primer3: not found at this Tm	N.d.	0.15
mm4_intron_SPIN1_chr9_91064245_R	Primer3: not found at this Tm	N.d.	0.15
mm4_intron_RP11-172E9.2_chr13_40923706_F	TCGTCGGCAGCGTC AGGACAATGCAGATGGACG	60.0	0.15
mm4_intron_RP11-172E9.2_chr13_40923706_R	GTCTCGTGGGCTCGG TCTTCTCTCTGGCCAGACA	59.8	0.15
mm3_intron_KLHL29_chr2_23920962_F	Primer3: not found at this Tm	N.d.	0.15
mm3_intron_KLHL29_chr2_23920962_R	Primer3: not found at this Tm	N.d.	0.15
mm4_exon_CAPN2_chr1_223963260_F	Primer3: not found at this Tm	N.d.	0.15
mm4_exon_CAPN2_chr1_223963260_R	Primer3: not found at this Tm	N.d.	0.15
mm4_exon_SDAD1_chr4_76882390_F	Primer3: not found at this Tm	N.d.	0.14
mm4_exon_SDAD1_chr4_76882390_R	Primer3: not found at this Tm	N.d.	0.14
mm4_exon_SDAD1P2_chr20_10367631_F	TCGTCGGCAGCGTC ACAGCTCGATGTTCTCTGGC	60.1	0.14
mm4_exon_SDAD1P2_chr20_10367631_R	GTCTCGTGGGCTCGG TCAGTGTTCGGAAGAGCTGA	58.6	0.14
mm4_exon_ZNF747_chr16_30544329_F	TCGTCGGCAGCGTC CTCTCCCGCACAGTAGC	60.8	0.13
mm4_exon_ZNF747_chr16_30544329_R	GTCTCGTGGGCTCGG GGCTGAAGGCTCCCAAG	60.0	0.13
mm4_intergenic_AC002978.1 CUX2_chr12_111515265_F	TCGTCGGCAGCGTC CAATCCATCAGGCTCACCAGC	60.1	0.12
mm4_intergenic_AC002978.1 CUX2_chr12_111515265_R	GTCTCGTGGGCTCGG GAATCCAGTGACCTGCTCC	60.0	0.12
mm3_intergenic_RNU4-12P RPL7P27_chr6_86780078_F	Primer3: not found at this Tm	N.d.	0.11
mm3_intergenic_RNU4-12P RPL7P27_chr6_86780078_R	Primer3: not found at this Tm	N.d.	0.11
mm4_intron_ZC3H14_chr14_89067871_F	Primer3: not found at this Tm	N.d.	0.10
mm4_intron_ZC3H14_chr14_89067871_R	Primer3: not found at this Tm	N.d.	0.10
mm4_exon_HNRNPA1P73_chr7_27368857_F	Primer3: not found at this Tm	N.d.	0.08
mm4_exon_HNRNPA1P73_chr7_27368857_R	Primer3: not found at this Tm	N.d.	0.08
mm4_intergenic_AP002856.6 AP002856.7_chr11_131099530_F	Primer3: not found at this Tm	N.d.	0.08
mm4_intergenic_AP002856.6 AP002856.7_chr11_131099530_R	Primer3: not found at this Tm	N.d.	0.08
mm4_intron_AC144521.1_chr3_18912521_F	TCGTCGGCAGCGTC ACAGAACGAACCAATCTGCCA	60.2	0.08

<http://crispor.tefor.net/crispor.py?batchId=1RnwELiEMrr1nxzM8pD8&pamId=s67%2B&otPrimers=1>

Table E.1 (continued): The list of the most possible 3 different off-targets determined by the CRISPOR web tool.

PCR primers for off-targets of GGTTGCCGGGTCATGTTAAT TGG

In the table below, Illumina Nextera Handle sequences have been added and highlighted in bold. Primers for the on-target have been added for convenience. The table below is sorted by the CFD off-target score. Sites with very low CFD scores < 0.02 are unlikely to be cleaved, see our study [Haeussler et al. 2016](#), Figure 2.

In the protocol by Matthew Canver, Harvard, two PCRs are run: one PCR to amplify the potential off-target, then a second PCR to extend the handles with Illumina barcodes. Please [click here](#) to download the protocol. Alternatively, you can have a look at [Fu et al. 2014](#).

Name	Primer Sequence	Tm CFD Score
ontarget_mm0_exon_BAG1_chr9_33261091_F	TCGTCGGCAGCGCTCTGATTCTGAGGATTCTGATGGA	57.0 1.00
ontarget_mm0_exon_BAG1_chr9_33261091_R	GTCTCGTGGGCTCGGGGAAACACCGTTGTGACGAC	59.9 1.00
mm2_exon_AF241726.2_chrX_38338750_F	Primer3: not found at this Tm	N.d. 0.64
mm2_exon_AF241726.2_chrX_38338750_R	Primer3: not found at this Tm	N.d. 0.64
mm3_intron_DPP10_chr2_115815065_F	Primer3: not found at this Tm	N.d. 0.31
mm3_intron_DPP10_chr2_115815065_R	Primer3: not found at this Tm	N.d. 0.31
mm4_intergenic_AC007682.1 AC097463.2_chr2_52130029_F	TCGTCGGCAGCGTCCAGTCAGTCATCTCAGAGAAGACA	59.5 0.24
mm4_intergenic_AC007682.1 AC097463.2_chr2_52130029_R	GTCTCGTGGGCTCGGTGTGCTCTGCCAATTGTGT	58.5 0.24
mm4_intergenic_TDGF1P6 MSL2_chr3_135909466_F	TCGTCGGCAGCGCTCTGTCCGAGTTTGACCATGA	59.5 0.17
mm4_intergenic_TDGF1P6 MSL2_chr3_135909466_R	GTCTCGTGGGCTCGGTCAAAATGATGTAACAAAGTCTGATGT	57.0 0.17
mm4_intron_RAPGEF1_chr9_134563802_F	TCGTCGGCAGCGTCCAAATAAACAGCAACAAGTGTC	59.2 0.13
mm4_intron_RAPGEF1_chr9_134563802_R	GTCTCGTGGGCTCGGGCCAGAAATGTAGGCCCTTG	58.8 0.13
mm4_intergenic_RN7SKP225 KRT8P11_chr9_102055912_F	TCGTCGGCAGCGTCTCTGGAAGATCCATATGCTCTTCA	57.8 0.13
mm4_intergenic_RN7SKP225 KRT8P11_chr9_102055912_R	GTCTCGTGGGCTCGGCCAGCAACGGTGTGACTA	60.2 0.13
mm4_exon_MCF2L_chr13_113696607_F	TCGTCGGCAGCGTCCACACCTTCTGCAGCTGAG	60.9 0.08
mm4_exon_MCF2L_chr13_113696607_R	GTCTCGTGGGCTCGGTCTCAACGAGAAGGACGGAC	59.1 0.08
mm4_intergenic_RBMV2DP REREP2Y_chrY_28289824_F	TCGTCGGCAGCGTCTCCCACTGGCTAAGTGGA	59.8 0.06
mm4_intergenic_RBMV2DP REREP2Y_chrY_28289824_R	GTCTCGTGGGCTCGGGGAAGATGGCATTCTCCCC	60.1 0.06
mm4_intergenic_RBMV2BP AC016694.2_chrY_24815388_F	TCGTCGGCAGCGTCTCCCACTGGCTAAGTGGA	59.8 0.06
mm4_intergenic_RBMV2BP AC016694.2_chrY_24815388_R	GTCTCGTGGGCTCGGGGAAGATGGCATTCTCCCC	60.1 0.06
mm4_intergenic_AC009947.2 AC009947.3_chrY_25673062_F	TCGTCGGCAGCGTCCGGAAGATGGCATTCTCCCC	60.1 0.06
mm4_intergenic_AC009947.2 AC009947.3_chrY_25673062_R	GTCTCGTGGGCTCGGTCCCACTGGCTAAGTGGA	59.8 0.06
mm4_intron_LINC01162_chr7_21017485_F	TCGTCGGCAGCGTCTGTGTCTGCTCATTTTCGGA	59.9 0.06
mm4_intron_LINC01162_chr7_21017485_R	GTCTCGTGGGCTCGGAATATCTGGAGCTAAAAGGTGA	57.0 0.06
mm4_intergenic_AC007967.3 AC007967.4_chrY_8795899_F	Primer3: not found at this Tm	N.d. 0.04
mm4_intergenic_AC007967.3 AC007967.4_chrY_8795899_R	Primer3: not found at this Tm	N.d. 0.04
mm4_intergenic_RP11-390E23.6 RNU6-11P_chr7_123395701_F	TCGTCGGCAGCGTCAGGGCTTGACAAACAGCAA	60.3 0.02
mm4_intergenic_RP11-390E23.6 RNU6-11P_chr7_123395701_R	GTCTCGTGGGCTCGGCTGGACACCTGAGCTCACTC	59.7 0.02
mm4_intergenic_TRDN AL354936.1_chr6_123961241_F	Primer3: not found at this Tm	N.d. 0.02
mm4_intergenic_TRDN AL354936.1_chr6_123961241_R	Primer3: not found at this Tm	N.d. 0.02
mm4_intergenic_AC016716.2 AC016716.1_chr2_80315521_F	TCGTCGGCAGCGTCTCCGAACCTCACTGCTACC	59.8 0.02
mm4_intergenic_AC016716.2 AC016716.1_chr2_80315521_R	GTCTCGTGGGCTCGGAGGACAAGACAGCCTTAGG	58.7 0.02
mm4_intron_FARS2_chr6_5419670_F	Primer3: not found at this Tm	N.d. 0.02
mm4_intron_FARS2_chr6_5419670_R	Primer3: not found at this Tm	N.d. 0.02
mm4_intergenic_RP11-398J5.1 RBFOX3_chr17_77177418_F	TCGTCGGCAGCGTCAAAACAGAGCTACGCTTCC	60.0 0.00
mm4_intergenic_RP11-398J5.1 RBFOX3_chr17_77177418_R	GTCTCGTGGGCTCGGCTCCGGTTTCCATGAGCA	60.3 0.00

Table E.1 : The list of the most possible 3 different off-targets determined by the CRISPOR web tool.

PCR primers for off-targets of AAGGAAAGGCTTGGTAAAAA AGG

In the table below, Illumina Nextera Handle sequences have been added and highlighted in bold. Primers for the on-target have been added for convenience. The table below is sorted by the CFD off-target score. Sites with very low CFD scores < 0.02 are unlikely to be cleaved, see our study [Haeussler et al. 2016](#), Figure 2.

In the protocol by Matthew Canver, Harvard, two PCRs are run: one PCR to amplify the potential off-target, then a second PCR to extend the handles with Illumina barcodes. Please [click here](#) to download the protocol. Alternatively, you can have a look at [Fu et al, 2014](#).

Name	Primer Sequence	Tm	CFD Score
ontarget_mm0_exon_BAG1_chr9_33255867_F	TCGTGGGAGCGTCACACCTTGTGTCGACTATT	57.4	1.00
ontarget_mm0_exon_BAG1_chr9_33255867_R	GTCTCGTGGGCTCGGACTCTTCATAGATCTGCCAG	59.0	1.00
mm2_intergenic_RPL31P12 RNU6-1246P_chr1_73056650_F	TCGTGGGAGCGTCACCTGGCAATATTCTCAGCCA	59.4	0.78
mm2_intergenic_RPL31P12 RNU6-1246P_chr1_73056650_R	GTCTCGTGGGCTCGGACATGAACCTCTCTGTGGCA	57.0	0.78
mm4_exon_NHSL2_chrX_71131204_F	Primer3: not found at this Tm	N.d.	0.56
mm4_exon_NHSL2_chrX_71131204_R	Primer3: not found at this Tm	N.d.	0.56
mm3_intergenic_TPBGUUBE3D_chr6_83241003_F	Primer3: not found at this Tm	N.d.	0.54
mm3_intergenic_TPBGUUBE3D_chr6_83241003_R	Primer3: not found at this Tm	N.d.	0.54
mm4_intron{EIF4G3_chr1_21138501_F	Primer3: not found at this Tm	N.d.	0.39
mm4_intron{EIF4G3_chr1_21138501_R	Primer3: not found at this Tm	N.d.	0.39
mm4_intergenic_KCND2 RNU6-581P_chr7_120065035_F	Primer3: not found at this Tm	N.d.	0.37
mm4_intergenic_KCND2 RNU6-581P_chr7_120065035_R	Primer3: not found at this Tm	N.d.	0.37
mm4_intergenic_NKTR RP4-613B23.1_chr3_42665046_F	Primer3: not found at this Tm	N.d.	0.36
mm4_intergenic_NKTR RP4-613B23.1_chr3_42665046_R	Primer3: not found at this Tm	N.d.	0.36
mm4_intergenic_Y_RNA TDPX2_chr9_12963877_F	Primer3: not found at this Tm	N.d.	0.35
mm4_intergenic_Y_RNA TDPX2_chr9_12963877_R	Primer3: not found at this Tm	N.d.	0.35
mm4_intergenic_RNA5SP189 CTC-278L1.1_chr5_105567456_F	TCGTGGGAGCGTCTCTCCAGTCTCACTATGTGGG	58.2	0.34
mm4_intergenic_RNA5SP189 CTC-278L1.1_chr5_105567456_R	GTCTCGTGGGCTCGGAGAGCATGTACCTACAGACAAATGT	59.5	0.34
mm4_intron_RP11-115J16.1_chr8_9185642_F	Primer3: not found at this Tm	N.d.	0.32
mm4_intron_RP11-115J16.1_chr8_9185642_R	Primer3: not found at this Tm	N.d.	0.32
mm4_intron_PCDP1_chr2_120352999_F	TCGTGGGAGCGTGGCTTTAGGATTATAGCCACTTCCC	58.9	0.32
mm4_intron_PCDP1_chr2_120352999_R	GTCTCGTGGGCTCGGCTTCAGCTCAACTACCCCTCTCT	60.0	0.32
mm4_intergenic_LINC00448 LINC00376_chr13_63525951_F	TCGTGGGAGCGTCACAGCCTGAATAAGAGGACAA	57.3	0.30
mm4_intergenic_LINC00448 LINC00376_chr13_63525951_R	GTCTCGTGGGCTCGGAGTCCACTTACCTATAAACTGGCA	59.2	0.30
mm4_exon_RGS17_chr6_153332543_F	TCGTGGGAGCGTCTGGCAATTCATTGTTTGTGTGG	59.1	0.29
mm4_exon_RGS17_chr6_153332543_R	GTCTCGTGGGCTCGGCTTCCTCAGGCTACTGTGA	59.7	0.29
mm4_intron_AUTS2_chr7_69257650_F	Primer3: not found at this Tm	N.d.	0.29
mm4_intron_AUTS2_chr7_69257650_R	Primer3: not found at this Tm	N.d.	0.29
mm4_intron_AF240627.2_chr21_20003392_F	TCGTGGGAGCGTCAACTTGGAAATTTGGCAACCTT	57.4	0.29
mm4_intron_AF240627.2_chr21_20003392_R	GTCTCGTGGGCTCGGACACACACTCAGCATATGC	58.9	0.29
mm3_intergenic_TENM2 CTB-178M22.1_chr5_167496874_F	Primer3: not found at this Tm	N.d.	0.29
mm3_intergenic_TENM2 CTB-178M22.1_chr5_167496874_R	Primer3: not found at this Tm	N.d.	0.29
mm4_intron_ABCC5_chr3_183734575_F	Primer3: not found at this Tm	N.d.	0.28
mm4_intron_ABCC5_chr3_183734575_R	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_TMEM106B VWDE_chr7_12289759_F	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_TMEM106B VWDE_chr7_12289759_R	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_HSPA13 SAMSNI_chr21_15767755_F	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_HSPA13 SAMSNI_chr21_15767755_R	Primer3: not found at this Tm	N.d.	0.28
mm4_intergenic_RP11-1L9.1 EGFL6_chrX_13561914_F	TCGTGGGAGCGTCTGAAAATAAGAGTAGAAGCAGGGT	57.6	0.27
mm4_intergenic_RP11-1L9.1 EGFL6_chrX_13561914_R	GTCTCGTGGGCTCGGTCTCCCTGAAGATAACCACTGT	58.2	0.27
mm3_intron_MDS2_chr1_23934544_F	Primer3: not found at this Tm	N.d.	0.26
mm3_intron_MDS2_chr1_23934544_R	Primer3: not found at this Tm	N.d.	0.26
mm3_intergenic_ATXN8OS RNU6-54P_chr13_70878232_F	Primer3: not found at this Tm	N.d.	0.26
mm3_intergenic_ATXN8OS RNU6-54P_chr13_70878232_R	Primer3: not found at this Tm	N.d.	0.26
mm4_intergenic_CYBAP5 NOVA1_chr14_26897468_F	TCGTGGGAGCGTCACAGAAAGTATGACTGAGGCAGA	59.1	0.25

<http://crispor.tefor.net/crispor.py?batchId=EXumdCQ0w0bE5mAGt2ak&pamId=s55%2B&otPrimers=1>

APPENDIX F: The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19

Table F.1 : The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19.

Geneid	WT Count-1	WT- Count 2	Bag-1 KO 1	Bag-1 KO 2
hsa-miR-200b-5p	2	2	1	1
hsa-miR-30e-5p	107	116	49	54
hsa-miR-30e-3p	8	9	10	12
hsa-miR-186-5p	77	82	49	62
hsa-miR-760	3	3	2	2
hsa-miR-190b	1	1	2	2
hsa-miR-92b-5p	3	3	0	1
hsa-miR-92b-3p	77	82	21	29
hsa-miR-181b-5p	7	8	5	5
hsa-miR-181a-3p	6	9	2	3
hsa-miR-1296-5p	1	1	2	2
hsa-miR-107	30	33	7	8
hsa-miR-146b-5p	97	104	26	27
hsa-miR-1307-3p	42	45	34	37
hsa-miR-1307-5p	2	3	8	8
hsa-miR-210-3p	2	2	1	1
hsa-miR-192-5p	55	59	30	31
hsa-miR-548k	5	7	3	3
hsa-miR-708-5p	5	6	6	7
hsa-miR-708-3p	1	1	1	1
hsa-let-7a-5p	19	19	9	10
hsa-miR-196a-5p	2	2	15	20
hsa-miR-148b-3p	72	81	69	69
hsa-let-7i-5p	1071	1187	40	47
hsa-miR-331-3p	1	1	7	7
hsa-miR-15a-5p	7	7	4	6
hsa-miR-17-5p	24	27	81	89
hsa-miR-18a-5p	1	1	13	14
hsa-miR-19a-3p	5	5	85	93
hsa-miR-20a-5p	21	22	49	52
hsa-miR-92a-1-5p	3	3	15	16
hsa-miR-92a-3p	6	7	6	9
hsa-miR-342-5p	32	35	1	1
hsa-miR-342-3p	14	15	2	2
hsa-miR-345-5p	34	38	2	2
hsa-miR-629-5p	6	6	15	15
hsa-miR-7706	2	3	1	1
hsa-miR-1276	1	1	1	1
hsa-miR-7-5p	2	3	2	2
hsa-miR-940	1	1	4	5
hsa-miR-193b-3p	6	8	4	5

Table F.1 (continued): The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19.

hsa-miR-484	6	7	2	3
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hsa-miR-328-3p	1	1	1	1
hsa-miR-140-3p	12	13	9	9
hsa-miR-1910-5p	3	4	1	1
hsa-miR-22-3p	189	201	19	22
hsa-miR-132-3p	3	3	3	3
hsa-miR-324-5p	2	2	5	5
hsa-miR-324-3p	1	1	4	5
hsa-miR-744-5p	28	32	20	23
hsa-miR-1180-3p	3	2	9	9
hsa-miR-152-3p	8	10	1	1
hsa-miR-10a-5p	36	43	2251	2499
hsa-miR-454-5p	7	7	1	2
hsa-miR-454-3p	75	82	4	4
hsa-miR-301a-3p	94	104	3	3
hsa-miR-21-5p	19843	21669	138	168
hsa-miR-21-3p	335	366	3	3
hsa-miR-6516-5p	1	1	1	1
hsa-miR-3187-3p	1	1	2	2
hsa-miR-7974	2	2	3	4
hsa-miR-181c-5p	28	27	4	4
hsa-miR-181c-3p	1	1	1	1
hsa-miR-181d-5p	18	22	6	7
hsa-miR-769-5p	30	32	18	19
hsa-miR-99b-5p	134	139	19	21
hsa-miR-99b-3p	13	13	5	5
hsa-let-7e-5p	734	820	64	65
hsa-miR-125a-5p	198	231	24	25
hsa-miR-125a-3p	4	3	1	1
hsa-miR-3679-5p	1	1	1	1
hsa-miR-128-1-5p	3	3	2	2
hsa-miR-10b-5p	8	11	1453	1613
hsa-miR-26b-5p	213	231	21	28
hsa-miR-375	8	7	1	1
hsa-miR-149-5p	8	8	7	8
hsa-let-7c-5p	87	100	74	77
hsa-miR-125b-2-3p	3	3	1	1
hsa-miR-185-5p	7	7	9	10
hsa-miR-301b	2	2	4	4
hsa-miR-130b-5p	3	3	5	5
hsa-miR-130b-3p	3	3	7	7
hsa-miR-33a-5p	2	2	1	1
hsa-let-7b-5p	604	659	16	17

Table F.1 (continued): The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19.

hsa-miR-425-5p	61	65	28	30
hsa-miR-425-3p	9	10	5	6
hsa-miR-191-5p	360	392	105	117
hsa-miR-191-3p	1	1	1	1
hsa-let-7g-5p	536	595	68	73
hsa-miR-15b-5p	16	18	6	6
hsa-miR-15b-3p	4	4	2	2
hsa-miR-28-5p	2	2	1	1
hsa-miR-28-3p	16	17	5	4
hsa-miR-340-5p	14	15	11	11
hsa-miR-1275	2	2	1	1
hsa-miR-30a-5p	44	47	79	87
hsa-miR-30a-3p	2	1	8	10
hsa-miR-339-5p	2	2	2	2
hsa-miR-339-3p	13	14	2	2
hsa-miR-589-5p	12	14	6	6
hsa-miR-148a-5p	2	2	7	9
hsa-miR-148a-3p	58	63	45	50
hsa-miR-196b-5p	6	6	202	218
hsa-miR-3609	1	1	1	1
hsa-miR-25-5p	11	10	5	4
hsa-miR-25-3p	434	472	195	197
hsa-miR-93-5p	370	385	174	193
hsa-miR-106b-5p	31	37	14	15
hsa-miR-106b-3p	38	39	24	26
hsa-miR-182-5p	982	1057	83	89
hsa-miR-183-5p	78	91	8	8
hsa-miR-29a-3p	2	2	3	3
hsa-miR-671-5p	4	4	2	3
hsa-miR-671-3p	1	1	1	1
hsa-miR-598-3p	6	8	2	3
hsa-miR-320a	196	228	99	121
hsa-miR-7705	1	1	1	1
hsa-miR-30b-5p	26	29	12	14
hsa-miR-30d-5p	334	358	48	52
hsa-miR-151a-3p	32	40	4	4
hsa-let-7f-5p	70	79	2	2
hsa-let-7d-5p	523	584	7	7
hsa-miR-23b-3p	12	13	2	2
hsa-miR-27b-5p	13	13	1	1
hsa-miR-27b-3p	297	314	8	10
hsa-miR-181a-2-3p	1	1	3	3
hsa-miR-221-3p	22	23	24	26

Table F.1 (continued): The sequence counts for 144 miRNAs that were observed in either two biological replicates of wt and Bag-1 KO samples annotated in the hg19.

hsa-miR-222-3p	6	7	3	3
hsa-miR-532-5p	11	11	10	10
hsa-miR-500a-3p	1	1	1	1
hsa-miR-501-3p	1	1	2	2
hsa-miR-660-5p	6	9	1	1
hsa-miR-98-5p	576	640	16	15
hsa-miR-421	14	13	1	1
hsa-miR-361-5p	3	4	4	4
hsa-miR-361-3p	1	1	2	2
hsa-miR-652-3p	4	4	4	4
hsa-miR-652-5p	1	1	0	1
hsa-miR-542-3p	1	1	1	1
hsa-miR-503-5p	4	4	4	4
hsa-miR-424-3p	4	4	0	1
hsa-miR-504-5p	1	1	2	2
hsa-miR-505-3p	3	3	4	5

APPENDIX G: The sequence counts for 88 miRNAs of two biological replicates of wt samples annotated in the hg19

Table G.1 : The sequence counts for 88 miRNAs of two biological replicates of wt samples annotated in the hg19.

Geneid	WT Count-1	WT- Count 2	Bag-1 KO 1	Bag-1 KO 2
hsa-miR-200b-3p	83	89	0	0
hsa-miR-200a-5p	13	12	0	0
hsa-miR-200a-3p	178	190	0	0
hsa-miR-429	16	16	0	0
hsa-miR-34a-5p	53	56	0	0
hsa-miR-3115	1	1	0	0
hsa-miR-942-5p	1	1	0	0
hsa-miR-3121-3p	1	1	0	0
hsa-miR-205-5p	2	4	0	0
hsa-miR-664a-3p	1	1	0	0
hsa-miR-3124-5p	1	1	0	0
hsa-miR-1287-5p	3	6	0	0
hsa-miR-2110	1	1	0	0
hsa-miR-6743-5p	1	1	0	0
hsa-miR-200c-5p	1	1	0	0
hsa-miR-200c-3p	174	190	0	0
hsa-miR-141-5p	3	4	0	0
hsa-miR-141-3p	281	305	0	0
hsa-miR-1293	1	1	0	0
hsa-miR-148b-5p	6	5	0	0
hsa-miR-1251-5p	1	1	0	0
hsa-miR-4707-3p	1	1	0	0
hsa-miR-1247-5p	2	2	0	0
hsa-miR-1247-3p	1	1	0	0
hsa-miR-190a-5p	1	1	0	0
hsa-miR-193b-5p	3	4	0	0
hsa-miR-365a-5p	2	5	0	0
hsa-miR-195-5p	1	1	0	0
hsa-miR-33b-5p	3	3	0	0
hsa-miR-193a-5p	2	5	0	0
hsa-miR-4725-3p	1	1	0	0
hsa-miR-365b-5p	4	5	0	0
hsa-miR-6780a-5p	1	1	0	0
hsa-miR-301a-5p	7	9	0	0
hsa-miR-4525	1	1	0	0
hsa-miR-4526	1	1	0	0
hsa-miR-4746-5p	2	2	0	0
hsa-miR-3940-5p	1	1	0	0
hsa-miR-24-2-5p	2	2	0	0
hsa-miR-27a-5p	4	5	0	0
hsa-miR-27a-3p	16	20	0	0

Table G.1 (continued): The sequence counts for 88 miRNAs of two biological replicates of wt samples annotated in the hg19.

hsa-miR-23a-3p	8	10	0	0
hsa-miR-3188	1	2	0	0
hsa-miR-330-3p	4	4	0	0
hsa-let-7e-3p	1	1	0	0
hsa-miR-1301-3p	1	1	0	0
hsa-miR-3127-5p	2	2	0	0
hsa-miR-128-3p	1	1	0	0
hsa-miR-26b-3p	1	1	0	0
hsa-miR-1292-5p	1	1	0	0
hsa-miR-296-3p	15	15	0	0
hsa-miR-99a-5p	2	2	0	0
hsa-miR-4760-5p	1	1	0	0
hsa-miR-185-3p	2	2	0	0
hsa-miR-6818-5p	1	1	0	0
hsa-miR-3200-5p	1	1	0	0
hsa-miR-3909	3	3	0	0
hsa-miR-4446-3p	5	5	0	0
hsa-miR-16-2-3p	4	5	0	0
hsa-miR-95-3p	2	2	0	0
hsa-miR-1255a	1	1	0	0
hsa-miR-4458	1	1	0	0
hsa-miR-2277-3p	1	1	0	0
hsa-miR-143-3p	7	7	0	0
hsa-miR-4466	1	1	0	0
hsa-miR-589-3p	1	1	0	0
hsa-miR-548n	1	1	0	0
hsa-miR-590-3p	1	1	0	0
hsa-miR-653-5p	24	29	0	0
hsa-miR-653-3p	4	6	0	0
hsa-miR-489-3p	47	50	0	0
hsa-miR-96-5p	8	8	0	0
hsa-miR-335-5p	2	3	0	0
hsa-miR-335-3p	3	4	0	0
hsa-miR-597-3p	2	2	0	0
hsa-miR-30b-3p	1	1	0	0
hsa-miR-937-3p	1	2	0	0
hsa-miR-4665-5p	1	1	0	0
hsa-let-7d-3p	4	6	0	0
hsa-miR-32-3p	5	5	0	0
hsa-miR-126-5p	7	6	0	0
hsa-miR-651-5p	1	1	0	0
hsa-miR-98-3p	2	2	0	0
hsa-miR-1468-5p	1	1	0	0
hsa-miR-374a-5p	1	1	0	0

Table G.1 (continued): The sequence counts for 88 miRNAs of two biological replicates of wt samples annotated in the hg19.

hsa-miR-374a-3p	2	2	0	0
hsa-miR-363-3p	1	1	0	0
hsa-miR-424-5p	1	1	0	0



APPENDIX H: The sequence counts for 77 miRNAs of two biological replicates of wt samples annotated in the hg19

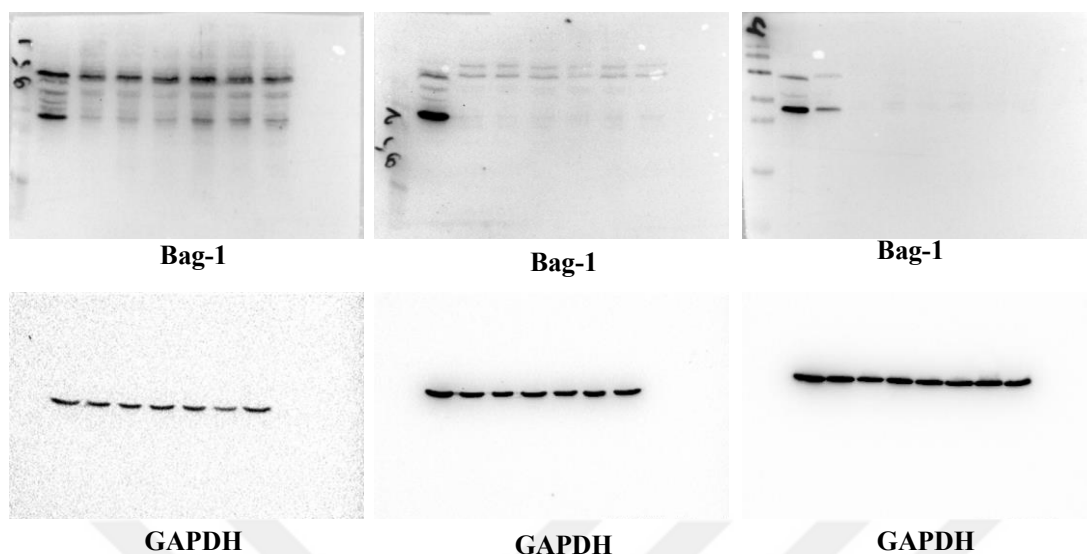
Table H.1 : The sequence counts for 77 miRNAs of two biological replicates of wt samples annotated in the hg19.

Geneid	WT Count-1	WT Count-2	Bag-1 KO- 1	Bag-1 KO-2
hsa-miR-197-3p	0	0	8	8
hsa-miR-29b-2-5p	0	0	2	2
hsa-miR-194-5p	0	0	1	1
hsa-miR-4677-3p	0	0	1	1
hsa-miR-5699-5p	0	0	1	1
hsa-miR-346	0	0	2	2
hsa-miR-3944-3p	0	0	1	1
hsa-miR-483-5p	0	0	1	1
hsa-miR-196a-3p	0	0	1	1
hsa-miR-615-3p	0	0	23	28
hsa-miR-6761-3p	0	0	1	1
hsa-miR-17-3p	0	0	3	3
hsa-miR-18a-3p	0	0	2	2
hsa-miR-4713-5p	0	0	1	1
hsa-miR-628-5p	0	0	1	1
hsa-miR-6882-5p	0	0	1	1
hsa-miR-3176	0	0	2	2
hsa-miR-4519	0	0	1	1
hsa-miR-4521	0	0	5	7
hsa-miR-193a-3p	0	0	2	2
hsa-miR-10a-3p	0	0	1	1
hsa-miR-4745-3p	0	0	1	1
hsa-miR-330-5p	0	0	1	1
hsa-miR-769-3p	0	0	2	2
hsa-miR-320e	0	0	1	1
hsa-miR-935	0	0	1	2
hsa-miR-1257	0	0	1	1
hsa-miR-4326	0	0	1	1
hsa-miR-1306-3p	0	0	1	1
hsa-miR-33a-3p	0	0	1	1
hsa-miR-3619-5p	0	0	2	2
hsa-miR-1226-5p	0	0	1	1
hsa-miR-4787-3p	0	0	3	3
hsa-miR-551b-5p	0	0	1	1
hsa-miR-1224-5p	0	0	1	1
hsa-miR-218-1-3p	0	0	1	1
hsa-miR-574-3p	0	0	1	1
hsa-miR-887-3p	0	0	6	6
hsa-miR-887-5p	0	0	1	1

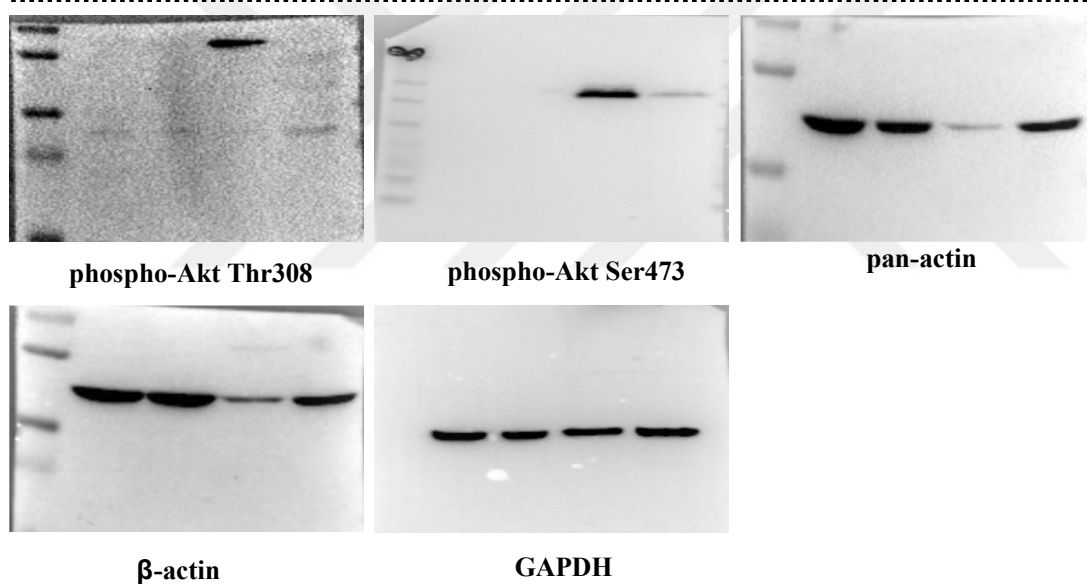
Table H.1 (continued): The sequence counts for 77 miRNAs of two biological replicates of wt samples annotated in the hg19.

hsa-miR-449a	0	0	2	2
hsa-miR-3607-3p	0	0	1	1
hsa-miR-874-5p	0	0	1	1
hsa-miR-378a-5p	0	0	1	1
hsa-miR-1294	0	0	1	1
hsa-miR-585-3p	0	0	1	1
hsa-miR-340-3p	0	0	2	2
hsa-miR-3691-5p	0	0	1	1
hsa-miR-30c-2-3p	0	0	1	1
hsa-miR-548b-3p	0	0	1	1
hsa-miR-196b-3p	0	0	1	1
hsa-miR-4467	0	0	1	1
hsa-miR-592	0	0	1	1
hsa-miR-183-3p	0	0	1	1
hsa-miR-383-5p	0	0	3	3
hsa-miR-4661-5p	0	0	2	2
hsa-miR-31-5p	0	0	6	6
hsa-miR-31-3p	0	0	1	1
hsa-miR-873-5p	0	0	5	6
hsa-miR-873-3p	0	0	1	1
hsa-miR-204-5p	0	0	1	1
hsa-miR-455-5p	0	0	1	1
hsa-miR-6877-5p	0	0	1	2
hsa-miR-221-5p	0	0	1	1
hsa-miR-362-5p	0	0	1	1
hsa-miR-501-5p	0	0	1	1
hsa-miR-502-3p	0	0	1	1
hsa-miR-6894-5p	0	0	1	1
hsa-miR-676-5p	0	0	1	1
hsa-miR-766-3p	0	0	1	1
hsa-miR-452-5p	0	0	1	1
hsa-miR-767-5p	0	0	5	6

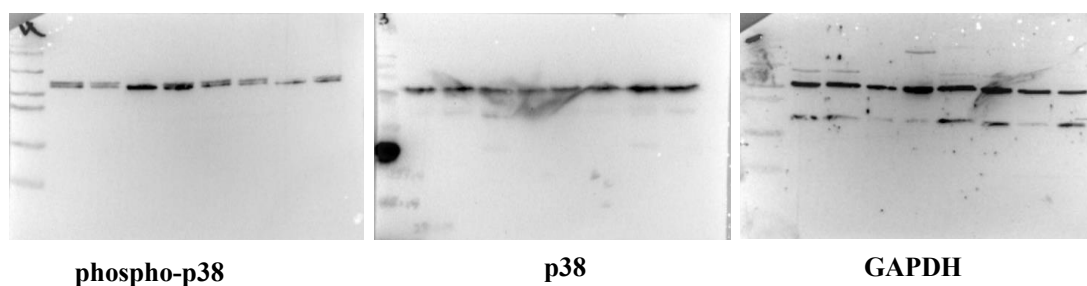
APPENDIX I: Uncropped western blotting images of the results



Uncropped Western Blot Figures of Figure 2.23.

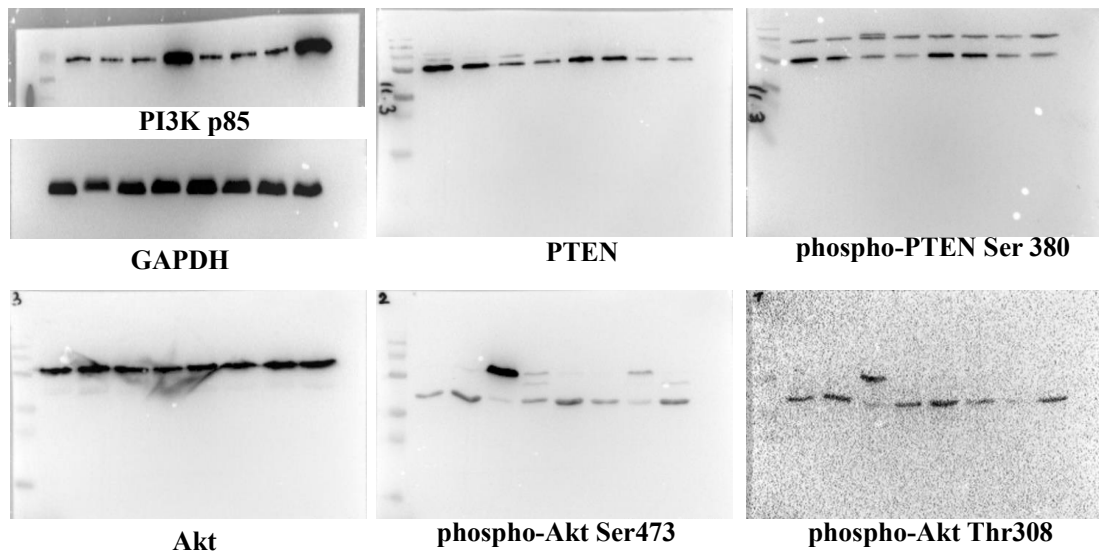


Uncropped Western Blot Figures of Figure 2.40.

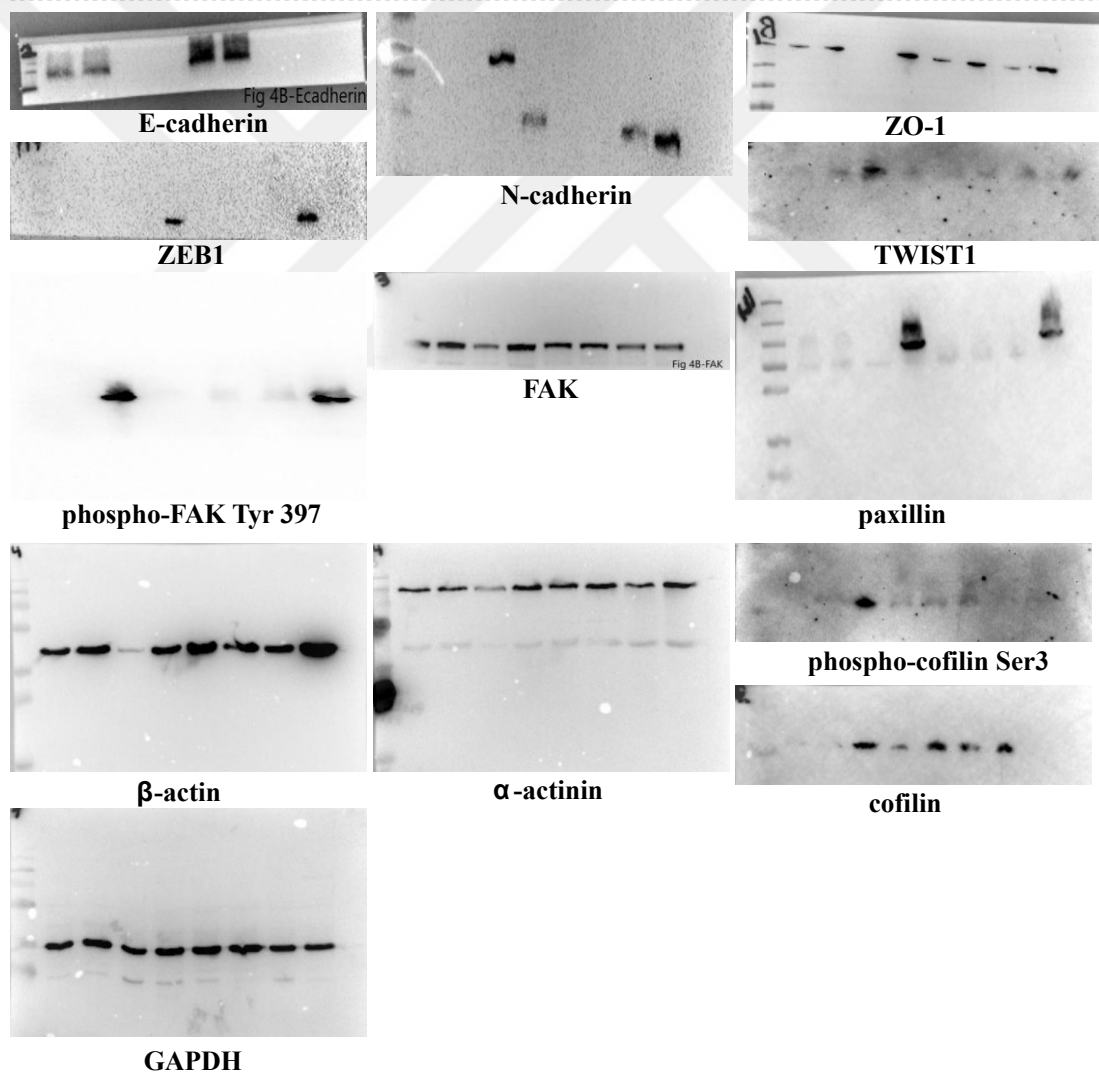


Uncropped Western Blot Figures of Figure 2.44.

Figure I.1 : Uncropped western blotting images of the results.

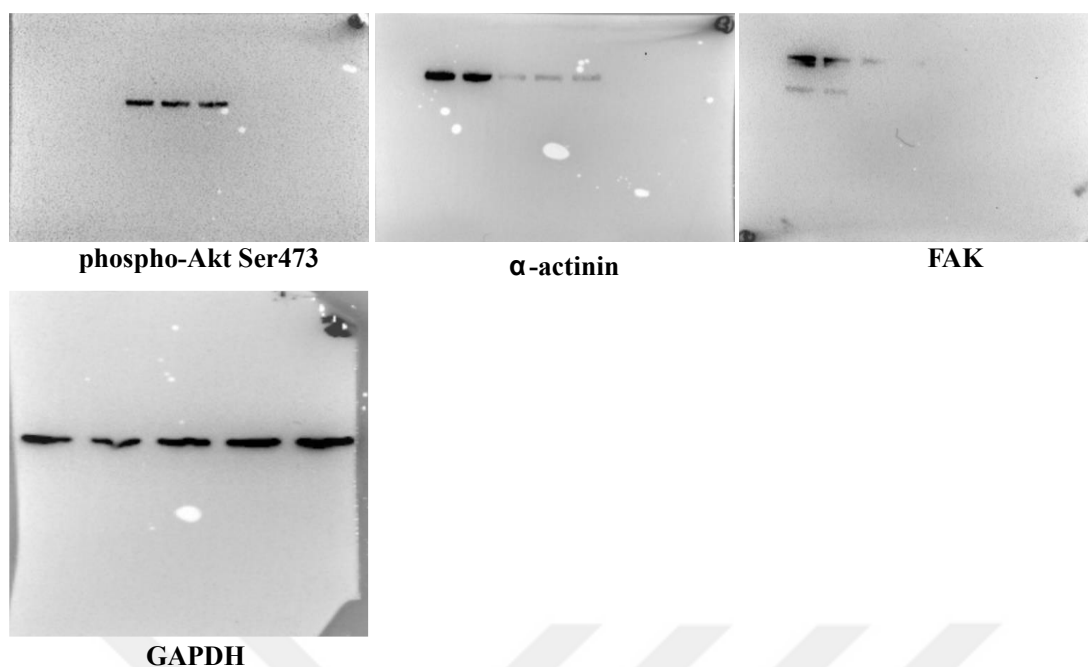


Uncropped Western Blot Figures of Figure 2.45.

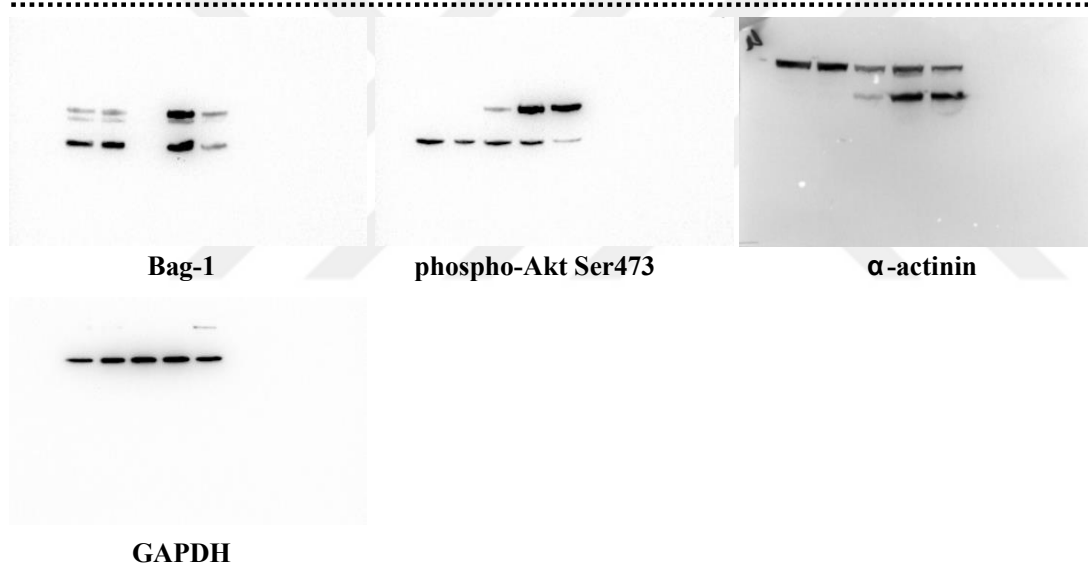


Uncropped Western Blot Figures of Figure 2.46.

Figure I.1 (continued): Uncropped western blotting images of the results.

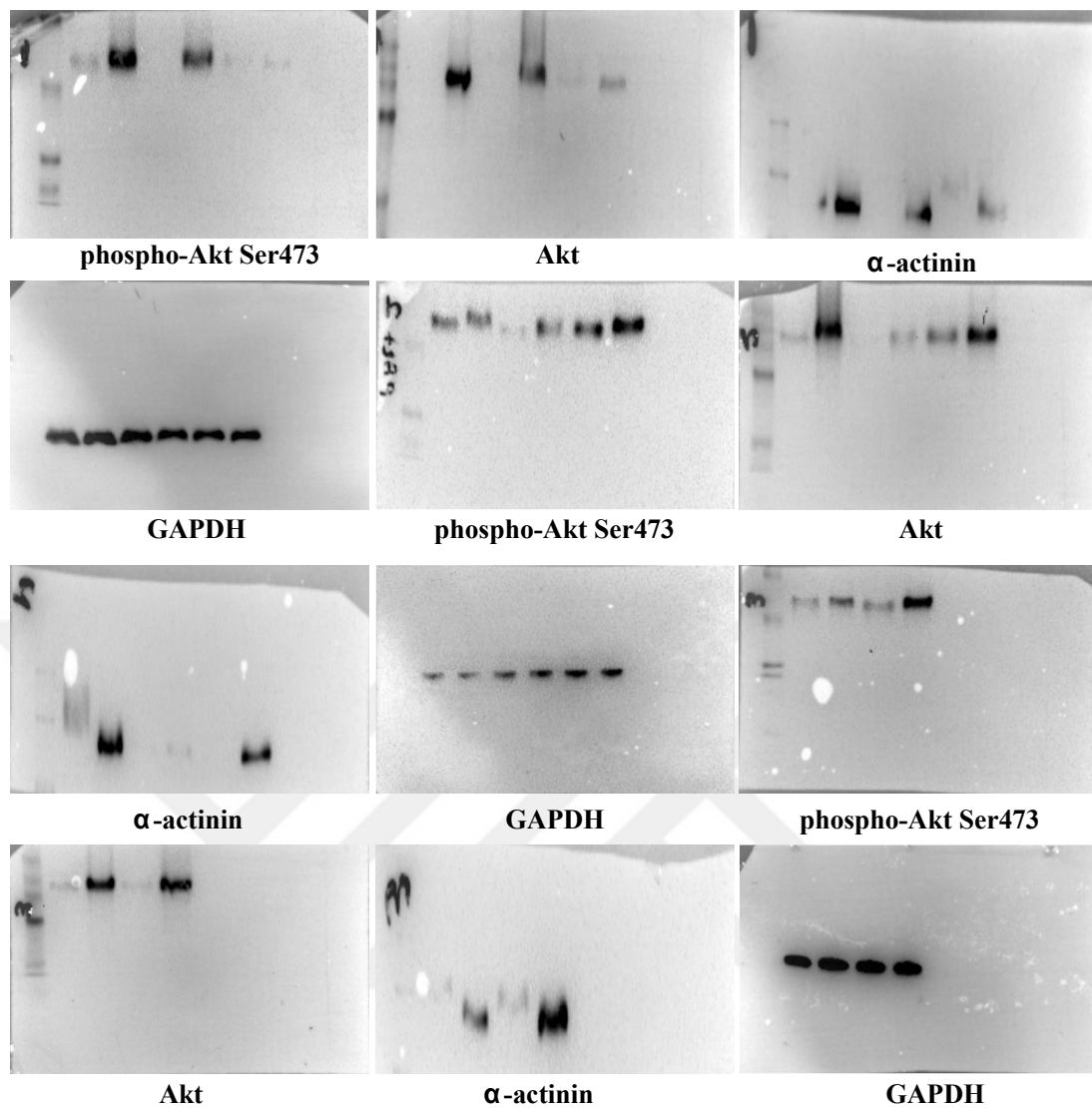


Uncropped Western Blot Figures of Figure 2.50.



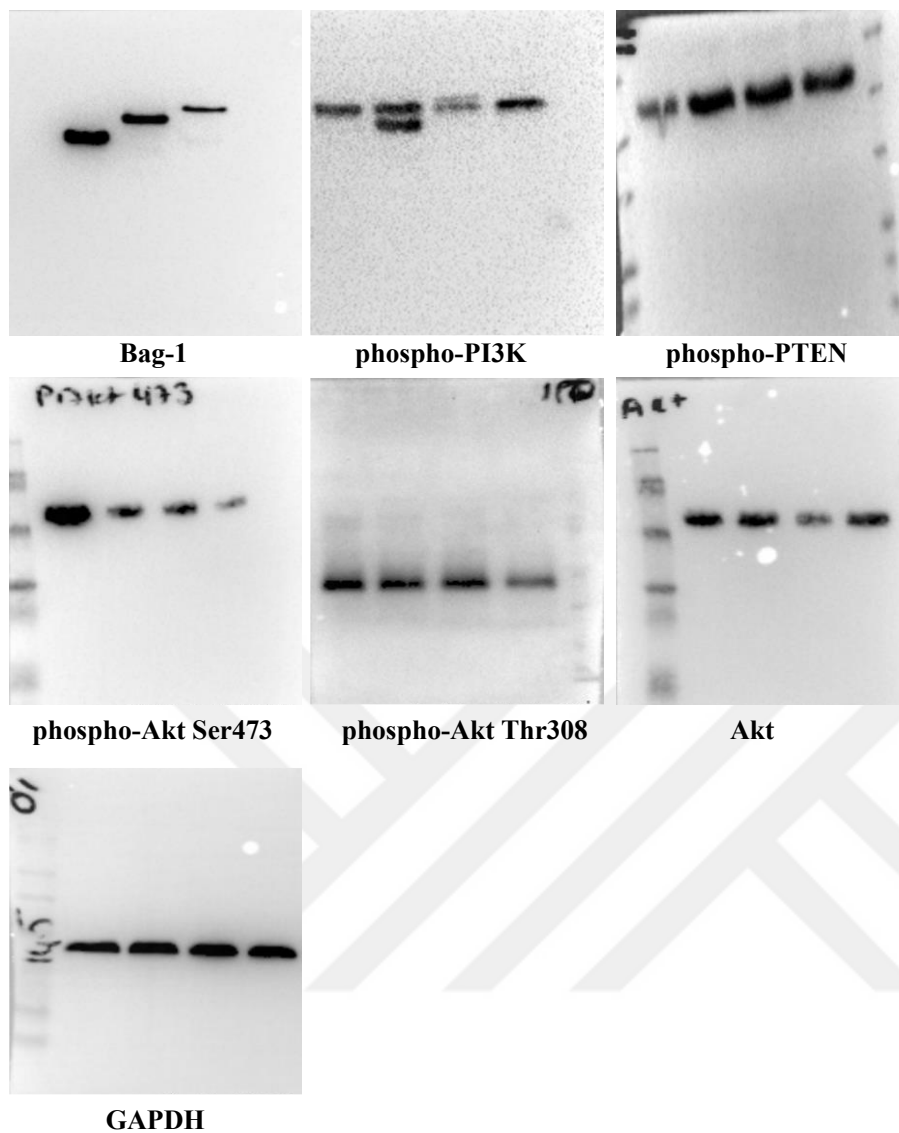
Uncropped Western Blot Figures of Figure 2.51.

Figure I.1 (continued): Uncropped western blotting images of the results.



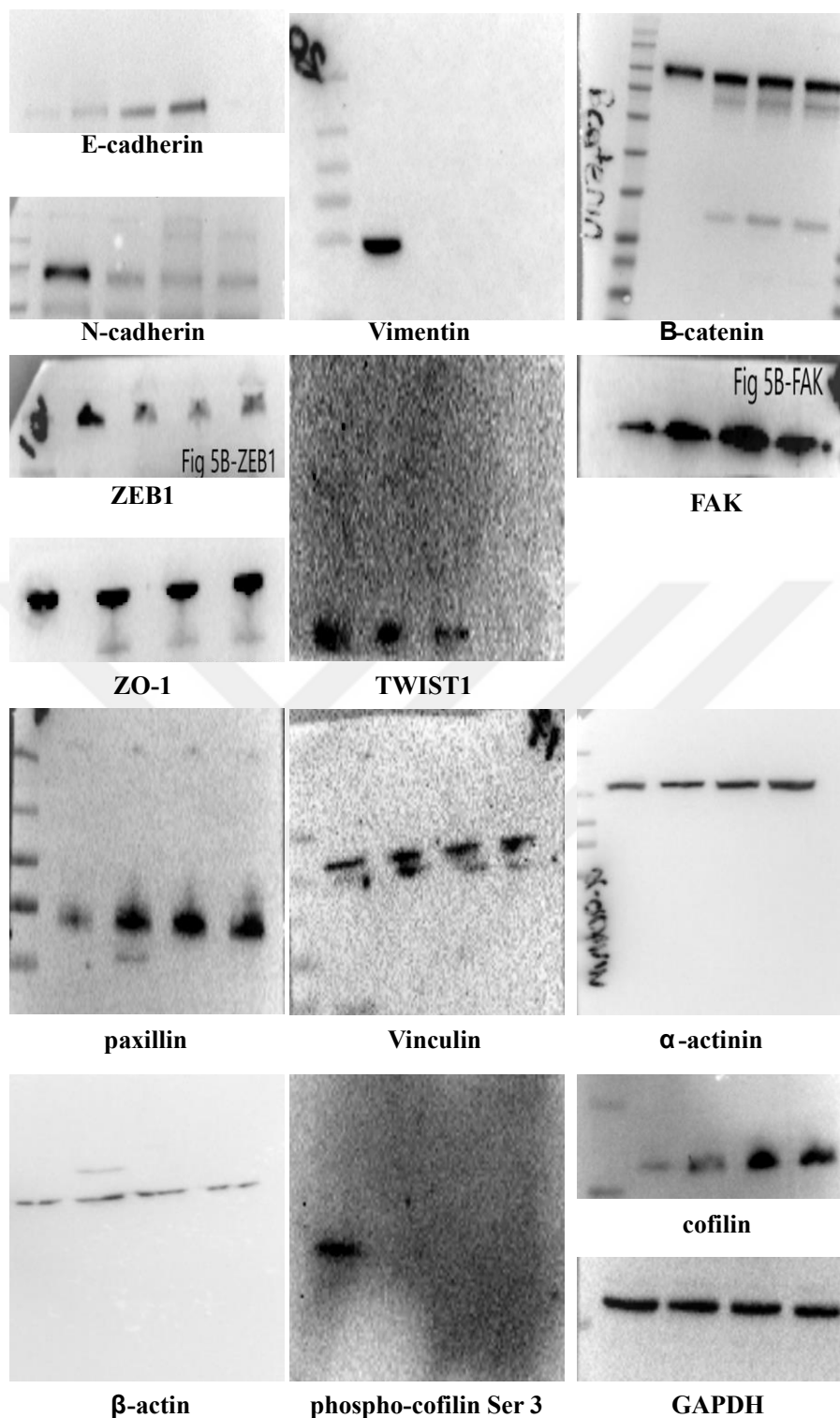
Uncropped Western Blot Figures of Figure 2.52.

Figure I.1 (continued): Uncropped western blotting images of the results.



Uncropped Western Blot Figures of Figure 2.54.

Figure I.1 (continued): Uncropped western blotting images of the results.



Uncropped Western Blot Figures of Figure 2.55.

Figure I.1 (continued): Uncropped western blotting images of the results.

CURRICULUM VITAE

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EDUCATION :

- **B.Sc.** : 2009, Istanbul University, Faculty of Science, Department of Biology
- **M.Sc.** : 2013, Istanbul Technical University, Graduate School of Science Engineering and Technology, Molecular Biology Genetics and Biotechnology

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2011- Research assistant at Istanbul Kultur University, Molecular Biology and Genetics Department

PUBLICATIONS AND PRESENTATIONS ON THE THESIS:

- **Ozfiliz-Kilbas P.**, Can ND., Kizilboga T., Ezberci F., Doganay HL., Arisan ED., Dinler-Doganay G. 2021: CRISPR/Cas9-Mediated Bag-1 knockout increased mesenchymal characteristics of MCF-7 cells via Akt hyperactivation-mediated actin cytoskeleton remodeling, *PLoS One*, (Research Article)
- **Ozfiliz-Kilbas P.**, Alkurt G., Obakan-Yerlikaya P., Coker-Gurkan A., Dinler-Doganay G., Arisan ED. 2021: The comparison of differentially expressed microRNAs in Bag-1 deficient and wild type MCF-7 breast cancer cells by small RNA sequencing, *Turk J Biol*, 10.3906/biy-2109-48. (Research Article)
- **Ozfiliz-Kilbas P.**, Arisan ED., Dinler-Doganay G. 2019: Actin disorganization is the major outcome of CRISPR-Cas9- mediated Bag-1 knockout MCF-7 breast cancer cells. International Congress- 7th International Congress of the Molecular Biology Association of Turkey, September 27-29 2019, Istanbul, Turkey. (Poster Presentation)
- **Ozfiliz-Kilbas P.**, Arisan ED., Dinler-Doganay G. 2019: Revealing the functional role of Bag-1 in MCF-7 breast cancer cells through CRISPR/Cas9-mediated gene knockout. International Congress- The 44th FEBS Congress, July 6-11 2019, Krakow, Poland. (Poster Presentation)
- **Ozfiliz-Kilbas P.**, Arisan ED., Dinler-Doganay G., Palavan-Unsal N. 2017: Bag-1 silencing enhanced apoptosis via altering the ubiquitination process of Foxo3a in MCF-7 breast cancer cells. International Congress- 22nd World Congress on

Advances in Oncology & the 20th Symposium on Molecular Medicine, October 5-7 2017, Athens, Greece. (Oral Presentation)

- **Özfiliz-Kilbas P.**, Arisan ED., Dinler-Doganay G. 2016: Knock-down of anti-apoptotic Bag-1 modulated the regulation of cell survival pathways in breast cancer. International Congress- 41st FEBS Congress, Molecular and Systems Biology for a Better Life, September 3-8, 2016, Kuşadası, Turkey. (Poster Presentation)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

Articles published in international peer-reviewed journals:

- **Özfiliz Kilbaş Pelin**, Sönmez Özlem, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Ünsal Zeynep Narçin, Uysal-Onganer Pinar, Arisan Elif Damla (2021). In Vitro Investigations of miR-33a Expression in Estrogen Receptor-Targeting Therapies in Breast Cancer Cells. *Cancers*, 13, Doi: 10.3390/cancers13215322 (Kontrol No: 7257193)
- Can Nisan Denizce, Baştürk Ezgi, Kizilboga Tuğba, Akçay İzzet Mehmet, Dingiloğlu Baran, Tatlı Özge, Acar Sevilay, **Özfiliz Kilbaş Pelin**, Elbeyli Efe, Muratçioğlu Serena, Tarbin Januzzi Ayşe, Gürsoy Atilla, Keskin Özkaya Zehra Özlem, Doğanay Hamdi Levent, Karademir Yılmaz Betül, Dinler Doğanay Gizem (2021). Interactome analysis of Bag-1 isoforms reveals novel interaction partners in endoplasmic reticulum-associated degradation. *PLoS One*, Doi: 10.1371/journal.pone.0256640
- Turk Miray, Tatlı Özge, Alkan Hamza Furkan, Alkurt Gizem, **Özfiliz Kilbaş Pelin**, Dinler Doğanay Gizem (2021). Co-Chaperone Bag-1 Plays a Role in the Autophagy-Dependent Cell Survival through Beclin 1 Interaction. *Molecules*, 26, Doi: 10.3390/molecules26040854
- **Özfiliz Kilbaş Pelin**, Sönmez Özlem, Uysal Onganer Pinar, Çoker Gürkan Ajda, Obakan Yerlikaya Pinar, Arisan Elif Damla (2020). Specific c-Jun N-terminal kinase inhibitor, JNK-IN-8 suppresses mesenchymal profile of PTX-resistant MCF-7 cells through modulating PI3K/Akt, MAPK, and Wnt signaling pathways. *Biology*, 10.3390/biology9100320
- **Özfiliz Kilbaş Pelin**, Akçay İzzet Mehmet, Dinler Doğanay Gizem, Arisan Elif Damla (2019). Bag-1 silencing enhanced chemotherapeutic drug-induced apoptosis in MCF-7 breast cancer cells affecting PI3K/Akt/mTOR and MAPK signaling pathways. *Molecular Biology Reports*, 46(1), 847-860., Doi: 10.1007/s11033-018-4540-x
- **Özfiliz Kilbaş Pelin**, Sarıkaya Bahar, Obakan-Yerlikaya Pinar, Çoker-Gürkan Ajda, Arisan Elif Damla, Temizci Benan, Ünsal Zeynep Narçin (2018). Cyclin-dependent kinase inhibitors, roscovitine, and purvalanol induce apoptosis and autophagy-related to unfolded protein response in HeLa cervical cancer cells. *Molecular Biology Reports*, 45(5), 815-828., Doi: 10.1007/s11033-018-4222-8
- **Özfiliz Pelin**, Kizilboğa Tuğba, Demir Salih, Alkurt Gizem, Ünsal Zeynep Narçin, Arisan Elif Damla, Dinler Doğanay Gizem (2015). Bag 1 promotes cell survival through c Myc mediated ODC upregulation that is not preferred under apoptotic stimuli in MCF 7 cells. *Cell Biochemistry and Function*, 33(5), 293-307., Doi: 10.1002/cbf.3114

- Çoker Gürkan Ajda, Arisan Elif Damla, Obakan Yerlikaya Pinar, **Özfiliz Pelin**, Kose Betsi, Bickici Guven, Palavan-Unsal Narcin (2015). Roscovitine-treated HeLa cells finalize autophagy later than apoptosis by downregulating Bcl-2. *Molecular Medicine Reports*, 11(3), 1968-1974., Doi: 10.3892/mmr.2014.2902
- **Özfiliz Pelin**, Arisan Elif Damla, Çoker Gürkan Ajda, Obakan Yerlikaya Pinar, Eralp Tuğçenur, Dinler Doğanay Gizem, Palavan-Ünsal Narçin (2014). Bag-1L is a stress-withstand molecule that prevents the downregulation of Mcl-1 and c-Raf under the control of heat shock proteins in cisplatin-treated HeLa cervix cancer cells. *Asian Pacific journal of cancer prevention : APJCP*, 15(11), 4475-82.
- Obakan Yerlikaya Pinar, **Özfiliz Pelin**, Arisan Elif Damla, Çoker Gürkan Ajda, Ünsal Zeynep Narçin (2014). Purvalanol A is a strong apoptotic inducer via activating polyamine catabolic pathway in MCF 7 estrogen receptor positive breast cancer cells. *Molecular Biology Reports*, 41(1), 145-154., Doi: 10.1007/s11033-013-2847-1.

Papers presented at international scientific meetings and published in proceedings books:

- **Özfiliz Kilbaş Pelin**, Sonmez Ozlem, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Ünsal Zeynep Narçin, Uysal-Onganer Pinar, Arisan Elif Damla (2021). In Vitro Investigations of miR-33a Expression in Estrogen Receptor-Targeting Therapies in Breast Cancer Cells. 5th Congress of SDIR: Translational Potential of Cancer Research in Serbia
- Kashani Sina, **Özfiliz Kilbas Pelin**, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Arisan Elif Damla (2019). Cisplatin and Paclitaxel Modulated the Cell Survival Potential of Prostate Cancer Cells. 3rd International Conference on Natural Products for Cancer Prevention and Therapy
- **Özfiliz Kilbas Pelin**, Arisan Elif Damla, Dinler Doğanay Gizem (2019). Actin disorganization is the major outcome of CRISPR-Cas9-Mediated Bag-1 Knockout MCF-7 breast cancer cells. 7th International Congress of the Molecular Biology Association of Turkey
- **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Dinler Doğanay Gizem (2019). Revealing the functional role of Bag-1 in MCF-7 breast cancer cells through CRISPR/Cas9-mediated gene knockout. The 44th FEBS Congress
- **Özfiliz Kılbaş Pelin**, Sönmez Özlem, Arisan Elif Damla, Çoker Gürkan Ajda, Obakan Yerlikaya Pinar, Ünsal Zeynep Narçin (2018). Investigation of the relationship between cholesterol metabolism and breast cancer through miR-33a in estrogen receptor targeting therapies. 6th International BAU Drug Design Congress
- **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Dinler Doğanay Gizem, Ünsal Zeynep Narçin (2017). Bag-1 silencing enhanced apoptosis via altering the ubiquitination process of Foxo3a in MCP-7 breast cancer cells. *International Journal Of Molecular Medicine*
- Aras Sahra, Özdemir Berilsu, Arisan Elif Damla, Rencüzoğulları Özge, **Özfiliz Kılbaş Pelin**, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Ünsal Zeynep Narçin

(2017). Orlistat as a fatty acid synthase inhibitor blocks cell survival mechanism via activating PTEN, which controls PI3K/AKT/MAPK axis in PC3 prostate cancer cells. Biotechnology Congress, 256, 31, Doi: 10.1016/j.jbiotec.2017.06.654

- **Özfiliz Pelin**, Arisan Elif Damla, Dinler Doğanay Gizem (2016). Knockdown of anti-apoptotic Bag 1 modulated the regulation of cell survival pathways in breast cancer. 41st FEBS Congress on Molecular and Systems Biology for a Better Life
- **Özfiliz Pelin**, Dinler Doğanay Gizem, Arisan Elif Damla, Ünsal Zeynep Narçin (2015). Suppression of polyamine biosynthesis decreased Bag 1 expression levels to alter cell proliferation survival in HeLa cervical cells. Gordon Conference Polyamines
- **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Dinler Doğanay Gizem, Ünsal Zeynep Narçin (2014). Silencing of Bag-1 enhanced the cisplatin or paclitaxel-induced apoptosis via regulating PI3K, Akt, and MAPK signaling axis in MCF-7 breast cancer cells. 2nd EACR Sponsored 2nd Anticancer Agents Congress 5th Multidisciplinary Cancer Research Congress, 145
- Obakan Yerlikaya Pinar, **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Çoker Gürkan Ajda, Ünsal Zeynep Narçin (2012). Purvalanol A is a Strong Apoptotic Inducer via Activating Polyamine Catabolic Pathway in MCF-7 Estrogen Receptor Breast Cancer Cells. EMBO Young Scientist Forum
- **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Dinler Doğanay Gizem (2012). The Potential Role of Bag-1 in cisplatin or paclitaxel-induced Apoptosis. First International Congress of the Molecular Biology Association of Turkey
- Çoban Mervener, Sevgin Börteçine, Şentürk Kübra, Rencüzoğulları Özge, **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Obakan Yerlikaya Pinar, Çoker Gürkan Ajda, Ünsal Zeynep Narçin (2017). Celastrol'ün prostat kanseri hücrelerinde PI3K/Akt sinyal yolağı ile ilişkili olarak epitelyal-mezenkimal hücre tranzisyonuna etkisi. 6. Kök Hücre Sempozyumu
- İlkhan Dilara, **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Çoker Gürkan Ajda, Obakan Yerlikaya Pinar, Ünsal Zeynep Narçin (2017). Serotonin, MCF-7 ve MDA-MB-231 meme kanseri hücrelerinde hücre canlılığını, sağkalımını ve klonojenik oluşumu azaltır. 6. Kök Hücre Sempozyumu
- Sarıkaya Bahar, **Özfiliz Kılbaş Pelin**, Arisan Elif Damla, Çoker Gürkan Ajda, Obakan Yerlikaya Pinar, Ünsal Zeynep Narçin (2016). CDK Inhibitors Roscovitine and purvalanol induced ER stress-mediated apoptosis in HeLa Cervical Cancer Cells Related with induction of oxidative stress. 6. Multidisipline Kanser Araştırma Kongresi

Book Chapter:

- Functional Foods and Cancer: Functional Foods in Integrative Oncology: Volume 5, First Edition, Bölüm adı: (Phytochemicals and Herbal Medicine in Oncology) (2017)., OBAKAN YERLİKAYA PINAR, **ÖZFİLİZ KILBAŞ PELİN**, Food Science Publisher, Editör: Danik Martirosyan, Jin-Rong Zhou, Basım sayısı:1, ISBN:978-1976255342

Projects:

- Investigation of anti-apoptotic pathways affected by Bag-1 protein in breast cancer cells through its interaction with the transcription factor FoxO3a. (ITU BAP, Coordinator: Dinler Doğanay Gizem, Researcher: **Özfiliz Kılbaş Pelin**, 20/11/2017-)
- Investigation of the relationship between cholesterol metabolism and cancer through miR-33 in estrogen receptor targeting therapies in breast cancer cells. (Coordinator: **Özfiliz Kılbaş Pelin**, Soroptimist International Europe (SIE), 01/03/2017 - 01/01/2019)
- Prostat kanseri ve obezite arasındaki ilişkinin hedef miRNA'lar aracılı olarak moleküler düzeyde incelenmesi, Tübitak 1002 project. (Coordinator: Palavan Unsal Narçin, Researcher: Arisan Serdar, Scholar: **Özfiliz Kılbaş Pelin**, 2016-2017)
- Investigation of the effect of Bag-1 overexpression and silencing on drug-induced apoptotic mechanism in MCF-7 breast cancer cells. (ITU BAP, Coordinator: Dinler Doğanay Gizem, Researcher: **Özfiliz Kılbaş Pelin**, 2012-2013)

Awards and Grants

- 2015- Participation Fellowship: Gordon Research Seminars: Role of Polyamines in Biology and Disease, 13-14 June 2015, Waterville Valley, NH, USA.
- 2014- Poster Presentation 2nd Award: **Ozfiliz P**, Arisan ED, Obakan P, Coker-Gurkan A, Doganay-Dinler G, Palavan-Unsal N, "Silencing of Bag-1 enhanced the cisplatin or paclitaxel-induced apoptosis via regulating PI3K, Akt and MAPK signaling axis in MCF-7 breast cancer cells" EACR Sponsored 2nd Anticancer Agents Congress& 5th Multidisciplinary Cancer Research Congress, PP59, Page: 145, 23-27 April 2014, Bodrum, Turkey

Certifications

- KOSGEB-Entrepreneurship Certificate, National Experimental animals use in vivo