ANALYSIS OF THE EFFECT OF ERBIN ON THE EPITHELIAL TO MESENCHYMAL TRANSITION RELATED GENES IN BREAST CANCER

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

ANALYSIS OF THE EFFECT OF ERBIN ON THE EPITHELIAL TO MESENCHYMAL TRANSITION RELATED GENES IN BREAST CANCER

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M.S. in Molecular Biology and Genetics

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Breast cancer is a heterogeneous disease and has complex mechanisms, which brings the need to come up with different approaches for its various types. Erbin is a member of the LAP family and directly interacts with ErbB2 (HER2), which is a crucial component for breast cancer sub-typing and treatment. Research on the relationship between Erbin and breast cancer showed different results since Erbin was seen to be able to act as both a tumor suppressor and a tumor promoter. There are multiple findings that Erbin has a role in the epithelial to mesenchymal transition in different cancer types. The role of Erbin in EMT is said to be multi-faceted, it affects multiple pathways that act on EMT progress. However, like Erbin's contradictory role in breast cancer, there are different findings that support the conflicting claims on whether Erbin is an EMT promoter or not. These findings in the literature lead us to analyze how Erbin affects EMT in breast cancer. To investigate if there is a role of Erbin in EMT regulation, we have overexpressed Erbin in MCF7 and silenced Erbin in MDA-MB-231 with MDA-66 cell lines. With this experimental setup, we aimed to see how Erbin plays a role in EMT in both

epithelial and mesenchymal cells as well as Luminal A, TNBC, and ER positive breast cancer types.

Our results showed that in TGFB induced EMT, Erbin levels are decreased in epithelial cells. Erbin overexpressed MCF7 cells show significant changes in the EMT markers compared to the control group. Experiments conducted with Erbin silenced MDA-MB-231 and MDA-66 cells also demonstrated significant changes in EMT marker levels. Our results suggest that Erbin has a role in EMT in breast cancer, and this role is not limited to one pathway and not streamlined, but possibly a big orchestration of different effects.

Keywords: Erbin, Breast Cancer, EMT

ÖZET

ERBIN GENİNİN MEME KANSERİNDE EPİTEL MEZENKİMAL DÖNÜŞÜMDE ROL OYNAYAN GENLER ÜZERİNDEKİ ETKİSİNİN ANALİZİ

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Meme kanseri heterojen bir hastalık olmasıyla birlikte karmaşık mekanizmalara da sahip olması, meme kanserinin çeşitli türleri için farklı yaklaşımlar geliştirme ihtiyacını beraberinde getirmektedir. Erbin, LAP ailesinin bir üyesidir ve meme kanseri tip ayrımı ve tedavisi için önemli bir bileşen olan ErbB2 (HER2) ile doğrudan etkileşime girer. Erbin ve meme kanseri arasındaki ilişki üzerine yapılan araştırmalar, Erbin'in hem tümör baskılayıcı hem de tümör destekleyici olarak hareket edebildiğini göstermiştir. Erbin'in farklı kanser türlerinde epitel mezenkimal dönüşümde (EMD) rolü olduğuna dair birçok bulgu vardır. Erbin'in EMD'deki rolünün çok yönlü olduğu ve EMD'de görev yapan bir çok yolağı etkilediğine dair bulgular elde edilmiştir. Bununla birlikte, tıpkı Erbin'in meme kanserindeki çelişkili rolü gibi, Erbin'in bir EMT destekleyicisi olup olmadığı konusundaki çelişkili iddiaları destekleyen farklı bulgular vardır. Literatürdeki bu bulgular, Erbin'in meme kanserinde EMT'yi etkilediği hipotezini öne sürmemize yol açmıştır. Erbin'in EMT regülasyonunda bir rolü olup olmadığını araştırmak için

MCF7'de Erbin'in ifadesini arttırdık ve MDA-MB-231 ile MDA-66 hücre hatlarında Erbin'i susturduk. Bu deneysel düzenek ile Erbin'in hem epitelyal hem de mezenkimal hücrelerde ve ayrıca Luminal A, üçlü negatif ve ER pozitif meme kanseri tiplerinde EMT'de nasıl bir rol oynadığını görmeyi amaçladık.

Sonuçlarımız, TGFB ile indüklenen EMD'de epitel hücrelerinde Erbin seviyelerinin azaldığını gösterdi. Erbin ekspresyonu arttırılan MCF7 hücreleri, kontrol grubuna kıyasla EMT ile ilgili genlerde önemli değişiklikler gösterdi. Erbin susturulmuş MDA-MB-231 ve MDA-66 hücreleri ile yapılan deneyler de EMD genlerinin seviyelerinde önemli değişiklikler gösterdi. Bulgularımız, Erbin'in meme kanserinde EMD'de bir rolü olduğunu ve bu rolün tek bir yolakla sınırlı olmadığını, ancak muhtemelen farklı etkilerin büyük bir orkestrasyonu olduğunu göstermektedir.
Anahtar Kelimeler: Erbin, Meme Kanseri, EMD

To my family and friends

who were always with me

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ABBREVIATIONS

AKT: V-akt murine thymoma viral oncogene homolog

APS: Ammonium peroxodisulfate

BSA: Bovine Serum Albumin

CDH1: E-Cadherin

CDH2: N-Cadherin

CEBPA: CCAAT Enhancer Binding Protein Alpha

ddH_2O: double distilled H_2O

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

EHF: ETS Homologous Factor

ELF3: E74 Like ETS Transcription Factor 3

EPCAM: Epithelial cellular adhesion molecule

ERBB2: Proto-oncogene erb-b2 receptor tyrosine kinase 2

ERBIN: Erbb2 Interacting Protein

ECL: Enhanced chemiluminescence

EGFR: Epidermal growth factor receptor 1

EMT: epithelial-mesenchymal transition

ER: estrogen receptor

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

FN: Fibronectin

FOXC2: Forkhead box proteini C2

FSP1: Ferroptosis Suppressor Protein 1

IF: Immunofluorescence

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GRHL3: Grainyhead like transcription factor 3

HER2: human epidermal growth factor receptor 2

HNF4A: Hepatocyte nuclear factor-4 alpha

MAPK: Mitogen-activated protein kinase

MET: mesenchymal-epithelial transition

OVOL2: Ovo Like Zinc Finger 2

PBS: phosphate buffer saline

PR: progesterone receptor

RT: Room temperature

SDS: Sodium Dodecyl Sulfate

SLUG: Snail Family Transcriptional Repressor 2

siRNA: Small interfering RNA

SNAIL: Snail Family Transcriptional Repressor 1

TBS: Tris Buffered Saline

TBS-T: Tris buffer saline Tween20

TCA: Trichloroacetic acid

TGF-β: Transforming growth factor beta

TN: Triple negative breast cancer

VIM: Vimentin

ZEB2: Zinc Finger E-Box Binding Homeobox 2

ZO1: Zonula occludens-1

CHAPTER 1. INTRODUCTION

1.1. Cancer

Cancer is known to be a heterogeneous disease that can be seen in any tissue in the body. Cells start to proliferate uncontrollably by evading or changing the checkpoints that healthy cells have. These cells create tumors that are heterotypic and have an abnormal environment in which they interact. Cancer cells have common traits regardless of their type. They are able to defy cell death, deregulate proliferation and growth signaling pathways, and resist growth suppressors. In addition to changes that enable the increase in cancer cell numbers, they also become immortal, invasive, and angiogenesis promoting. When these changes are combined in the multi-step progress, cancer cells become hard to destroy by the body. These changes can arise from inherited or accumulated genetic instability and inflammation. [1,2]

Despite the accumulating research on cancer, it is still one of the leading death causes in the world. The chance of being diagnosed with cancer is 40.2% for men and 38.5% for women. [3] In 2020, approximately 19.3 million new cancer cases and 10 million cancer deaths were reported. [4] In 2022, it is predicted to have 1.3 million cancer deaths in the European Union [5] and 1,918,030 diagnosed cancer cases with 609,360 cancer related deaths in the United States. [3]

1.2 Breast Cancer

Breast cancer is reported to be %11 percent of the diagnosed cancer cases annually and is one of the three most common cancers. [6,7] Globally, breast cancer is the most

common cancer among women. [8] In their lifetime, 1 in 8-10 women develop breast cancer. [7] In 2020, there were approximately 2.3 million new cases of female breast cancer. [4] Risk factors for breast cancer are various: genetic and epigenetic makeup, obesity, physical activity level, alcohol, and hormonal contraceptives can be counted as examples. [9]

Breast cancer is a complex disease with three main subtypes according to the hormone receptors: hormone receptor (HR) positive and ErbB2 negative (Luminal A), ErbB2 positive and HR-positive or negative (Luminal B), and triple negative (Basal) breast cancer. [10] HR+/ErbB2- type is approximately %70 of breast cancer cases. If the breast cancer is HR-positive, patients get endocrine therapy, and ErbB2 positive patients receive chemotherapy with ErbB2 targeted antibody or inhibitor treatments. The only option for TNBC patients is, as of today, chemotherapy. [8] ErbB2 negative and HR-positive breast cancer type occurs after ERα activates the oncogenic pathways. Almost %70 percent of breast cancer cases are this kind of subtype. The prognosis can be counted as the best out of other subtypes with fewer metastasis occurrences. ErbB2 positive cases have amplified ErbB2 gene or high level of ErbB2 protein; thus, overactivity of ErbB2 oncogene is seen in this subtype. Approximately %15-20 of the breast cancer belong to this group. Prognosis and metastasis can be worse than the ErbB2 negative subtype. [8] TNBC subtype does not have estrogen, progesterone receptor, or ErbB2 (HER2) amplification. [8,9] The mechanism is highly unknown as it is thought to be a complex disease. %15 percent of the breast cancer cases belong to this subtype and have the worst prognosis, and most metastatic properties. [8]

TNBC is a heterogeneous disease with intertwined networks. In 2015, Lehmann et al. created a categorization system after analyzing a large dataset. According to this study, there are six TNBC subtypes: basal-like 1(BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal stem-like (MSL), mesenchymal-like (M), and luminal androgen receptor (LAR). The BL1/2 subtypes constitute 47-88% of TNBC and 10-25% of invasive breast

cancers. [9] BL1 subtype is found to have high expression of cell cycle and DNA damage response genes; BL2 has elevated growth factor and MAPK signaling. IM is enriched in immune response genes, and M and MSL subtypes have a high level of cell differentiation, cell motility, cell proliferation, EMT, and growth factor pathways. LAR subtype highly expresses androgen receptors and hormonally regulated pathways. [9,11]

Table 1.0.1 Breast cancer classification according to the hormone receptors

Table adapted from [10]

Subtype	Alias	Biomarker Status	Outcome	Prevalence
Luminal	Luminal A	ER+ PR+ HER2- KI67-	Good	23.7%
	Luminal B	ER+ PR+ HER2-KI67+ ER+PR+HER2+ KI67+	Intermediate Poor	38.8% 14%
HER2 Positive	HER2 over-expression	ER-PR-HER2+	Poor	11.2%
Triple Negative	Basal	ER-PR-HER2-	Poor	10-25%

1.3 Epithelial to Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) happens when epithelial cells experience biochemical changes and acquire mesenchymal cell properties that include higher migration, invasiveness, and resistance to apoptosis. [12]

Epithelial cells are connected laterally to each other via adjunction structures and attached to the laminal layer by their basal surface. Mesenchymal cells usually do not have these contacts and have front-back end polarity. Morphologically, epithelial cells are monolayer and “cobblestone-like” with apical-basal polarity; whereas mesenchymal cells look like spindles, they are dispersed and have migratory protrusions. [13] During EMT, the basal

membrane degrades, and the mesenchymal cells that originated from epithelial cells gain the ability to migrate from their layer. [12]

Besides the morphological changes, a group of molecular changes occurs in the cells during EMT. These can be counted as changes in the cell surface protein expressions, activation of some transcription factors, production of extracellular matrix (ECM) degrading proteins, changes in the expressions of cytoskeleton proteins and particular microRNAs. [12] Genes that have roles in cell migration, invasion, and adhesion are modified by transcription factors. [13] Expression of FSP1; increased expression of N-cadherin, Vimentin, Snail, Slug, and Twist; loss or decreased expression of E-cadherin, ZO-1, and cytokeratin can be seen in EMT. [14]

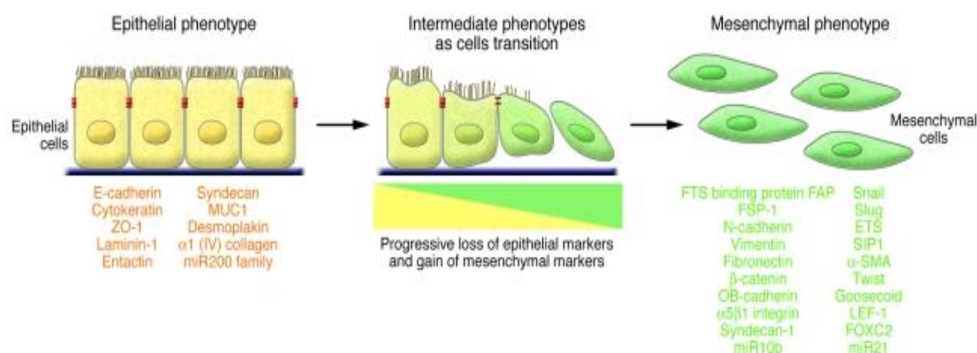


Figure 1.1 Epithelial to Mesenchymal Transition

EMT is classified into three groups. Type 1 EMT is seen during embryogenesis and organ development. After Type 1 EMT, the mesenchymal cells, called primary mesenchyme, can go through MET (Mesenchymal to Epithelial Transition) and create the secondary epithelia. [12] This type of EMT can be seen during mesoderm formation, neural crest formation, cardiac valve formation, and secondary palate formation. [13] Type 2 EMT has a role in tissue regeneration and wound healing. After an inflammatory injury or tissue trauma, type 2 EMT is initiated to heal the area by generating fibroblasts and other necessary cells. However, if this process is not terminated the organs can be destroyed. Type 3 EMT arises in genetically and epigenetically changed cells in which tumor suppressors and oncogenes have

been affected. These carcinoma cells can become invasive and metastatic after going through type 3 EMT, impacting the prognosis of the disease for the worse. [12]

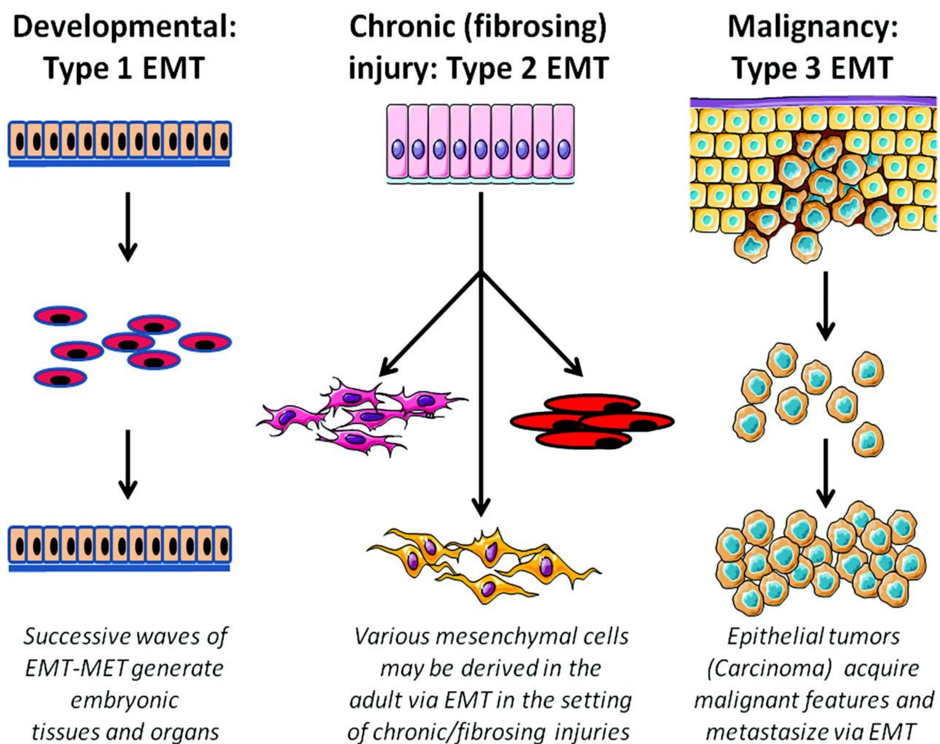


Figure 1.2 Classification of Epithelial to Mesenchymal Transition

1.3.1 EMT and Cancer

In many cancer types, the EMT program leads to invasiveness and metastatic properties that result in more aggressive phenotypes. [12] Decreased expression of E-cadherin is the hallmark of EMT, and this situation has been observed in various human carcinomas, potentially activating invasion. It has been shown that the forced expression of E-cadherin in some invasive tumor cells has been shown to suppress the invasiveness. Besides E-cadherin, other cell adhesion molecules are also downregulated, whereas the molecules favoring cell migrations are upregulated in many aggressive carcinomas. However, in many cases, the cancer cells do not go through EMT completely but carry both epithelial and mesenchymal

features. [1] Sarcomas are malign tumors that arise from mesenchymal origin cells. It has been seen that some epithelial markers are expressed in sarcomas, showing that they undergo EMT/MET partly. This combination of epithelial and mesenchymal traits in sarcomas is suggested to add to the aggressive clinical results. [15] One of the examples of this mixed phenotype is that most human breast carcinomas still express E-cadherin while they metastasize. [13]

Various studies established that activation of transforming growth factor-β (TGF-β) signaling is the inducer of EMT *in vitro* and *in vivo*. TGF-β signaling induces transcription factors such as Snail, Slug, Twist1, and FOXC2; in return, they repress the E-cadherin expression. For instance, in lobular breast carcinoma, Twist1 is overexpressed, and the cells are permanently in EMT phenotype with no E-cadherin expression. The Twist1 expression is crucial for the breast to lung metastasis of mouse breast carcinoma. [13]

It has been proposed by many studies that the EMT process is essential in metastasis, and the studies of pathways are still ongoing.

1.4 Erbin

Erbin is a member of the Leucine-rich repeat and PDZ domain (LAP) family, has 16 LRRs, 1 PDZ domain, and an intermediary area in between. It was identified by Borg et al. in 2000 as an interacting protein of ErbB2, and its role was found in the localization of the Erbb2 to the basolateral membrane of epithelial cells. [16] It was shown that Erbin directly interacts with ErbB2 via its only PDZ domain in its C terminus, which has six β-strands organized as two β-sheets and two α-helices. [16,17] Erbin binds to non-phosphorylated ErbB2 specifically, localizing it to the basolateral membrane of epithelial cells, since it was seen that mutation in the PDZ domain of Erbin disrupts this localization, which disturbs ErbB2 signaling. [16] It was shown that ErbB2 levels in Erbin-null mice were low, possibly

resulting from a posttranscriptional mechanism since there was no difference in ErbB2 mRNA level. In addition, ErbB2 was less stable, and less ErbB2 was internalized in sciatic nerves. [18]

1.4.1 Different Functions of Erbin

Brain is one of the organs that Erbin is highly expressed. [16,19] Right after the identification of Erbin, it was found that ErbB2 and Erbin are colocalized in post-synaptic membranes of the neuromuscular junctions, which act to transmit the signals from motoneurons to muscles. Results showing in vivo interaction between Erbin and ErbB2 further prove the previous findings and brings another location to the table that this interaction occurs other than in epithelial cells. Neuregulin 1 (NRG1) is a vital factor for myelination, the velocity of the synaptic signals, and it interacts with ErbB2. It was shown that Erbin is expressed highly in regions with a high number of myelinated axons. Erbin also acts on the neuromuscular junctions in the central nervous system. [20] Erbin is essential in neuronal networks and proper neuronal signal transmission.

Erbin can be found in various signaling pathways. One of these pathways has early findings in MAPK/ERK signaling pathway, also known as the Ras-Ref-ERK pathway. Mitogen-activated protein kinases (MAPK) family includes a subfamily of extracellular signal-regulated kinases (Erk), and they have various important roles such as cell differentiation, proliferation, and apoptosis. This cascade regulates gene expression by conveying cell surface receptors signals to transcription factors. It is also an important pathway in cancer and drug resistance, thus an attractive therapeutic target. [21,22,23] Results indicate that Erbin acts as an inhibitor of the Ras-Raf-Erk signaling pathway. Erbin was found to inhibit Erk activation by interacting with active Ras via its LRR domain and interfere with the interaction between Ras and Raf. [21,23] Another pathway that has been associated with

Erbin is NF- κB signaling pathway. This pathway is quite important for inflammatory response and DNA transcription and has been linked to cancer, viral infection, autoimmune diseases, and cardiomyopathy. [24,25]

Cell functions such as proliferation, differentiation, migration, cell cycle, and cell adhesion are regulated by various signaling pathways. A study on the cell cycle shows that Erbin is aggregated in the nuclei of cells during mitosis, rising during the S phase to its highest level G2/M stage. Erbin regulates the transition between phases during mitosis. [26] Cell adhesion helps tissue organization and keeps tissues intact. In addition, cell adhesion proteins are involved in signaling, recognition, and proliferation. [27] Cell junctions anchor cells to other cells or other areas.[28] One of the first findings on Erbin was its interaction with adhesion complex hemidesmosome-related proteins eBPAG1 and integrin β4 subunit in keratinocytes. This might also be a link between ErbB2 and the integrin β4 subunit, which was shown to interact before. [29]

These pathways and processes are vital in many tissues. The regulatory property of Erbin in signaling might be the reason why Erbin is essential in multiple biological processes.

1.4.2. Role of Erbin in Cancer

Erbin is associated with many diseases, particularly cancer. Erbin has been observed to have a role in renal interstitial fibrosis (RIF) which results in renal failure in many cases [30] and in heart-related diseases, especially cardiac hypertrophy-induced chronic heart failure. [31,32]

As cancer is associated with many signaling pathways, Erbin also plays a role in various signaling events. This makes Erbin a target for cancer related studies. β-catenin is found in adherens junctions; its misregulation is a hallmark for several cancer types. It was found that β-catenin and Erbin interact with their respective PDZ domains, and wild-type

Erbin negatively regulates β-catenin signaling in colon carcinoma cell lines. In contrast, an Erbin splice variant mimic which is found in the nucleus was found to increase β-catenin dependent gene expression. These findings highlight the possibility of Erbin's dual effects and functions in different environments. [33] Erbin knockdown increased proliferation and tumorigenesis of cervical cells, showing a tumor-suppressing function of Erbin. [34,35] There is evidence that Erbin acts as a skin tumor suppressor. [36] Erbin decreases the invasiveness of head and neck squamous cell carcinomas (HNSCC), cancer with a low survival rate. [37]

Conversely, the results that indicate Erbin as a tumor promoter came from colorectal cancer studies. Erbin was found to promote tumor growth in colorectal cancer mouse xenograft models, and elevated levels of Erbin were shown in colorectal cancer patient samples. [38] However, another colorectal cancer study describes Erbin as a tumor suppressor. In this contradictory study, it was seen in patient tissue samples, Erbin expression was downregulated. Erbin loss disrupted cell polarity, induced EMT, and increased cell proliferation. Knockout of Erbin in mice model showed increased tumor initiation and reduced survival rates via promoting Wnt signaling and Akt-Ras/Raf signaling pathways. [39] These studies add to the distinct functions of Erbin, even in the same cancer type.

Erbin inhibited ERα signaling by promoting ubiquitination via its PDZ domain, thus increasing tumorigenesis in hepatocellular carcinoma. Erbin silenced mice model showed increased sensitiveness to tamoxifen treatment by increasing the nuclear translocation of ERα, making it a possible target for efficient tamoxifen therapy. [40]

These studies showed that Erbin has distinctive roles in cellular mechanisms. This makes Erbin both an attractive and a puzzling target to study.

1.4.2.1 Erbin in Breast Cancer

One of the leading causes of breast cancer is inherited mutations in BRCA1 and BRCA2, tumor suppressor genes and components of DNA damage repair mechanism. [41, 42] BRCA1 mutations were reported to increase by %72 of the cumulative risk of breast cancer by the age of 80. [43] It has been shown that in breast cancer cell lines, BRCA1 upregulates the expression of Erbin. [41] Erbin is thought to be a negative apoptosis regulator by inducing ErbB2 expression in breast cancer cells. [42] In breast cancer cells, Erbin is downregulated, sometimes to the degree that Erbin expression is lost. In ErbB2 overexpressing cells, Erbin deficiency led to a significant increase in induced AKT activation. Erbin knockdown promoted cell migration and invasiveness and created resistance to the anti-proliferative therapeutic antibody, trastuzumab. PI3K/AKT inhibition was seen to reverse these effects. Hence, Erbin negatively regulates AKT activation, which participates in cell migration. [44] Erbin is described as a tumorigenesis promoting factor. Studies made with model mice showed that Erbin increases proliferation and tumor development dependent on ErbB2. Both Erbin and ErbB2 expression are found to be correlating with human breast cancer samples. [45]

Erbin is associated with almost all the mechanisms that promote tumorigenesis in the breast tissue; hence Erbin might be a key factor in understanding how breast cancer cells function and creating new therapies that target Erbin.

1.5 Aim of The Study

The objective of this study is to investigate the role of Erbin during EMT in different breast cancer types. Therefore, Erbin was overexpressed in MCF7 using prk5-myc-Erbin vector, and silenced in MDA-MB-231 and MDA-66 cell lines via siRNA. The results of the overexpression and knockdown were examined through q-RT-PCR, Western Blot, and Immunofluorescence staining along with in silico analyses.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. General Laboratory Materials

General laboratory materials are the chemicals, kits, substances, solutions, and equipment regularly used in common laboratory techniques. In Table 2.1 these materials are listed.

2.1.1.1. Chemicals and Reagents

Table 2.2 The list of general laboratory materials

Name	Catalog Number	Company
2-mercaptoethanol	M3148	Sigma Aldrich (USA)
30% Acrylamide/Bis solution	1610156	Bio-Rad (USA)
Agarose	BHE500	Prona (Spain)
Ammonium persulfate	A3678	Sigma Aldrich (USA)
Bovine Serum Albumin Fraction V (BSA)	10735078001	Roche (USA)
Bromophenol blue	B5525	Sigma Aldrich (USA)
DEPC Water	A0881	Applichem (Germany)
Ethidium Bromide	17898	Thermo Scientific (USA)
EDTA	A3562	Applichem (Germany)
ECL Prime System	RPN2232	Life Sciences (USA)
ECL		
Gene Ruler DNA Ladder (1 kb)	SM0311	Thermo Scientific (USA)

Glycine	G8898	Sigma Aldrich (USA)
KCl	12636	Sigma Aldrich (USA)
KH2PO4	4243	Sigma Aldrich (USA)
NaCl	31434	Sigma Aldrich (USA)
Proteinase K	P2308	Sigma Aldrich (USA)
Proteinase inhibitor (PI) cocktail	P8340	Sigma Aldrich (USA)
PageRuler Prestained Protein Ladder, (170kDa)	26616	Thermo Scientific (USA)
Rnase A	R6513	Sigma Aldrich (USA)
Roche PVDF Membranes (0.2uM)	3010040001	Roche (USA)
SDS	71725	Sigma Aldrich (USA)
Taq DNA Polymerase	EP0402	Sigma Aldrich (USA)
TEMED	1610801	Biorad (USA)
Triton X-100	T8787	Sigma Aldrich (USA)
Trizma Base	T1503	Sigma Aldrich (USA)
Tween-20	822184	Merck (Germany)
Prolong Diamond Anti-fade Mountant Solution	P36962	Thermo Scientific (USA)

2.1.1.2. Solutions and Buffers

Table 2.3 The list of used solutions and buffers

Solution	**Recipe**
4x Protein Loading Buffer (50 ml)	200 mM Tris-HCL (pH=6.8); 4 g SDS; %40 Glycerol; 200 mg Bromophenol Blue; %5 β-mercaptoethanol
5X Running Buffer (1 L)	15 g Trisma Base; 72 g Glycine; 5 g SDS in 1 L dH2O

10X TBS	24 g Trizma base; 88 g NaCl in 1 L H_2O. pH = 8.0
10X PBS	2 g KCl; 80 g NaCl; 14.4 g $Na_2HPO_4.2H_2O$; 2.4 g KH_2PO_4 in 1 L dH_2O; pH=7.4
50X TAE (100 ml)	24.2 g Tris-base; 5.71 ml glacial acetic acid; 10 mL 0.5M EDTA; pH=8.0
Blocking Solution (10 ml) (3%)	0.3 milk powder; 10 ml 1X TBS-T
Mild Stripping Buffer (10 ml)	1g SDS; 1.5 g glycine; 1 ml Tween-20 in dH2O pH = 2.2
Ponceau S Staining Solution	1g Ponceau S; 50 ml acetic acid; 100 mL ddH2O
RIPA buffer (1 ml)	1 µl 1 M Tris-HCL pH=8.0; 300 µl 5M NaCl ; 20 µl EDTA; 100 µl NP-40; 40 µl 25X protease inhibitor
TBS-T (0.2%) (500ml)	1 mL Tween-20; 50 ml 10X TBS diluted 450 mL ddH2O
Wet Transfer Buffer (1 L)	6 g Trisma Base ; 28.8 g Glycine; 10 % methanol in dH2O

2.1.1.3. Kits

Table 2.4 The list of Kits used in this study

Kit Name	Catalog Number	Company
Nucleospin RNA extraction Kit	740955	Macherey Nagel (Germany)
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific (USA)
DyNAmo HS SYBR Green qPCR Kit	F-410L	Thermo Scientific (USA)
Pierce™ BCA Protein Assay Kit	23225	Thermo Scientific (USA)
ZymoPURE™ Plasmid Miniprep Kit	D4208S	Zymo Research (USA)

DNA Clean & Concentrator^{TM}-5	D4014	Zymo Research (USA)
AMPIGENE® qPCR Probe Mix No-ROX	Enz-Nuc107-1000	Thermo Scientific (USA)

2.1.1.4 PCR Primers

PCR primers that were used in RT-qPCR and their Tm values are listed in Table 2.4. After the arrival of the primers, they were solved in nuclease-free water to make their final concentration 100 µM and stored at -20 °C.

Table 2.5 List of primers used in RT-qPCR

Primer	**Sequence**	**Probe**
ERBIN F	CTAATCAGATTGAAGAGCTTCC	-
ERBIN R	AACTCCTGTATTCCATTCTTGC	-
ERBIN F	TGACAGCTATGTGGCTCTCAG	115
ERBIN R	GTCCTTGGCTGTTGAGGGA	115
CDH1 F	ATCCTCGCCCTGCTGATT	13
CDH1 R	ACCACCGTTCTCCTCCGTA	13
ELF3 F	ACCGAACCCTGACACACCT	2
ELF3 R	AGCTGTACATGGCGTTGAAGT	2
GRHL3 F	AAGGAAGATGTCGAATGAACTTG	30
GRHL3 R	TCGTCCTCATTACTGTAGGGAAA	30
OVOL2 F	GTGAGGATTGCGGCTACAC	33
OVOL2 R	TGGTCACTGTTCACATGCAG	33
CEBPA F	AAACAACGCAACGTGGAGA	2

CEBPA R	GCGGTCATTGTCACTGGTC	2
HNF4A F	ACGGACAGATGTGTGAGTGG	27
HNF4A R	GCAGGAGCTTATAGGGCTCAG	27
EHF F	TCATTGTCAAGACTGAACAAACC	12
EHF R	GTCCAACAGATCTACTGTGCTACC	12
FN1 F	AGGTGGACCCCGCTAAAC	1
FN1 R	TGCCGCAACTACTGTGATTC	1
SLUG F	ATCCTTGGGGCGTGTAAGT	7
SLUG R	TGAACCACTGTGATCCTTGG	7
SNAIL F	CTTGCTCCACAAGCACCA	10
SNAIL R	GAGGATGGGGAGGTAGCAG	10
ZEB2 F	TTGCTCCAGGATGTGTGG	85
ZEB2 R	CACACACTTGTTTGTGTGCATATC	85
GAPDH F	AGCTTGTCATCAACGGGAAG	60
GAPDH R	TTTGATGTTAGTGGGGTCTCG	60
GAPDH F	GGCTGAGAACGGGAAGCTTGTCAT	-
GAPDH R	CAGCCTTCTCCATGGTGGTGAAGA	-

2.1.1.5 Antibodies

Table 2.6 List of antibodies used in Western Blot and IF experiments

Antibody Name	**Catalog Number**	**Company**
Rabbit anti-ERBIN Antibody *	N/A	N/A
Rabbit anti-Beta-Actin	3700S	Cell Signaling Technology (USA)
Mouse anti-E-Cadherin	610181	BD Bioscience (USA)
Anti-Rabbit IgG HRP	A6154	Sigma-Aldrich

Anti-Mouse IgG-HRP	A0168	Sigma-Aldrich
Alexa Fluor™ 488 Phalloidin	A12379	Invitrogen (USA)
Alexa Fluor™ 594 goat anti-mouse IgG	A11005	Invitrogen (USA)

* ERBIN antibody was gifted by Dr. Jean-Paul Borg (INSERM, Marseille, France).

2.1.1.6. Equipment

Table 2.7The list of equipment used in the experiments

Equipment Name	**Company**
PCR Thermal cycler	Applied Biosystems (USA)
Centrifuges 5810 and 5810 R	Eppendorf (Germany)
Amersham Imager 600	Dharmacon (USA)
ChemiDoc MP Imaging System	BioRad (USA)
MultiSkan GO	Thermo Scientific (USA)
Nanodrop 2000	Thermo Scientific (USA)
AutoFlow NU-8500 Water Jacket CO2 Incubator	NuAire (USA)
Light Cycler 96	Roche (USA)
Applied Biosystems Fast 7500	Applied Biosystems (USA)

2.1.2 Cell Culture Reagents and Media

Table 2.8 Cell Culture Materials

Name of the Reagent	**Catalog Number**	**Company**
DMEM Low Glucose w/o L-Glutamine w/ Sodium Pyruvate	L0064-500	Biowest (USA)

DMEM, high glucose	41965039	Thermo-Fischer Scientific (USA)
Fetal Bovine Serum (FBS)	A4736201	Thermo-Fischer Scientific (USA)
Trypsin/EDTA (10X)	X0930-100 T	Biowest (USA)
Trypsin/EDTA (1X)	25200056	Thermo-Fischer Scientific (USA)
PBS	L0615-500	Biowest (USA)
PBS	10010031	Thermo-Fischer Scientific (USA)
Non-Essential Amino Acids	11140035	Thermo-Fischer Scientific (USA)
Penicillin/Streptomycin	SV30010	GE Healthcare (UK)
Insulin	I9278	Sigma Aldrich (USA)
Dimethyl sulfoxide (DMSO)	A1584	Applichem (Germany)
Opti-MEM I	11058021	Thermo-Fischer Scientific (USA)
Lipofectamine 2000 Transfection Reagent	11668027	Thermo Scientific (USA)
Lipofectamine RNAiMAX	13778075	Thermo-Fischer Scientific (USA)
FuGENE® HD Transfection Reagent	E2311	Promega (USA)

2.1.2.1 Cell Lines and Growth Mediums

Table 2.9 Cell culture mediums used in the experiments

Cell Line	Medium
MCF7	DMEM Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids ; 0.1% insulin
MDA-MB-231	DMEM/ Low Glucose; 10%FBS; 1%

	Penicillin/Streptomycin; 1% nonessential amino acids
MDA-66	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids
MCF10A	Basic DMEM/Ham's F12; 10µg/ml insulin; 20 ng/ml EGF; 0.5 mg/ml hydrocortisone
MDA-MB-157	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids, 1mM Sodium pyruvate
SK-BR-5	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids
CAL-51	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids

2.1.2.2 Nucleic Acids

Table 2.10 The list of nucleic acids that were used for transfection

Name	Catalog Number	Company	Target Sequence
siGENOME Non-Targeting siRNA #2	D-001210-02-20	Thermo Scientific (USA)	UAAGGCUAUGA AGAGAUAC
Hs_ERBB2IP_12 FlexiTube siRNA	SI03063704	Qiagen (USA)	CAGACTCTATAG GAGGGTTAA

2.1.2.3 Plasmids

Table 2.11 The list of plasmids used for transfection

pRK5-Myc
pRK5-Myc-Erbin

* Plasmids were gifted by Dr. Jean-Paul Borg (INSERM, Marseille, France).

2.2. METHODS

2.2.1. Cell Culture Techniques

2.2.1.1 Cell Culture Maintaining and Handling

Cell culture techniques were performed in sterile conditions entirely. Cells were incubated at 37 °C and 5% CO_2. Cell cryo-vials that were stored in nitrogen were taken and heated, transferred inside the growth medium, and centrifuged at 1500 RPM for 5 minutes to get rid of DMSO. After the supernatant was removed, the pellet was dissolved in the growth medium and transferred to the cell culture dish. All cells were passaged once they reached 80% confluency. To passage the cells, cells are washed with PBS and treated with Trypsin/EDTA to detach the cells from the surface. Cells are collected and centrifuged, the supernatant is removed, and the cells are divided into the fresh growth medium.

To freeze the cells, after collecting and centrifuging, cells are dissolved in the freezing medium containing 90% FBS and 10% DMSO and stored in cryo-vials in nitrogen for a long preserving time.

2.2.1.2 siRNA Transfection

In order to silence ERBIN, MDA-MB-231 and MDA-66 cells were transfected with Hs_ERBB2IP_12 FlexiTube siRNA (si12-Erbin) (Qiagen, USA). For control scramble RNA, siGENOME Non-Targeting siRNA #2 is used (Thermo Scientific, USA).

200.000 cells per well were seeded to 6-well plates. 2.5 µl of si12-Erbin, scramble RNA, and Lipofectamine RNAiMAX (Thermo-Fischer Scientific, USA) were diluted in 250

μl DMEM separately and incubated for 5 minutes at room temperature. The separate siRNAs and transfection reagents including DMEMs are combined, resulting in 500 μl of transfection mix, and incubated for 20 minutes at room temperature. The mixes are added to the wells, the growth medium is completed to make a total of 2 mL per well. The transfected cells are incubated for 72 hours.

2.2.1.3 Overexpression Plasmid Transfection

For the overexpression of ERBIN gene, prk5-myc-Erbin vector was used in MCF7 and HUH7 cell lines. prk5-myc backbone vector was used for control. For transfection, 250000 cells/well were seeded to 6-well plates for both cell lines. 3 mL Fugene transfection reagent (Promega, USA) is added to 100 μl empty DMEM and incubated for 5 minutes at room temperature. Then, 3 μg of vectors are added to the transfection mixes and incubated for 20 minutes at room temperature. The transfection mixes are added to the wells in 2 mL of total growth medium. Cells are incubated for 72 hours.

2.2.2. Laboratory Techniques

2.2.2.1 Total RNA Isolation and cDNA Synthesis

Cell pellets were collected with cold PBS and centrifuged at +4°C prior to RNA isolation. The total RNA isolation was performed using Nucleospin RNA extraction Kit Macherey Nagel (Germany). After the isolation, the quality of the RNA was measured using Nanodrop (Thermo Scientific, USA).

cDNA Synthesis was done using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with the manufacturer’s protocol.

2.2.2.2 qRT-PCR

qRT-PCR was done using DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific, USA) and AMPIGENE® qPCR Probe Mix No-ROX (Thermo Scientific, USA). For experiments done with DyNAmo HS SYBR Green qPCR Kit (Thermo Scientific, USA), the total reaction volume was 10 µl, and it included 5 µl master mix, 2.6 µl nuclease-free water, 0.2 µl 10 µM forward primer, 0.2 µl 10 µM reverse primer, and 2 µl cDNA. For experiments done with AMPIGENE® qPCR Probe Mix No-ROX (Thermo Scientific, USA), total volume was 10 µl, consisting of 6.25 µl buffer, 2.5 µl ddH_2O, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.25 µl probe, and 2 µl cDNA.The experiment conditions were as follows with 40 cycles:

Initial denaturation: 95°C, 15 minutes

Denaturation: 94°C, 20 seconds,

Annealing: Tm suitable for the primers, 20 seconds

Extension: 72°C, 15 seconds

Melting curve: 95°C, 1 minute

55°C, 10 seconds

95°C, 30 seconds

Obtained triplicate Ct values were averaged and normalized with respective housekeeping GAPDH Ct values. For analysis, $2^{-\Delta\Delta Ct}$ method was used.

2.2.2.3 Immunofluorescence Staining

Cells that transfection experiments were conducted used for immunofluorescence staining. Slides were positioned in the 6 wells on the day of transfection. After incubation, the slides were first washed with PBS 3 times, fixed with 4% formaldehyde (Sigma, USA) for 10 minutes, and washed with PBS again 3 times. By adding 0.25% Triton

X-100 (Sigma, USA)for 5 minutes, cells were permeabilized and washed with PBS 3 times. Cells were incubated with 50 µl of 5% BSA (1:200) primary antibody anti-E-cadherin (BD Bioscience,USA) for 1 hour at room temperature and in the dark. Later, the slides were washed with PBS again 3 times. Secondary antibody 488 Phalloidin (Invitrogen,USA) with Alexa Fluor™ goat anti-mouse IgG (Invitrogen, USA) were incubated for 1 hour in the dark at room temperature. To visualize the nuclei, 1:1000 DAPI (Invitrogen, USA) was added. The slides were mounted to the lams using Prolong Diamond Antifade Mountant Solution (Thermo Fisher Scientific, USA). Visualization was performed with LSM 880 Confocal Microscope (Zeiss, Germany).

2.2.2.4 Total Protein Isolation and Protein Quantification

Cell pellets were collected using cold PBS and centrifuged in +4C. RIPA Buffer (Table 2.2) was used as cell lysis buffer. Pellets were dissolved and incubated in +4C in a shaker. Later, they were centrifuged in 13.000 rpm +4C for 30 minutes. Supernatants were collected into new tubes. To quantify the proteins, Pierce™ BCA Protein Assay Reagent Kit was used according to the manufacturer's protocol. The absorbances was measured in 562 nm and a standart curve was created.

2.2.2.5 SDS-PAGE and Western Blot

SDS-PAGE gels were prepared according to the Table 2.13. Proteins were prepared using 4X Loading Buffer (Table 2.2) and boiled at 95°C for 5 minutes. Samples were loaded to gel, the setup was prepared in 1X Running Buffer (Table 2.2) The gels were run at 80V until the samples passed the stacking gel then at 120V until the end of the gel. The transfer was done with PVDF membrane at 250 mA, for 2.5 hours in 1X transfer buffer (Table 2.2)

Then, blockind was done using 5% milk powder in 1X TBS-T for 1 hour at RT. Membranes were incubated overnight at 4°C in the rotator. After, the membranes were washed 3 times for 10 minutes in 1X TBS-T. Next, secondary antibody incubation was done for an hour in room temperature. The membranes were washed again with 1X TBS-T 3 times for 10 minutes. To visualize the membranes ECL was used and the pictures were taken with Amersham Imager 600. Quantifications of the bands were done with ImageJ.

Table 2.12 SDS-PAGE Gel Preparation

8% Resolving Gel for 10 ml	4.7 mL ddH_2O, 2.7 mL 30% Acrlyamide-Bis Mix, 2.5 mL 1M Tris HCl (pH 8.8), 100 ul 10% SDS, 100 ul 10% APS, 8 ul TEMED
5% Stacking Gel for 5 mL	3.4 mL ddH_2O, 850 ul 30% Acrlyamide-Bis Mix, 625 uL 1M Tris HCl (pH 8.8), 50 ul 10% SDS, 50 ul 10% APS, 5 ul TEMED

CHAPTER 3. RESULTS

3.1 Determination of ERBIN Expression Levels in Different Breast Cancer Cell Lines

ERBIN gene expression levels were determined in epithelial-like MCF7, MCF10A, SK-BR-5, and mesenchymal-like CAL-51, MDA-MB-231, MDA-66 breast cancer cell lines by q-RT-PCR. (Figure 3.1) According to the results, cell lines that have mesenchymal characteristics have higher expression levels except for SK-BR-5. We checked the NCI-60 Dataset to see RNA-Seq results of breast cancer cell lines for their Erbin expressions. (Figure 3.1) MDA-MB-231 came up with the highest level of Erbin and MCF7 having lower expression of Erbin. TNBC and mesenchymal-like cell lines HS-578T and BT-549 has medium expression level just like epithelial cell line T47D. MCF7 and MDA-MB-231 cell lines were selected for the following experiments as the epithelial-like and mesenchymal-like examples respectively, since both of the data shown here is alike for these cell lines.

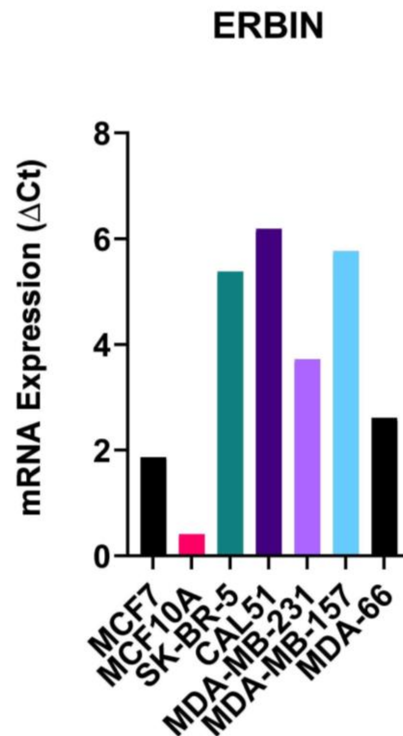


Figure 3.1 ERBIN gene expression in breast cancer cell lines

ERBIN gene expression levels in MCF7, MCF10A, CAL51, SK-BR-5, MDA-MB-231, MDA-MB-157, MDA-66 were shown by q-RT-PCR. Experiments were done in at least duplicates once. GAPDH gene is used for normalization in each experiment.

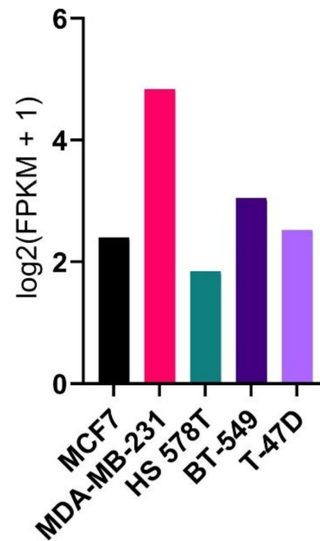


Figure 3.2 ERBIN gene expression in breast cancer cell lines from NCI-60 Dataset

ERBIN expression levels obtained from NCI-60 Data (U.S. National Cancer Institute) via CellMiner Analysis Tool.

3.2 Change In The Erbin Expression In TGF-β Induced EMT

In order to see the change of ERBIN expression after the cells go through EMT, we have selected two epithelial-like breast cancer cell lines MCF7 for adenocarcinoma and MCF10A as normal mammary cell representatives to investigate if there is a different outcome in tumor cells. MCF7 and MCF10A cells were treated with TGF-β for 72 hours to induce EMT. Later, q-RT-PCR was performed. To check if the cells have indeed gone through EMT, CDH1 expression was checked and seen that there was a significant decrease

in CDH1 in both cell lines. ERBIN expression levels were also significantly decreased in EMT induced MCF7 and MCF10A cells. (Figure 3.2)

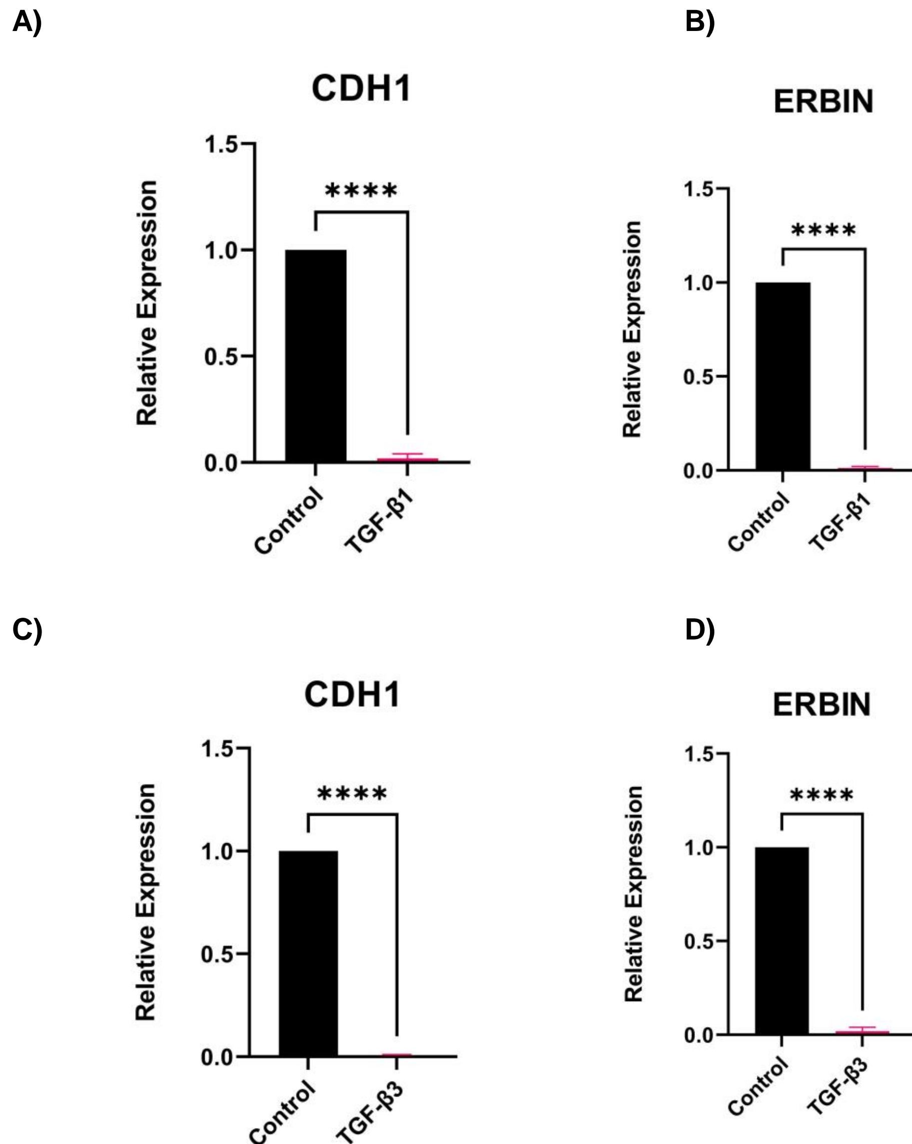


Figure 4.2 Erbin expression decreases in the TGF-β Induced EMT

MCF7 (A-B) and MCF10A (C-D) cells were treated with TGF-β for 72 hours. Gene expressions were checked with q-RT-PCR. CDH1 and ERBIN mRNA expressions were decreased in both cell lines. Experiments were done in triplicates, once. GAPDH gene was used to normalize the expression results. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$

3.3 Effect of ERBIN Overexpression on Epithelial to Mesenchymal Transition Related Genes in MCF7 Cell Line

3.3.1 Verification of ERBIN Overexpression in MCF7

To analyze if ERBIN has a role in EMT and the EMT pathways, first ERBIN was overexpressed in MCF7 cell line which normally has the lower ERBIN expression level than MDA-MB-231. After the prk5-myc-ERBIN plasmid transfection, the efficiency was checked with q-RT-PCR and Western Blot, in which a significant increase in both mRNA level and protein level was seen. (Figure 3.3)

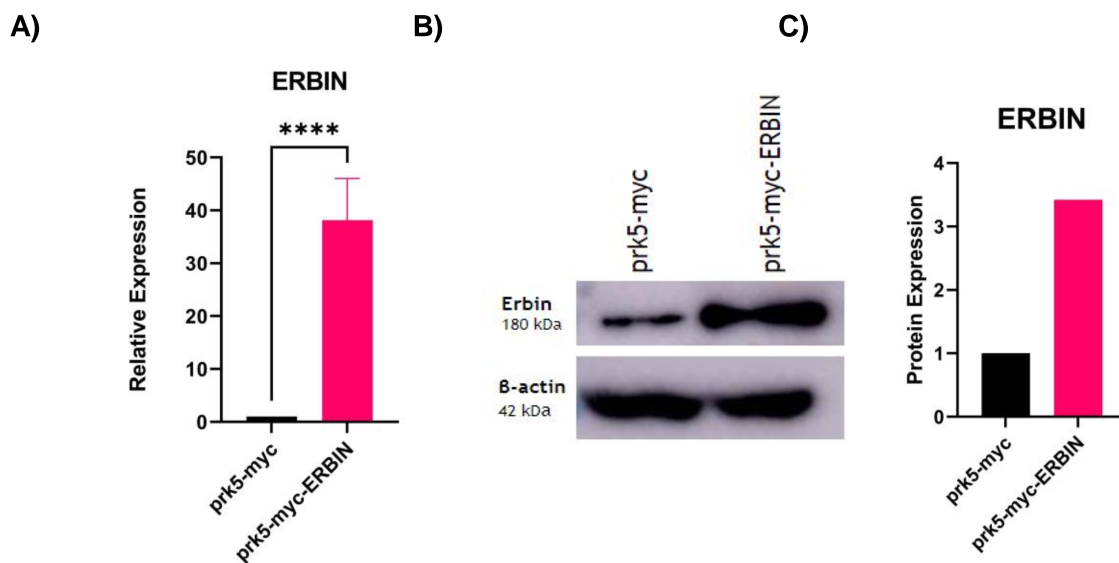
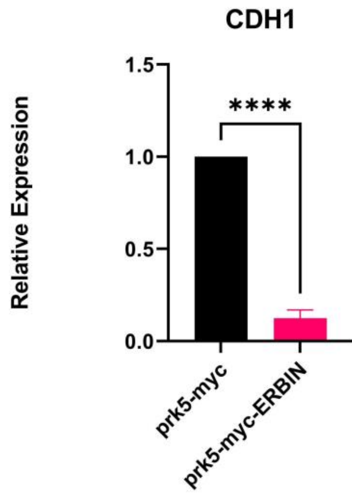


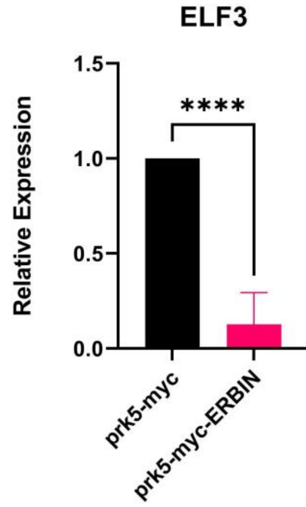
Figure 3.3. prk5-myc-ERBIN plasmid is successful in overexpressing ERBIN in MCF7

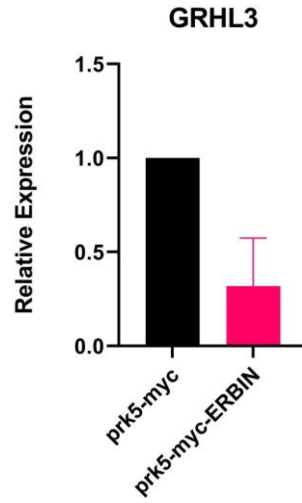
prk5-myc-ERBIN plasmid was used to transfect the cells, prk5-myc backbone plasmid was used as control. (A) After 72 hours, q-RT-PCR was performed to validate the transfection efficiency. q-RT-PCR was performed with triplicates, twice. GAPDH was used to normalize the results. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$ (B-C) After 72 hours, Western Blot was done and quantified with ImageJ and normalized with β-actin.

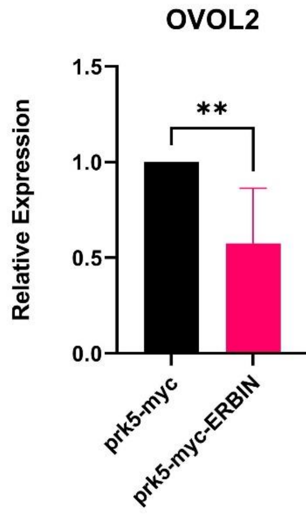
3.3.2 Changes in the EMT Related Genes Upon ERBIN Overexpression

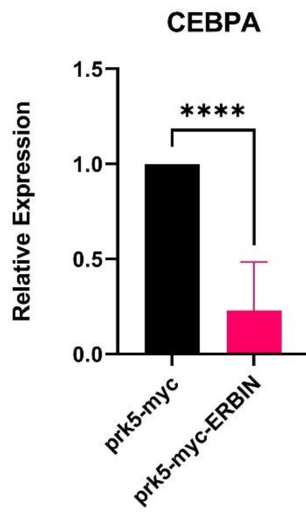
With the aim of investigating the effect of ERBIN overexpression in MCF7, we have focused on genes that are known to have a role in the EMT mechanism. As epithelial markers; CDH1, ELF3, GRHL3, OVOL2, CEBPA, HNF4A, EHF, and EPCAM were selected. As mesenchymal markers; FN, SNAIL, SLUG, ZEB2 and VIM were chosen to explore the changes in the cells upon ERBIN overexpression. To do so, after MCF7 cells were transfected with control and prk5-myc-ERBIN vectors succesfully were collected after 72 hours of incubation, and q-RT-PCR experiments were done. According to the mRNA levels of the transfected cells, out of the epithelial markers, CDH1, ELF3, OVOL2, CEBPA, HNF4A, EHF, and EPCAM were significantly decreased, whereas for GRHL3 there is a non-significant decrease. When we look at the mesenchymal markers, fibronectin was significantly increased. However, the other mesenchymal markers were decreased with ZEB2 and VIM decreases being significant even though these genes already show low expression in the epithelial cells. (Figure 3.4) These results tell us that when ERBIN is overexpressed in an epithelial cell, the epithelial markers show a significant decrease along with some of the mesenchymal markers being increased, some of them not significantly changing and some of them increasing. MCF7 cells start to differ from the normal epithelial gene expression upon ERBIN overexpression.

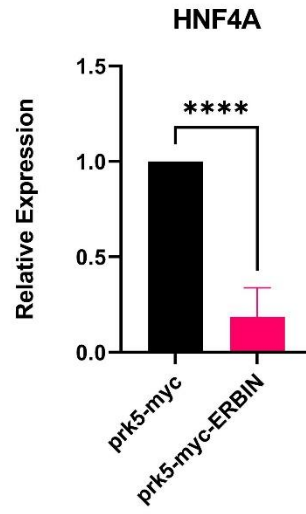

CDH1
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN

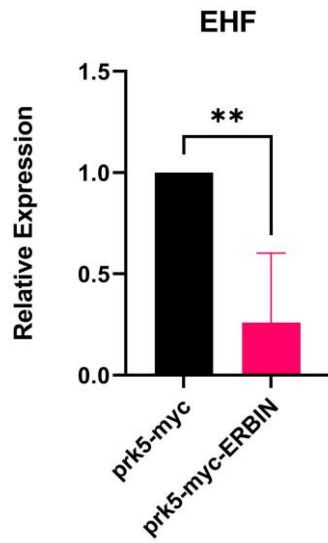

ELF3
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN

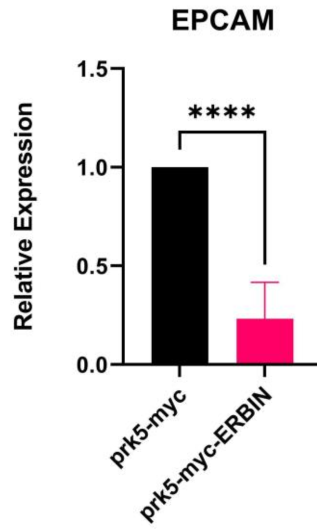

GRHL3
Relative Expression
1.5
1.0
0.5
0.0
prk5-myc
prk5-myc-ERBIN

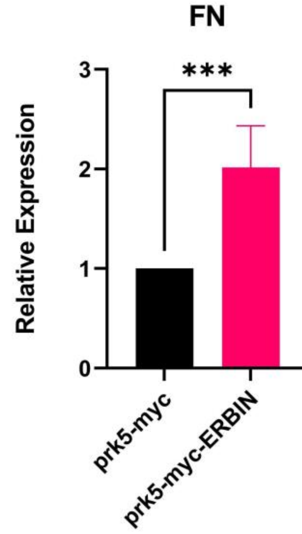

OVOL2
Relative Expression
1.5
1.0
0.5
0.0
**
prk5-myc
prk5-myc-ERBIN

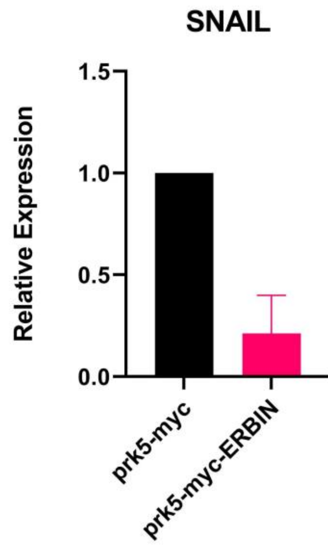

CEBPA
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN

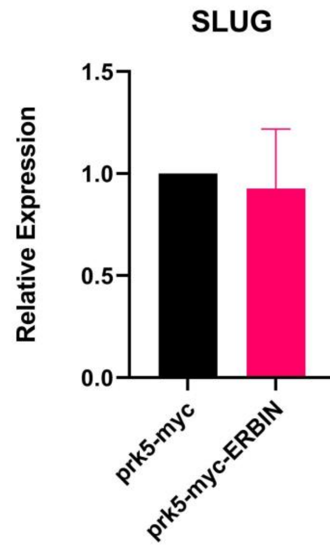

HNF4A
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN

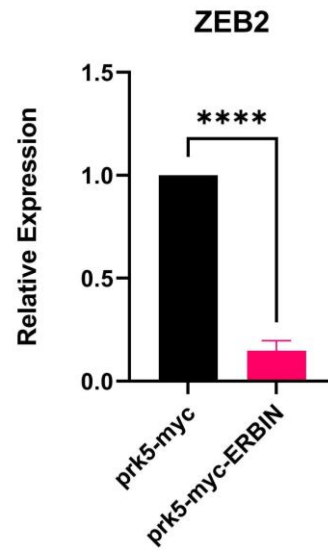

EHF
Relative Expression
1.5
1.0
0.5
0.0
**
prk5-myc
prk5-myc-ERBIN

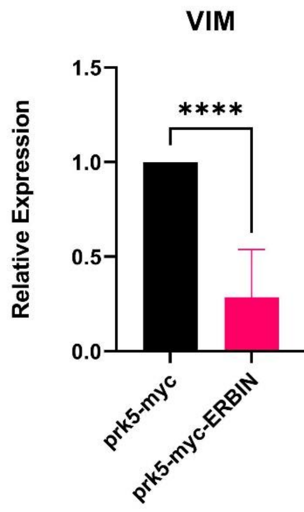

EPCAM
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN


FN
Relative Expression
3
2
1
0
***
prk5-myc
prk5-myc-ERBIN


SNAIL
Relative Expression
1.5
1.0
0.5
0.0
prk5-myc
prk5-myc-ERBIN


SLUG
Relative Expression
1.5
1.0
0.5
0.0
prk5-myc
prk5-myc-ERBIN


ZEB2
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN


VIM
Relative Expression
1.5
1.0
0.5
0.0
****
prk5-myc
prk5-myc-ERBIN

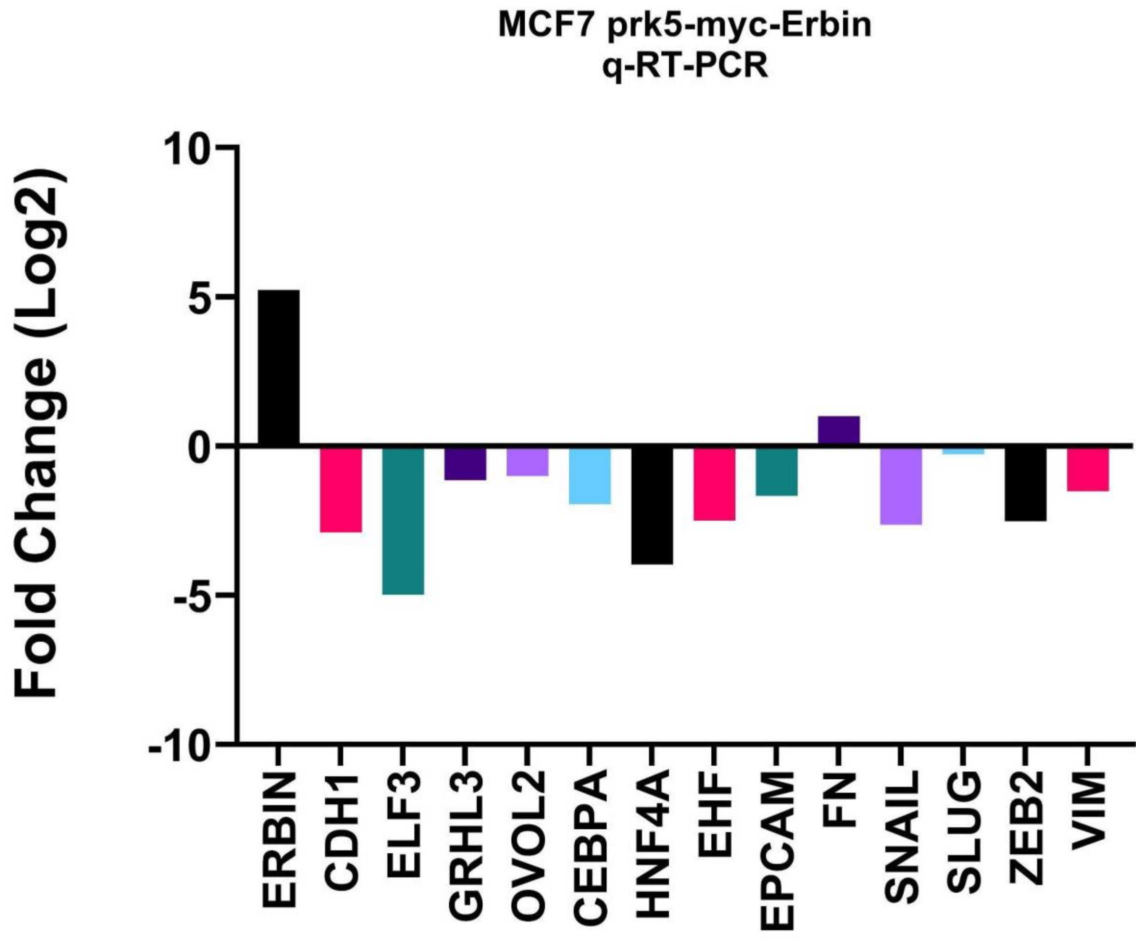


Figure 3.5 ERBIN Overexpression Effects EMT Genes' Expression Levels

Erbin overexpressed cells were collected, and q-RT-PCR was performed. Experiments were done in at least duplicates and twice. GAPDH was used to normalize each gene. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$

3.4 Effect of ERBIN Knockdown on Epithelial to Mesenchymal Transition Related Genes in MDA-MB-231 and MDA-66 Cell Lines

3.4.1 Validation of ERBIN Knockdown in MDA-MB-231 Cell Line

After examining how an epithelial cell changes when the Erbin level change, we looked into mesenchymal cells next. To do so, we have decided to knockdown ERBIN in the MDA-MB-231 cell line which is a mesenchymal characteristic line with high ERBIN expression. To achieve this non-target scramble RNA and siERBIN were transfected to MDA-MB-231 cells and collected after 72 hours to validate the knockdown. Western Blot and q-RT-PCR experiments confirmed that there is a significant decrease in both mRNA and protein levels. (Figure 3.5)

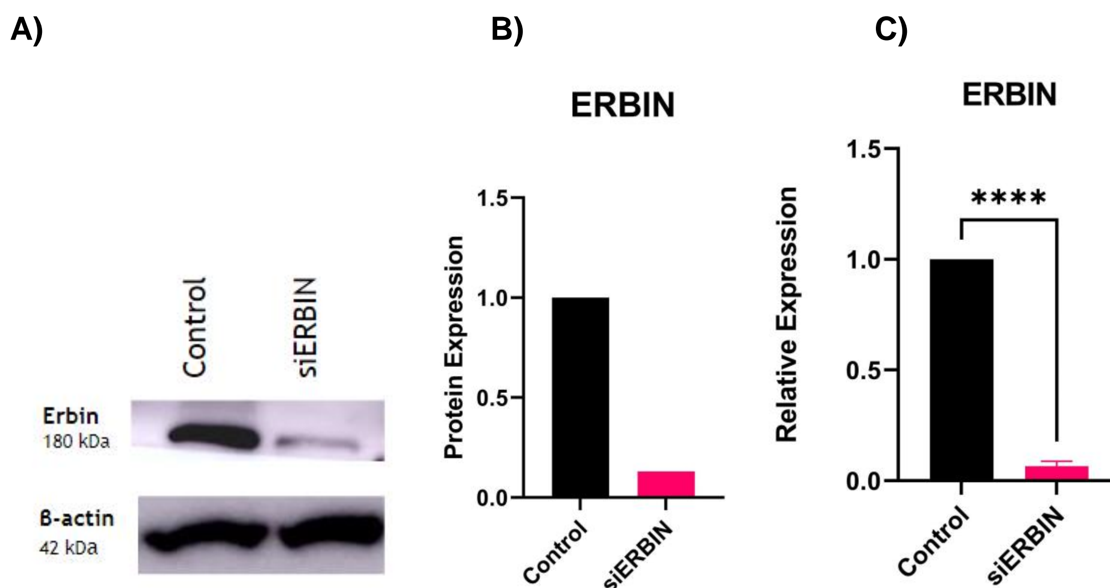
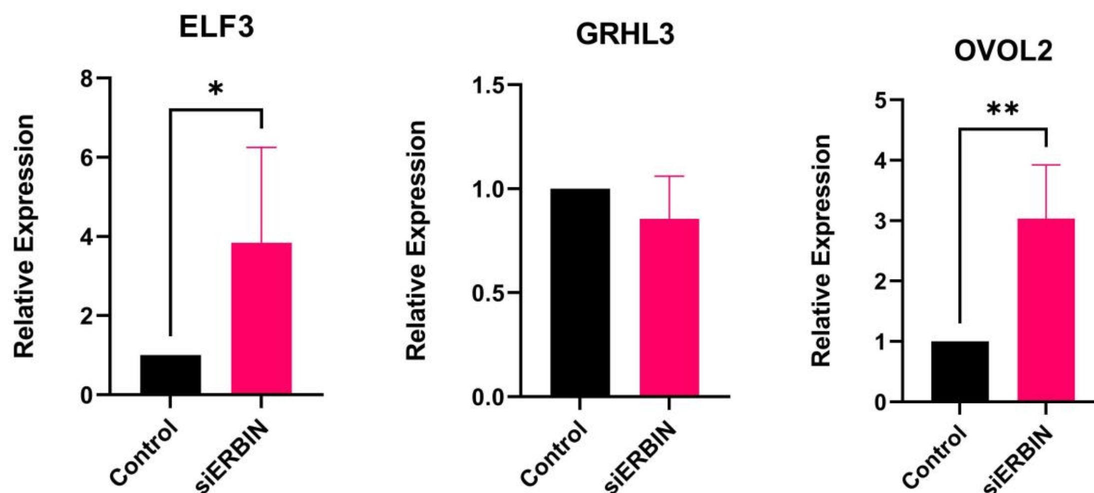


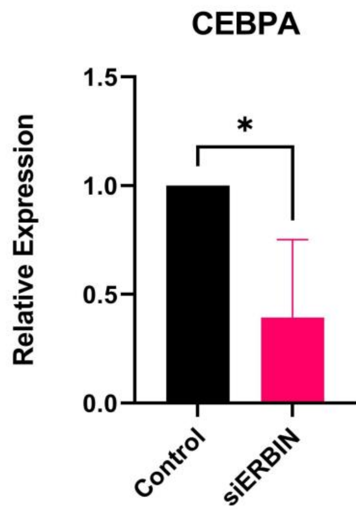
Figure 3.6 ERBIN was silenced with si-ERBIN

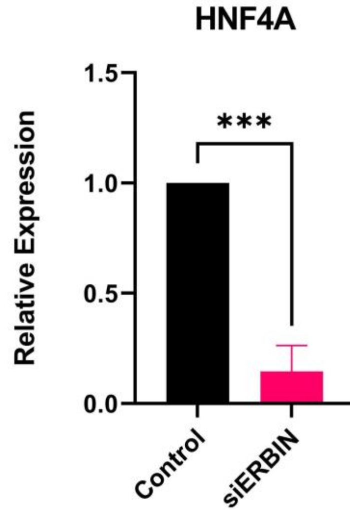
Efficiency of the siERBIN transfection was determined using Western Blot and q-RT-PCR after 72 hours post transfection. (A-B) Western Blot of treated MDA-MB-231 cells, quantified with ImageJ and normalized with β-actin. (C) q-RT-PCR validation of transfected MDA-MB-231 cells. Experiment was performed in triplicates, twice. GAPDH was used as control. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$

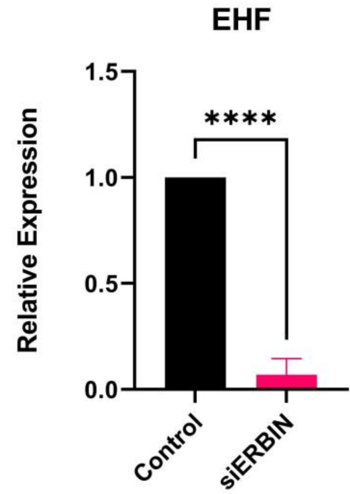
3.4.2 Assessing the Expressions of EMT Markers in ERBIN Silenced MDA-MB-231 Cells

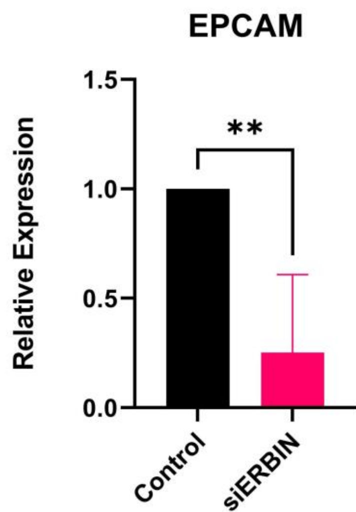
Upon ERBIN knockdown, we have performed q-RT-PCR experiments so that we could investigate the result of the lack of ERBIN in EMT marker expression levels. For this experiment ELF3, GRHL3, OVOL2, CEBPA, HNF4A, EHF, and EPCAM were selected as epithelial markers. Mesenchymal markers are CDH2, FN, SNAIL, SLUG, ZEB2, and VIM. The results of these experiments showed that when ERBIN is silenced in this mesenchymal-like cell line, the epithelial markers ELF3 and OVOL2 are significantly upregulated, GRHL3 is non-significantly increased. CEBPA, HNF4A, EHF, and EPCAM are downregulated significantly. When we look at mesenchymal markers, N-CAD and FN show significant decrease, accompanied by a non-significant decrease of SNAIL. On the other hand, SLUG and ZEB2 are significantly upregulated and there is an increase in VIM expression. (**Figure**) The loss of ERBIN in MDA-MB-231 cell line affects these EMT markers significantly with no distinction of epithelial or mesenchymal shift directly.

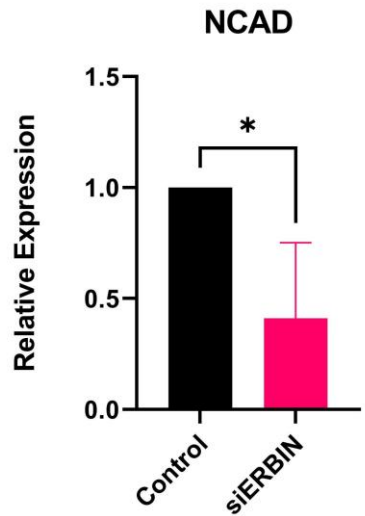


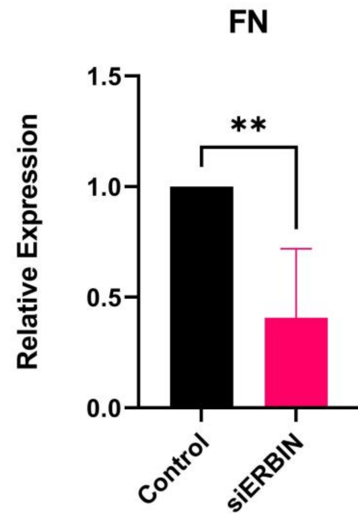

CEBPA
Relative Expression
1.5
1.0
0.5
0.0
*
Control
siERBIN

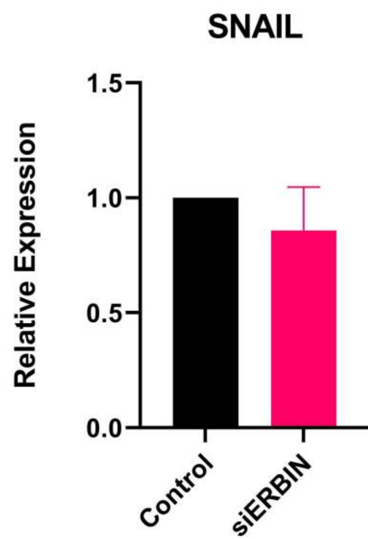

HNF4A
Relative Expression
1.5
1.0
0.5
0.0
***
Control
siERBIN

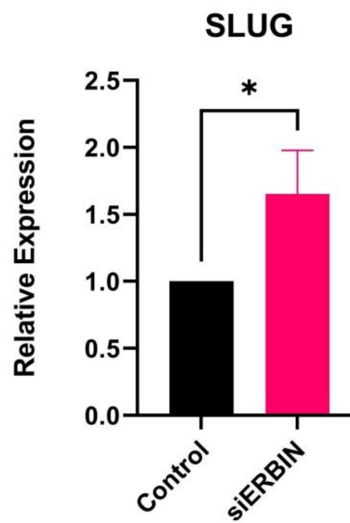

EHF
Relative Expression
1.5
1.0
0.5
0.0
****
Control
siERBIN

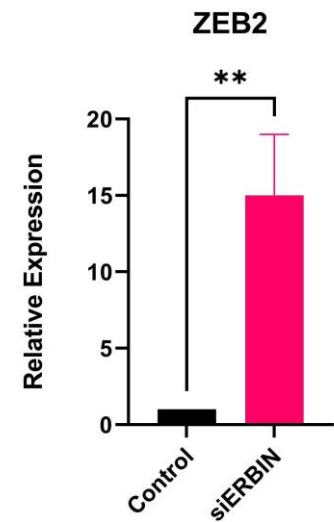

EPCAM
Relative Expression
1.5
1.0
0.5
0.0
**
Control
siERBIN

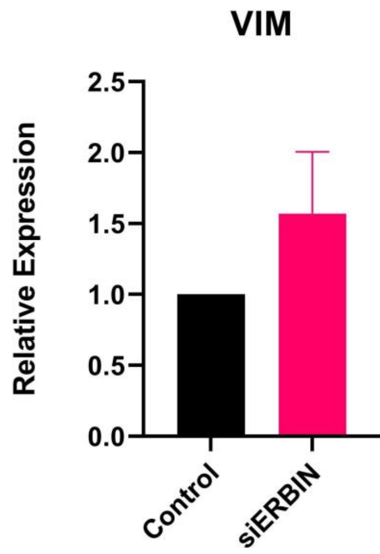

NCAD
Relative Expression
1.5
1.0
0.5
0.0
*
Control
siERBIN


FN
Relative Expression
1.5
1.0
0.5
0.0
**
Control
siERBIN


SNAIL
Relative Expression
1.5
1.0
0.5
0.0
Control
siERBIN


SLUG
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
siERBIN


ZEB2
Relative Expression
20
15
10
5
0
**
Control
siERBIN


VIM
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
siERBIN

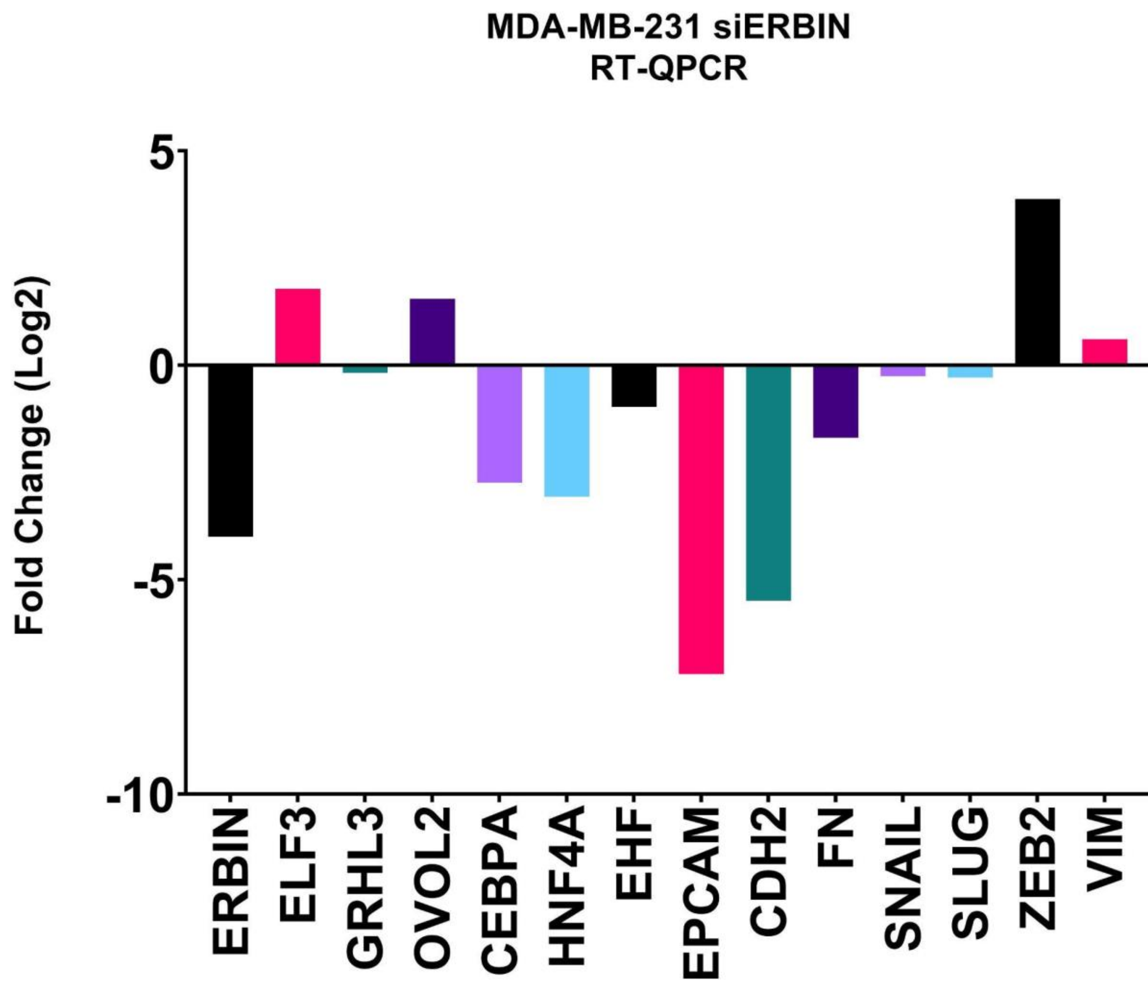

MDA-MB-231 siERBIN
RT-QPCR
Fold Change (Log2)
5
0
-5
-10
ERBIN
ELF3
GRHL3
OVOL2
CEBPA
HNF4A
EHF
EPCAM
CDH2
FN
SNAIL
SLUG
ZEB2
VIM

Figure 3.7 ERBIN Knockdown Effects EMT Marker Gene Expressions

Erbin silenced MDA-MD-231 cells were collected, and q-RT-PCR was done. Experiments were done in at least duplicates and twice. To normalize each sample GAPDH was chosen.. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$

3.4.3 ERBIN Knockdown of MDA-66 Cell Line

To further investigate the effect of ERBIN silencing in mesenchymal cells, we have decided to use MDA-66 cell line. This cell line is ERα-positive, created by stable transfection of pCMV-ERα vector of MDA-MB-231.[46] This cell line is courtesy of Alotaibi Lab, İzmir Biomedicine and Genome Center.

After siERBIN transfection of the MDA-66 cells, to demonstrate the knockdown, q-RT-PCR technique was used. The results validated the silencing, showing a highly significant downregulation. (Figure 3.7)

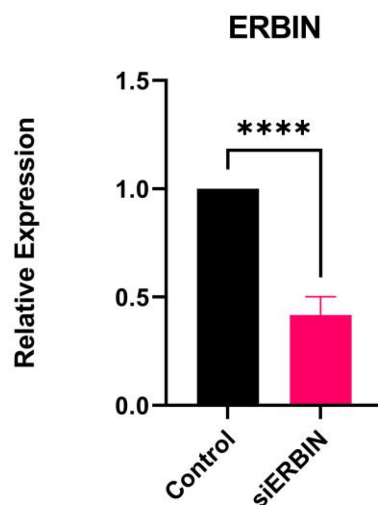
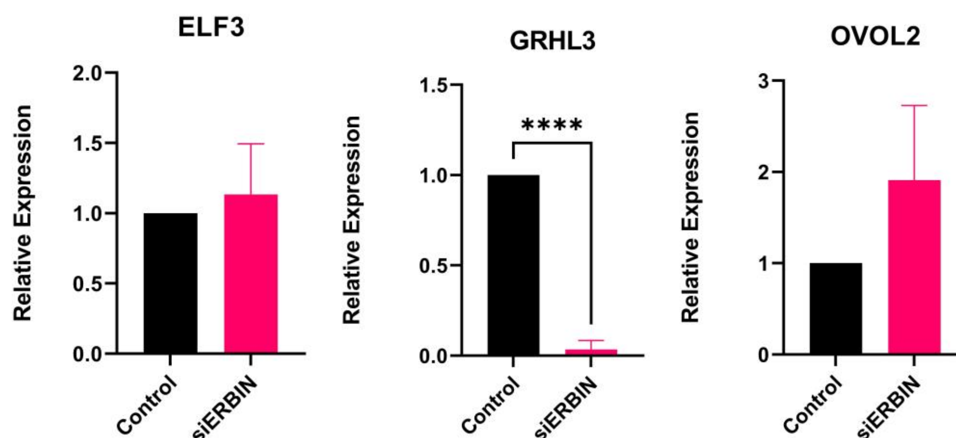


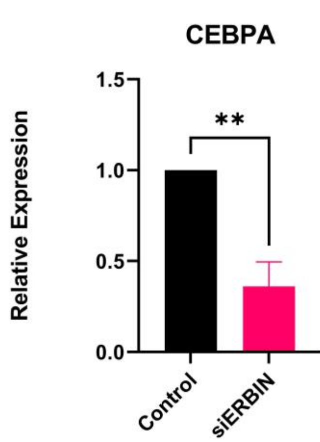
Figure 3.8 ERBIN knockdown was successful with si-ERBIN in MDA-66 Cells

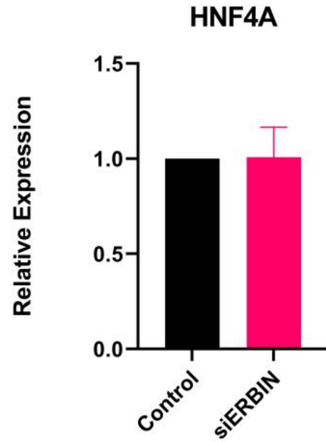
q-RT-PCR experiment of transfected MDA-66 cells. The experiment was done with at least duplicates, twice. q-RT-PCR results were normalized with GAPDH. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p < 0.001$

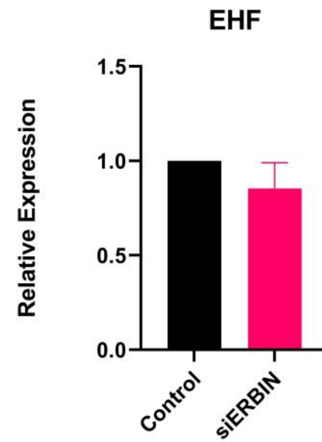
3.4.4 Knockdown of ERBIN Effects EMT Markers in MDA-66 Cell Line

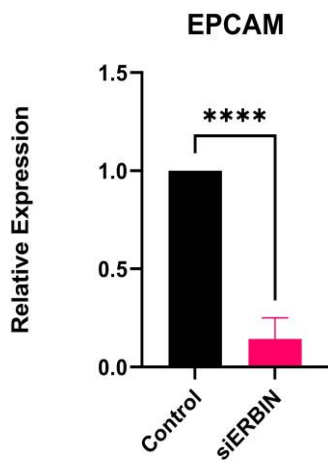
Our next aim was to see if there is any change in this ERα-positive and mesenchymal-like cell line regarding EMT markers after ERBIN silencing. Non-target scramble RNA and siERBIN was used to transfect the cells and they were incubated for 72 hours. After the cell pellets are collected and cDNA were obtained, we performed q-RT-PCR. For comparison of epithelial and mesenchymal markers and inspecting if there is a shift towards epithelial type, a set of EMT markers was used again. For epithelial markers ELF3, GRHL3, OVOL2, CEBPA, HNF4A, EHF and EPCAM were selected. In addition, FN, SNAIL, SLUG, ZEB2 and VIM were used as mesenchymal markers. q-RT-PCR experiments told us that epithelial markers GRHL3, CEBPA and EPCAM are significantly downregulated. Other epithelial markers of ERBIN silenced ELF3, OVOL2, HNF4A and EHF did not show significant differences from the controls. FN, SLUG and VIM downregulated significantly whereas SNAIL was upregulated. ZEB2 showed a decrease but not a significant one. In the light of these results, we cannot say there is an inclination towards epithelial type when ERBIN is silenced in mesenchymal-like MDA-66, but there is an effect on different EMT related genes.

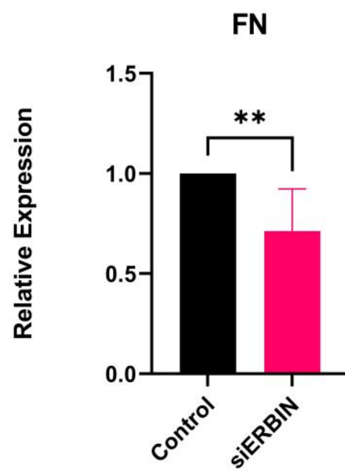


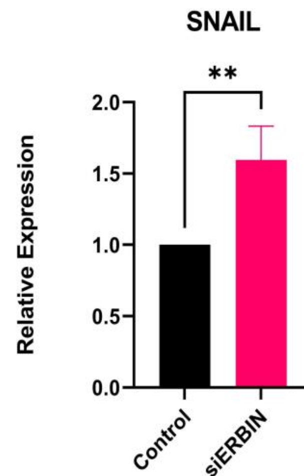

CEBPA
Relative Expression
1.5
1.0
0.5
0.0
**
Control
siERBIN

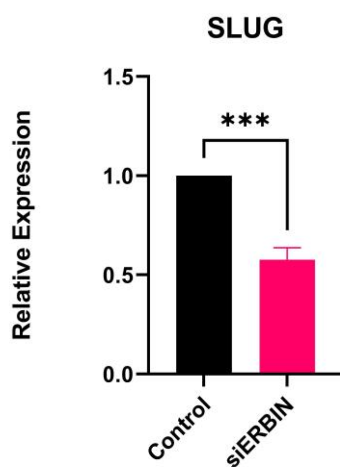

HNF4A
Relative Expression
1.5
1.0
0.5
0.0
Control
siERBIN

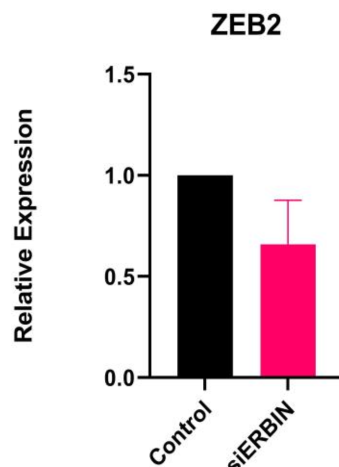

EHF
Relative Expression
1.5
1.0
0.5
0.0
Control
siERBIN

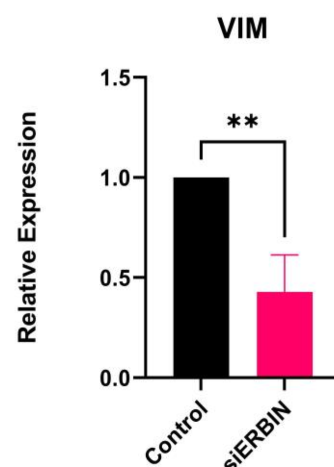

EPCAM
Relative Expression
1.5
1.0
0.5
0.0
****
Control
siERBIN


FN
Relative Expression
1.5
1.0
0.5
0.0
**
Control
siERBIN


SNAIL
Relative Expression
2.0
1.5
1.0
0.5
0.0
**
Control
siERBIN


SLUG
Relative Expression
1.5
1.0
0.5
0.0
***
Control
siERBIN


ZEB2
Relative Expression
1.5
1.0
0.5
0.0
Control
siERBIN


VIM
Relative Expression
1.5
1.0
0.5
0.0
**
Control
siERBIN

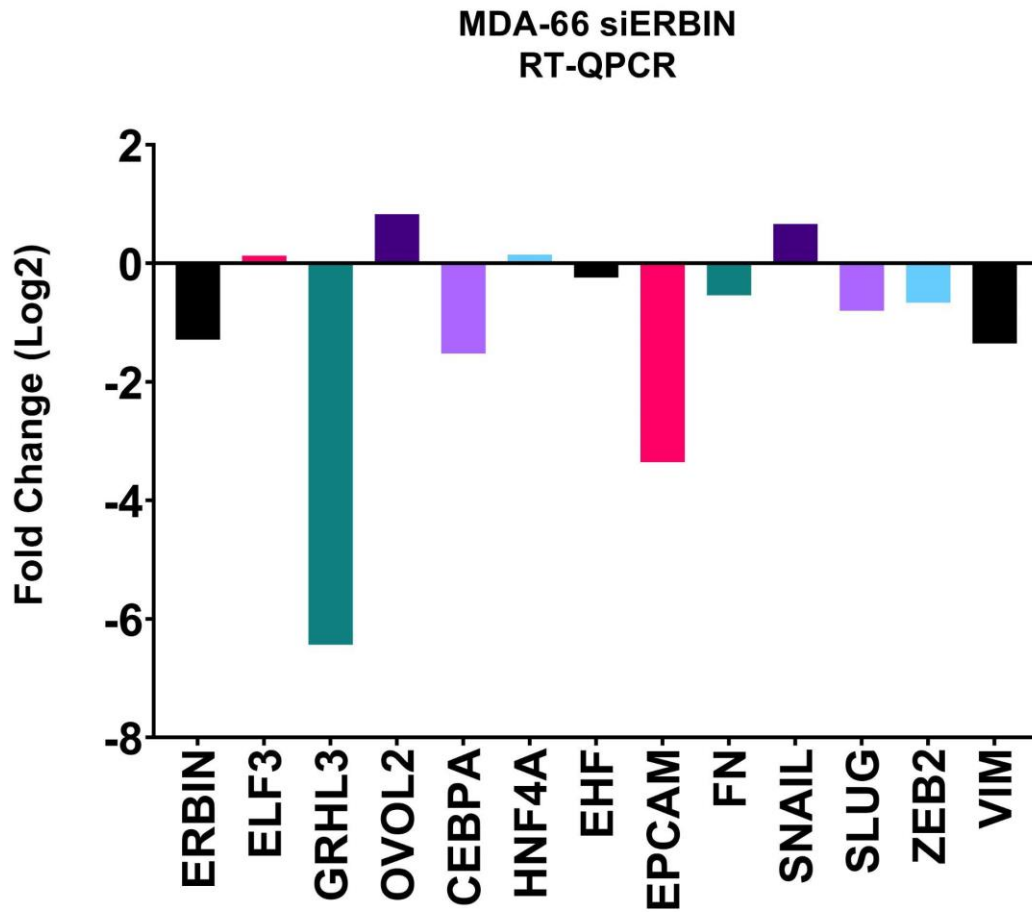


Figure 3.9 ERBIN Knockdown Results in Changes in EMT Marker Expressions in MDA-66 Cells

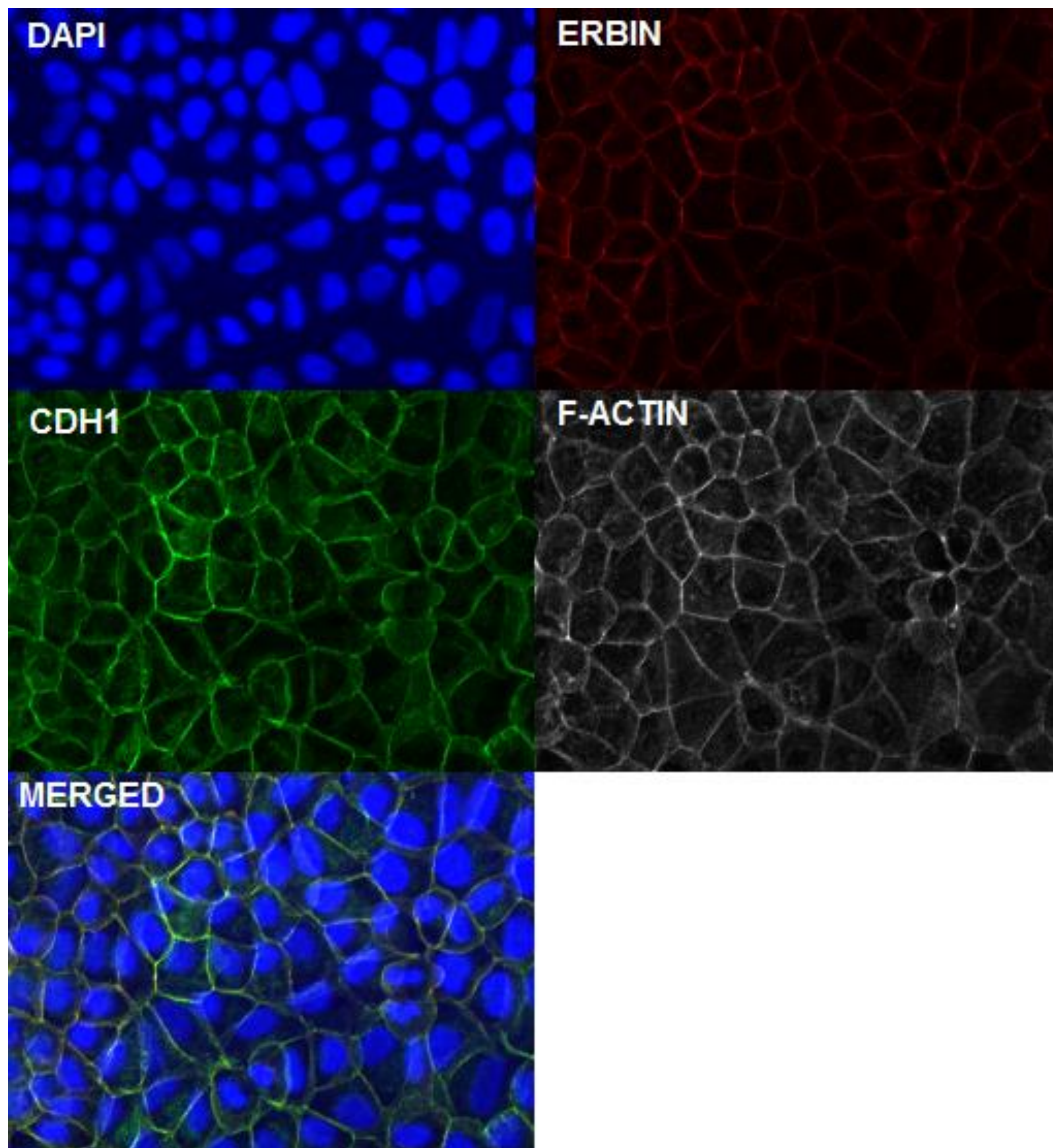
MDA-66 cells were transfected with non-target control RNA and siERBIN, then collected after 72 hours. q-RT-PCR were done in at least duplicate, twice. GAPDH was used to normalize each sample result. * for $p < 0.05$, ** for $p < 0.005$ ***, **** for $p <0.001$

3.5 Co-Immunofluorescence Staining of ERBIN Overexpressed MCF7 Cells

After the experiments that provided us with the results of mRNA expression levels, we decided to check the protein levels and the protein localization with co-immunofluorescence labeling followed by confocal microscopy. In this experiment, we have chosen Cdh1 to check if there is a shift in the cell towards any side of epithelial or mesenchymal cell type. We have also labeled the actin skeleton by using F-Actin (Phalloidin) to visualize if the cell went through a change in the cell skeleton as it happens in EMT, in relation to morphological change of the cells.

In Erbin overexpressed MCF7 cells we can see the ERBIN protein, mostly localized at the membrane as expected. Cdh1 appeared to be decreased, as well as F-Actin, they are no longer localized at the membranes as much as the control cells. These indicate that there is a shift towards the mesenchymal state in ERBIN overexpressed MCF7 cells. (Figure 3.10)

A)



B)

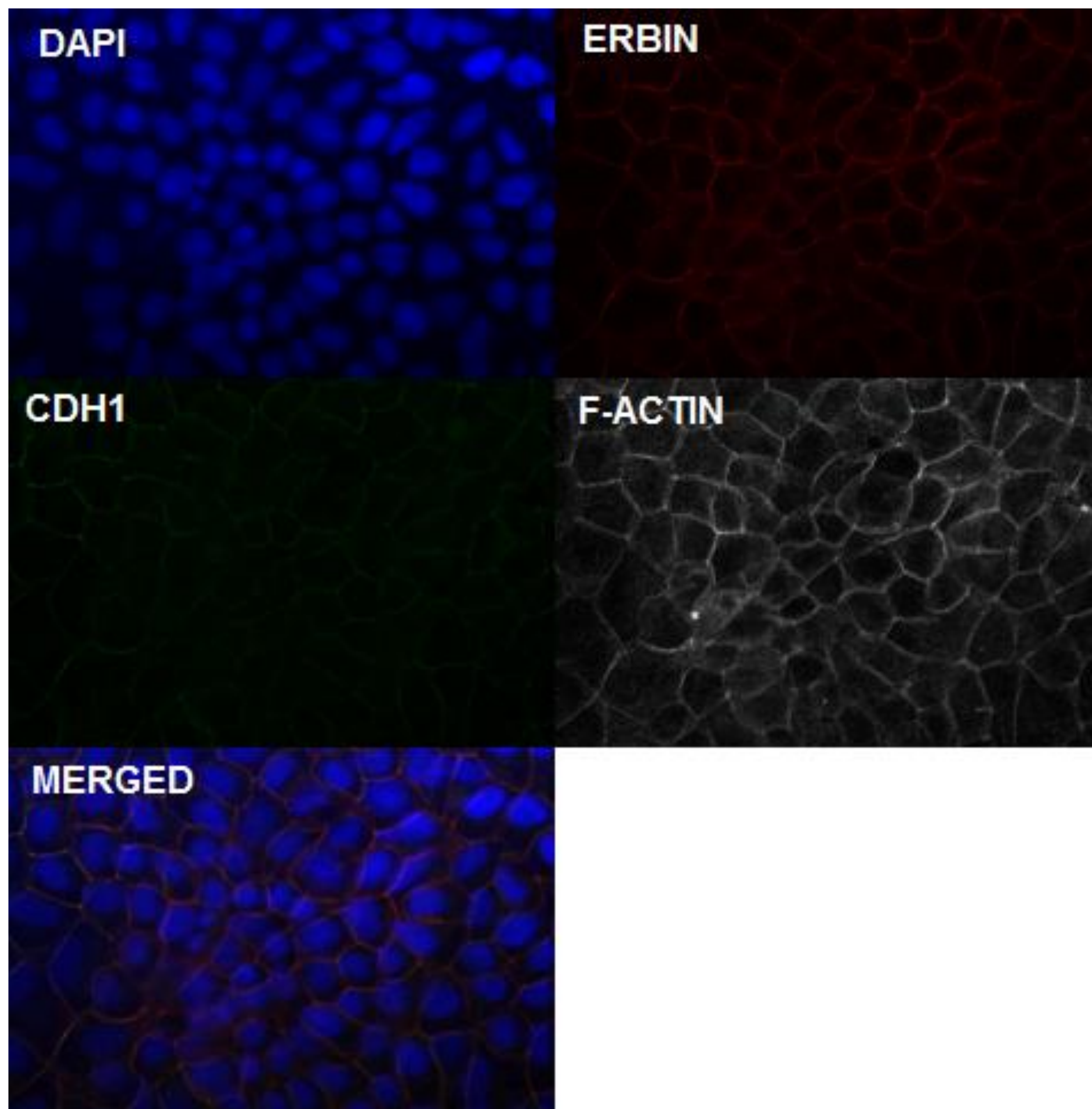


Figure 3.10 Co-Immunofluorescence Labeling of ERBIN Overexpressed MCF7 Cells Shows Decreased E-Cadherin Protein Level

MCF7 cells were transfected with (A) control plasmid and (B) prk5-myc-ERBIN vector to overexpress ERBIN prior to staining with ERBIN, CDH1, and F-ACTIN antibodies. Pictures were obtained with confocal microscopy. The pictures were taken at 25X. This labeling was done once.

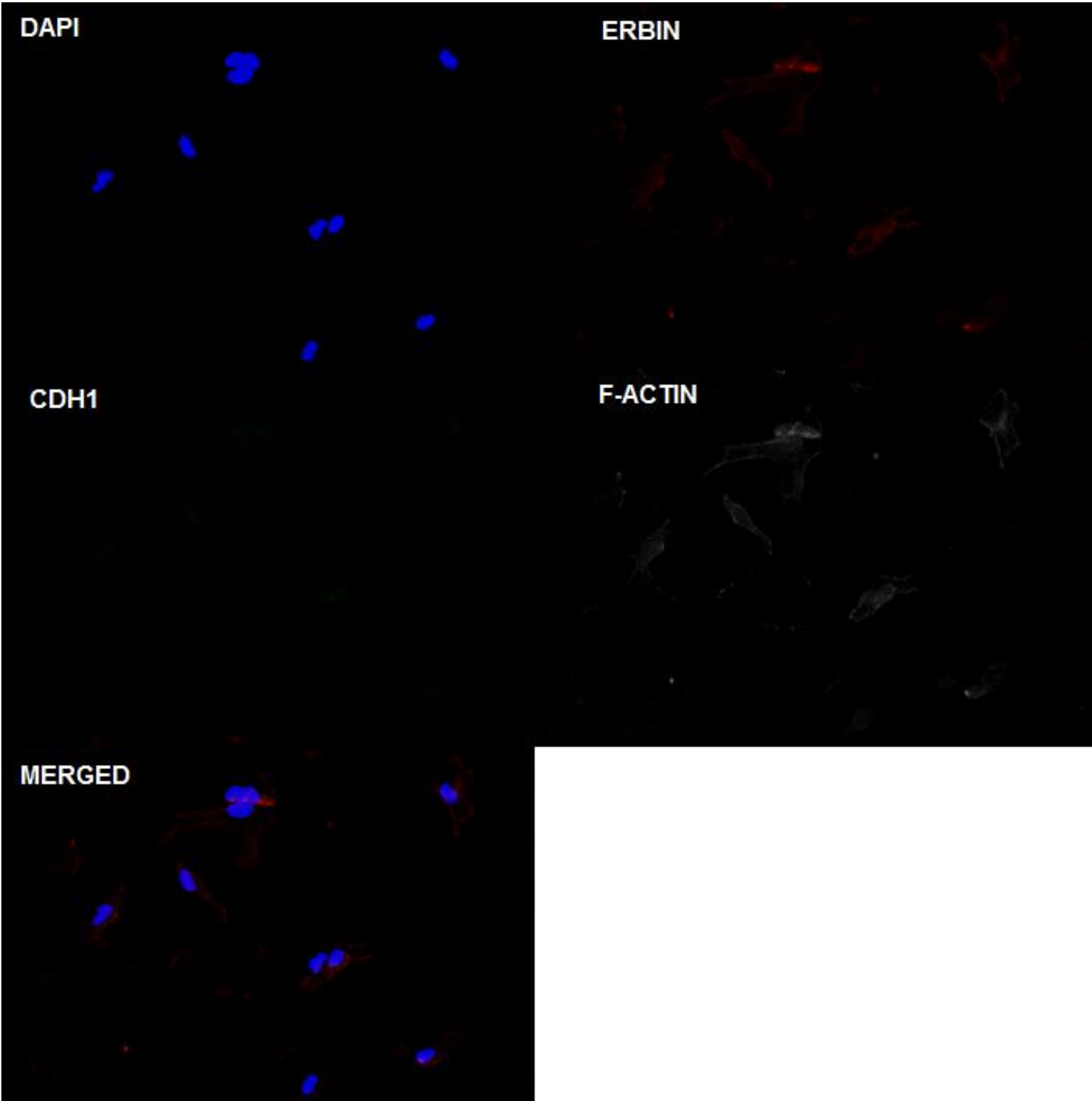
3.6 Co-Immunostaining of ERBIN Silenced MDA-MB-231 Cells

Our next step was visualizing the protein change in the siERBIN transfected MDA-MB-231 cells. For co-immunostaining Erbin, Cdh1, and F-actin were used again. In Erbin silenced cells, there is no detectable Erbin antibody signal. Plus, no Cdh1 was detected in the control group due to this cell line's mesenchymal nature and there was no increase of Cdh1 after Erbin knockdown. There is no visibly significant change in F-actin as well. (Figure 3.11)

3.7 Co-Immunostaining of ERBIN Silenced MDA-66 Cells

After transfection of non-target RNA and siERBIN in MDA-66 cells, the cells were stained with Erbin, Cdh1, F-Actin antibodies. Compared to the control group, in the knockdown group, there is a decrease in Erbin level. No Cdh1 was detected in either of the groups. There is a slight decrease of F-Actin in the membranes of Erbin silenced cells. (Figure 3.12)

A)



B)

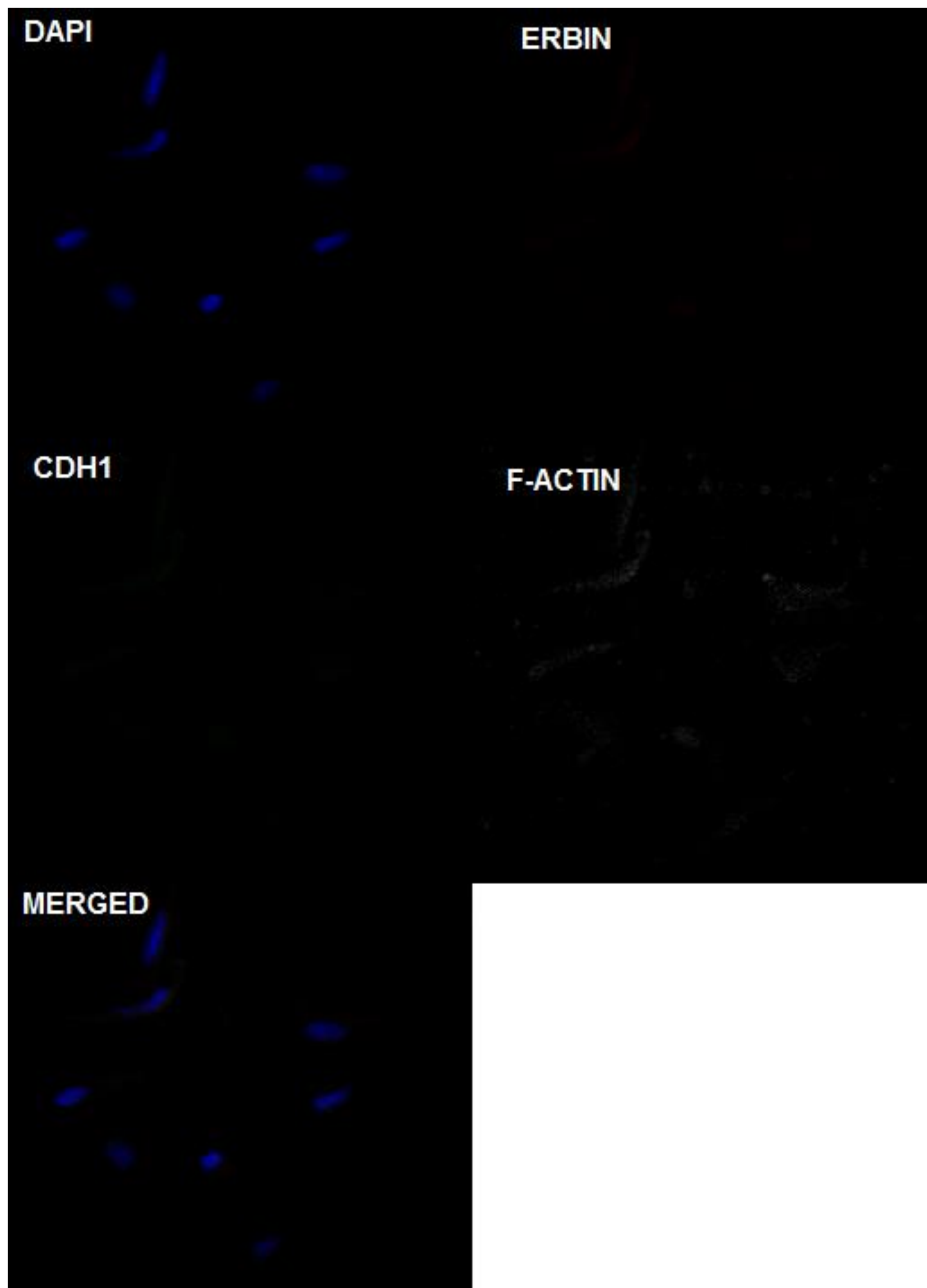
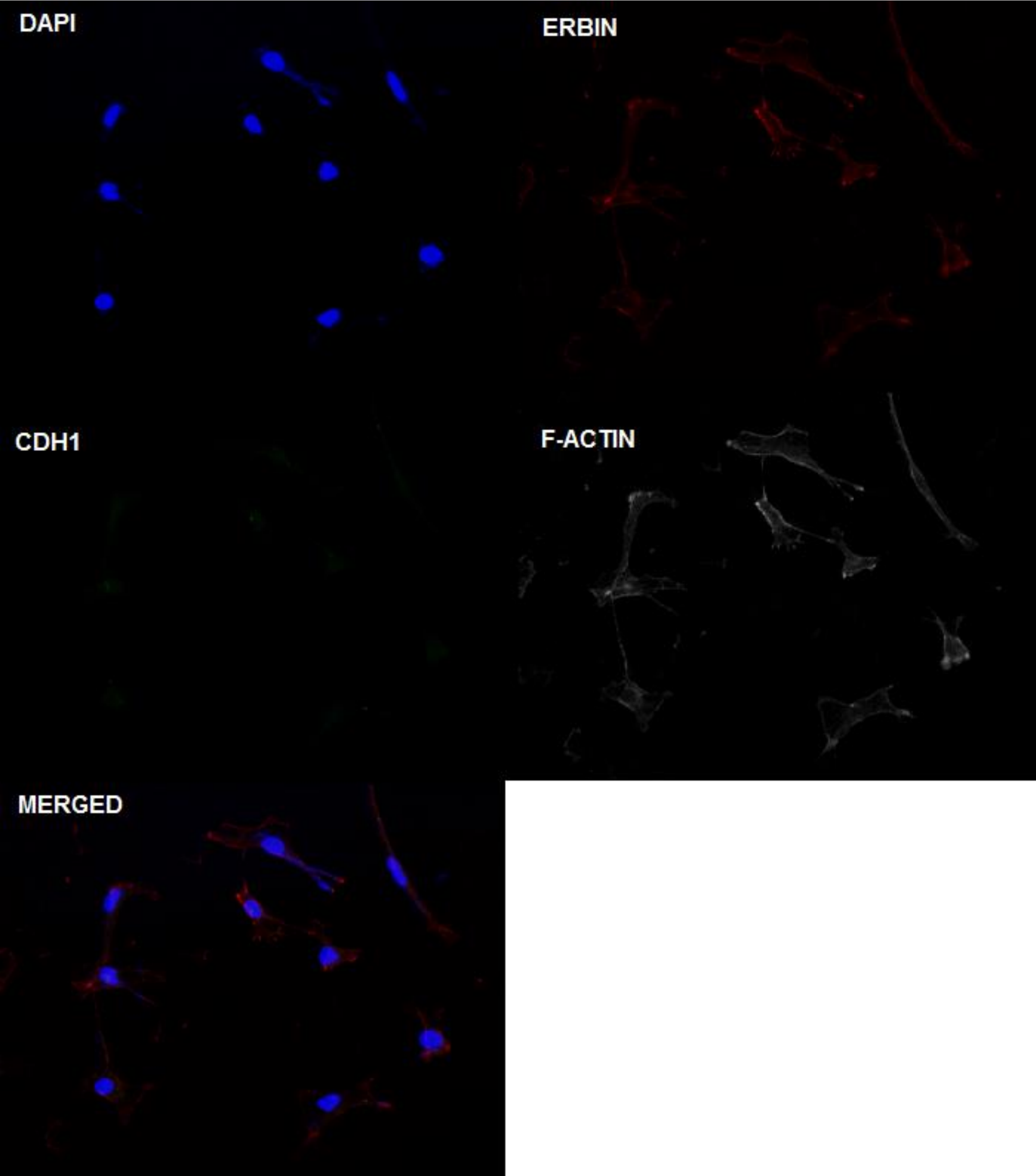


Figure 3.11 Co-Immunofluorescence Staining of ERBIN Silenced MDA-MB-231 Cells

MDA-MB-231 cells were transfected with (A) non-target RNA and (B) siERBIN to knockdown ERBIN and stained with ERBIN, CDH1, and F-ACTIN antibodies. The pictures were taken at 25X with confocal microscopy. This labeling was done once.

A)



B)

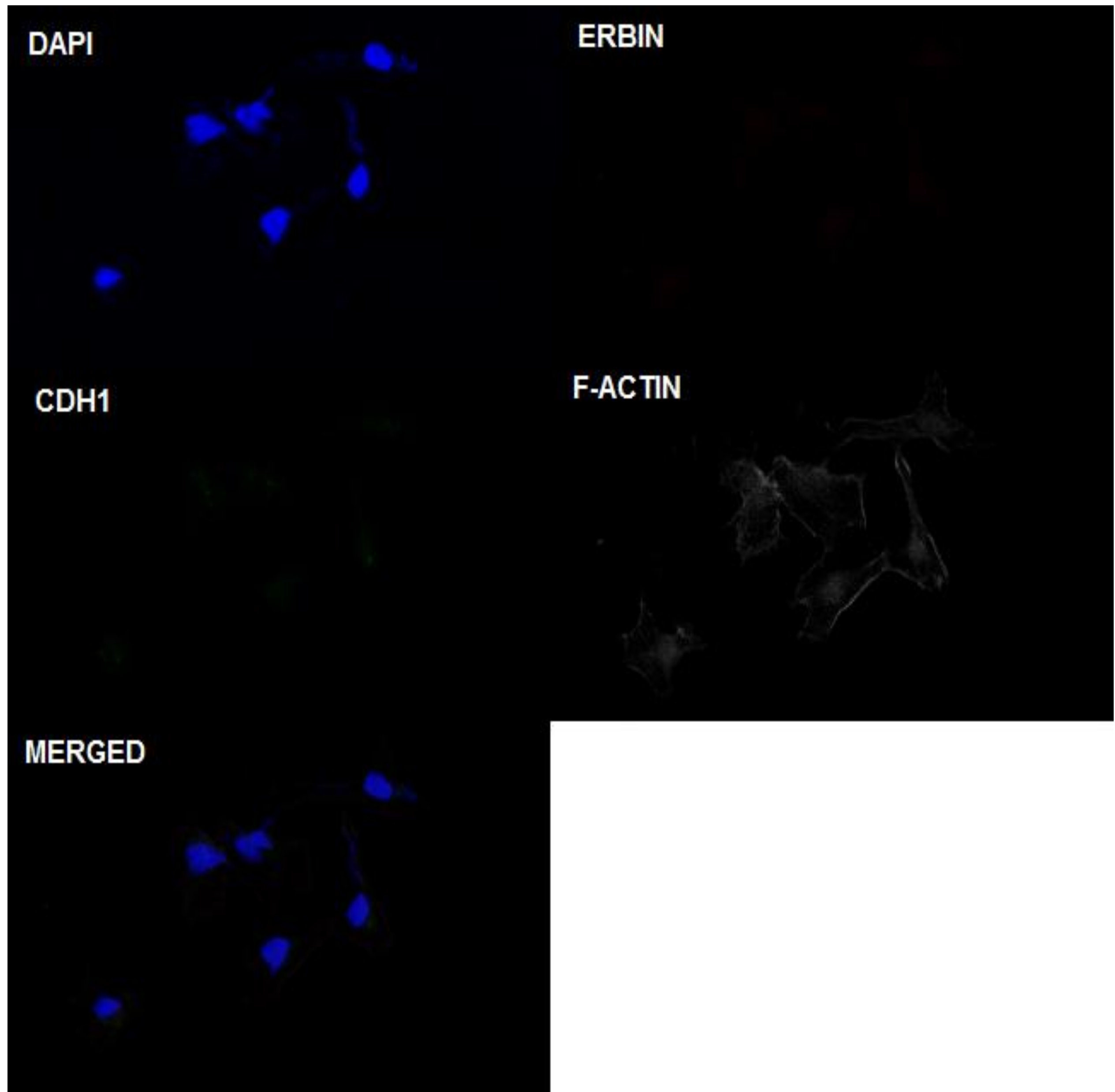


Figure 3.12 Co-Immunofluorescence Staining of ERBIN Silenced MDA-66 Cells

MDA-66 cells were transfected with (A) non-target RNA and (B) siERBIN and ERBIN silenced cells were stained with ERBIN, CDH1, and F-ACTIN antibodies. Confocal microscopy was used to obtain the pictures. The pictures were taken at 25X. This labeling was done once.

3.8 In Silico Analyses of Expression of Erbin in EMT

After obtaining our results, we wanted to check what happens to Erbin in EMT process from other data with bioinformatic analyses.

Differential expression analyses were done by Necati Kaan KUTLU with MCF7 cells that are ZEB1 induced to go through EMT/MET and NMuMG EMT/MET model which is a mouse mammary gland cell line. To obtain the results, FPKM values from RNA-Seq were filtered to be over 0.5. Then, to obtain EMT and MET log fold change values, the formulas log2((induced+1)/(uninduced+1)) and log2((deinduced+1)/(induced +1)) respectively. To visualize logFC(EMT) ve logFC(MET), ggplot2 package in R was used. In NMuMG dataset, Erbin is upregulated during MET by a small amount but it is not a differentially expressed gene during the EMT/MET progress. Erbin does not cluster with either epithelial or mesenchymal genes. (Figure) When we for Erbin at MCF7 data which is from ZEB2-induced cell, it is not a differentially expressed gene again. In this data, Erbin is placed closely with epithelial markers. (Figure)

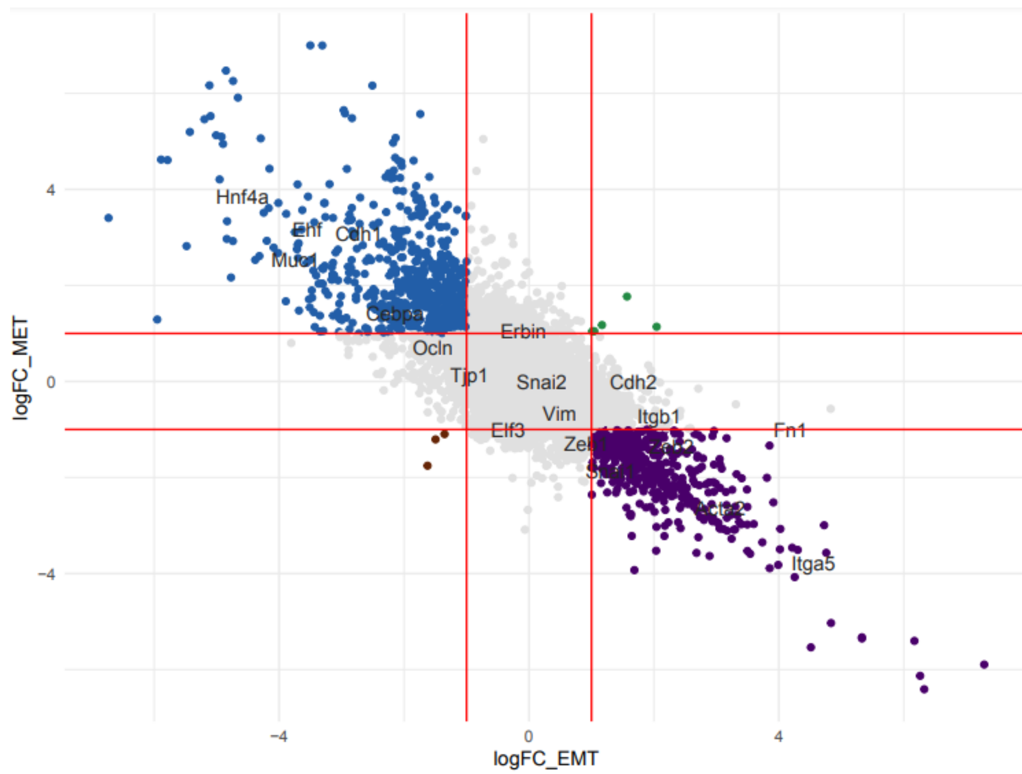


Figure 3.13 Differentially expressed genes in NMuMG EMT/MET time course

Differential gene expression analysis of RNA-Seq data from EMT/MET model of NmuMG cell line was done. The figure was created using Rstudio.

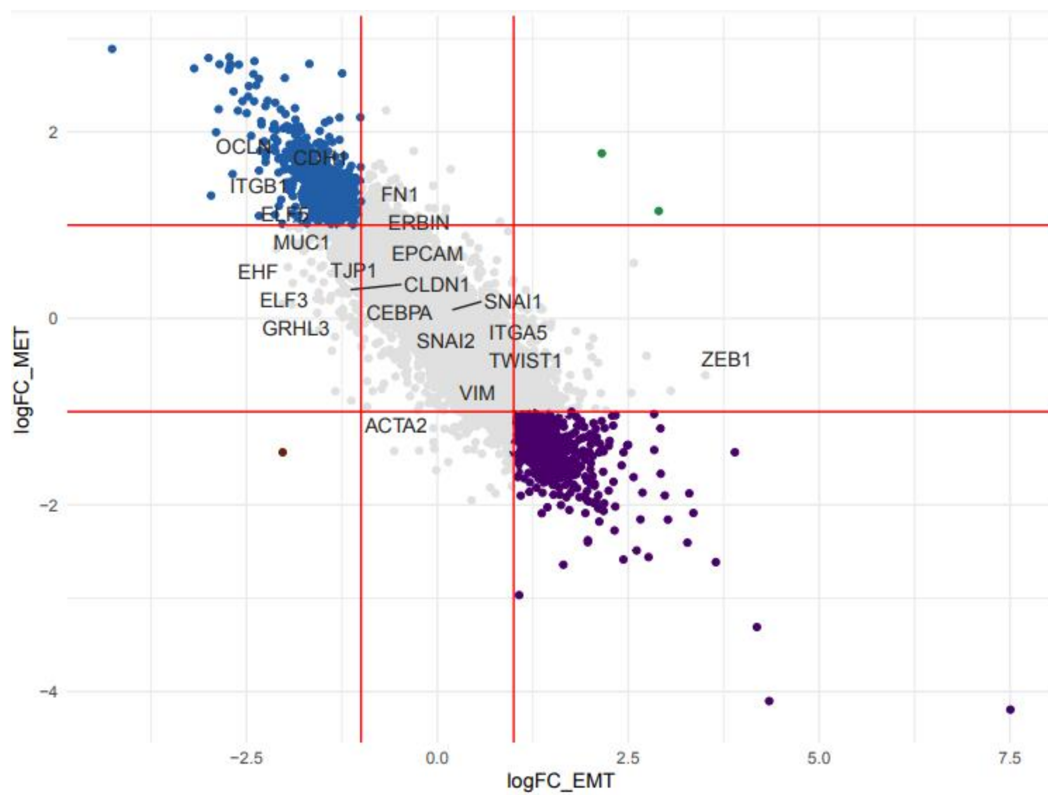


Figure 3.14 Differentially expressed genes in ZEB1 induced EMT in MCF7 cell line

Differential gene expression analysis of RNA-Seq data from ZEB1 induced EMT/MET model of MCF7 cell line was done. The figure was created using Rstudio with ggplot2 package.

Additionally, various datasets from Gene Expression Omnibus (NIH) were analyzed. The dataset GSE17708 is from A5A9 adenocarcinomic human alveolar basal epithelial cells, GSE20247 is from HK2 which is a normal kidney cell line, GSE23952 is from pancreatic cancer cell line PANC-1, and GSE8240 is obtained from MCF7 breast cancer cell line. All of the data in these datasets are from TGFβ induced EMT experiments and contains RNA-Seq data. Observing the hierarchical clustered heatmap figure created from the log fold change numbers, it is seen that Erbin is clustered with mesenchymal markers. Erbin level is increased in TGF-β treated cells except MCF7 cells. Also, in MCF7 cell line data here, contrary to other cell lines, the mesenchymal markers are not visibly increased except FN upon TGFβ treatment. (Figure 3.15)

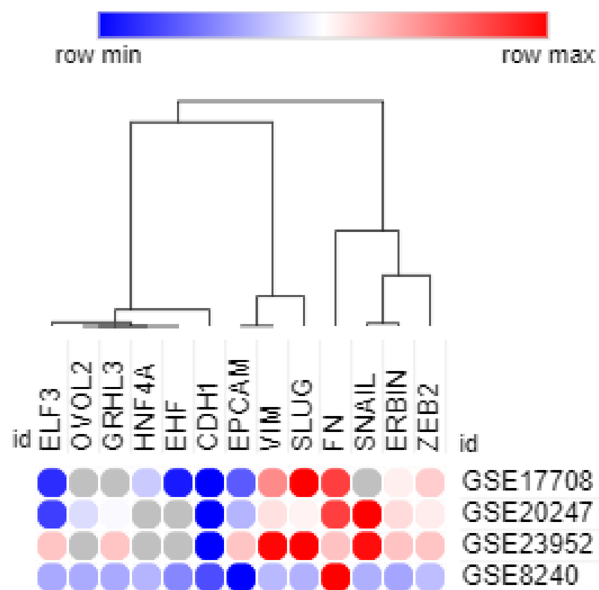


Figure 3.15 Heatmap consisting of the chosen EMT markers and Erbin

The RNA-Seq datasets GSE17708, GSE20247, GSE23952, GSE8240 were obtained from GEO (NIH) and the logFC was calculated from untreated and TGFβ treated groups. Heatmap was created using Morpheus (Broad Institute)

4. DISCUSSION

Erbin, a LAP family protein, has been a target of various research concerning various subjects and tissues. These studies paint a model where Erbin is a crucial aspect in critical cellular functions and mechanisms can lead to diseases. First function of Erbin has been described as association with ErbB2. [16] ErbB2/Her2 is a member of epidermal growth factor family and a component of cell proliferation, differentiation, migration, apoptosis as this family is essential for the growth of the multicellular organisms. [47] The relation between Erbin and ErbB2 that has critical functions in cells, can be the key of other interactions of Erbin.

Other pivotal function of Erbin is regulating many signaling pathways which ties every function of Erbin in different tissues together. It has been seen that Erbin regulates Ras-Raf-Erk signaling pathway, NF- κB signaling pathway and TGF-β signaling pathway. Regulatory property of Erbin in signaling might be the reason why Erbin is essential in multiple biological processes. In addition, the association between Erbin and mentioned signaling pathways partakes in the various disease development and mechanisms such as multiple cancer types. Erbin might be one of the of the essence components in the complexity of cancer mechanisms, since it regulates several signaling pathways and might be an element that makes communication between signaling pathways possible.

Breast cancer is a cancer with many subtypes and affected by many regulatory pathways. The diversity between breast cancer cases creates a need for specific treatments. This need has been tried to be met with various chemotherapeutic agents, surgical procedures, adjuvants therapy and monoclonal antibodies. [6] Even so, there is still a call for improvement for therapies and new approaches for the subtypes that cannot be treated properly. Breast cancer is closely related to ErbB2 regulation, in which one of the main regulators is Erbin. In addition, Erbin found to be an important regulator for many signaling pathways and cellular

processes that shown to be involved in breast cancer by numerous studies. On the other hand, Erbin's involvement in multiple crucial pathways can be a hindrance for finding specific targeting therapies as there might be unwanted outcomes.

With the controversy around the role of Erbin in breast cancer and the findings that indicate Erbin has a role in EMT, we have decided to investigate this subject further. In TGF-β pathway, TGF-β first binds to transmembrane receptors, then these receptors activate Smad proteins by phosphorylation, Smad proteins transmit signals from cytoplasm to the nucleus where Smads can regulate the transcription of target genes, in summary. This signaling cascade works in multiple cellular processes such as differentiation, motility, EMT, apoptosis and has been associated with multiple diseases including cancer. [48, 49] It has been shown that Erbin interacts with Smad proteins, specifically Smad3 and Smad2 via a domain (SID) upstream of its PDZ domain. [49,50] Erbin inhibits TGF-β signaling through disturbing Smad2/Smad3 association with Smad4. This inhibition results in decreased cell growth and TGF-β dependent transcriptional activation. [49] Another important factor of TGF-β is SARA, which is an endosomal protein that has the critical role of presenting Smad2/Smad3 for phosphorylation and mediates TGF-β receptor dephosphorylation. It has been shown that Erbin binds to SARA via the same domain it binds to Smad proteins. This indicated that Sara, Smad2 and Smad3 competes for binding, thus bringing the question that if Erbin regulates TGF-β more than one way. Indeed, overexpression of SARA diminished the Erbin dependent inhibition of TGF-β. [51] This suggest that Erbin regulates the TGF-β pathway depending on the concentration and affinities of its binding partners.

Evidence shows that a positive regulator of TGF-β pathway, Sema4C, interacts with Erbin. Knockdown of Erbin increased Sema4c induced EMT, whereas overexpression of Erbin diminished this effect. This is in line with Erbin being a negative regulator of TGF-β signaling, as TGF-β is a key inducer of EMT. TGF-β stimulation upregulated both Erbin and

Sema4C protein levels. These results can be interpreted as when TGF-β signaling promotes Sema4C induced EMT, Erbin level is increased as a response and Erbin inhibits EMT by hindering Sema4c function in HK2 cells. [52]

The results listed above indicates that Erbin has a regulatory role in EMT, but theres is no research on the relationship between Erbin, breast cancer and EMT even though Erbin is a very important protein in breast cancer. This led us to initiate this project.

We have started with determining the Erbin level in different breast cancer cells with various characteristics. When we group these cells as epithelial and mesenchymal, the Erbin levels are not in favor of either of the group. However, it might not be enough to look at this data with just EMT perspective, since these cells are different types of breast cancer. There is evidence that Erbin negatively regulates ERα [53], there might be a similar situation in breast cancer where ERα and Erbin is antagonistic since ER- cell lines such as MDA-MB-231, MDA-MB-157, and CAL-51 have higher Erbin levels. Additionally, when MDA-MB-231 and MDA-66 are compared, MDA-MB-231 has a higher Erbin expression and the only difference between these cells is that MDA-66 cell line is ER+. (Figure 3.1)

To induce EMT we have treated epithelial MCF7 and MCF10A with TGF-β, and validated EMT by E-Cadherin, and checked the change in Erbin levels. Our results say that upon EMT, Erbin expression decreases in breast cancer and normal breast cell lines. (Figure 3.3) This outcome is contradictory with the literature that suggests that Erbin increases in TGF-β induced EMT in normal kidney cells HK-2. [52] However, the in-silico analysis we have done also indicates that in MCF7 cells, Erbin expression is decreased when there is a TGF-β treatment. Yet, these decreases are not enough to claim Erbin is a differentially expressed gene in TGF-β induced EMT. (Figure 3.14, Figure 3.15)

When we checked EMT associated genes upon Erbin overexpression in MCF7, which is a Luminal A type breast cancer [54], we saw that the general trend in epithelial markers is

downregulation. E-Cadherin is an important indicator in EMT/MET, its loss led to EMT and metastasis in cancer. [55] In Erbin overexpressed MCF7 cells, E-Cadherin mRNA expression (Figure 3.5) and protein level (Figure 3.10) was decreased, showing that there is a change from epithelial type to mesenchymal. Elf3 is another epithelial marker which is necessary of MET progress [56] and negative regulator of EMT [57]. Grhl3 is a known regulator of Cdh1, but there is contradicting results in different cell types and cancers as in some of them Grhl3 negatively regulates Cdh1 and induces EMT [58, 59] whereas in some cases it can re-establish Cdh1 expression [60]. Here in our results, Grhl3 decreases along with other epithelial markers. Ovol2 also suppresses EMT [61] and promotes MET [62]. There is evidence that shows C/EBPα maintains epithelial state and decreases significantly when there is TGF-β treatment in breast cancer. [63] HNF4α is a major effector for MET [64] and negatively regulates EMT by a double-negative loop with Wnt/β-catenin signaling. [65] EHF is a transcriptional factor that can inhibit ZEBs which are EMT promoters, thus inhibits EMT. [66, 67] Also, it regulates Her2/Erbb2 positively in thyroid cancer [68], this could be linked to Erbin as Erbin positively regulates Erbb2. [18] But there is no evidence on if the regulation between Ehf and Erbb2 goes both ways, and our result does not indicate such an outcome since Erbin and Ehf seems to be negatively correlated. EpCAM expression is lost when the breast cancer cells go through EMT [69] and regulates EMT process via a double negative feedback loop with ERK. [70] These characteristically epithelial components were all downregulated in Erbin overexpressed MCF7 cells, which is normally an epithelial breast cancer cell line. Even so, except FN which is a EMT inducer [71], our mesenchymal markers were also downregulated. (Figure 3.5) This consequence was also seen in the in-silico analysis of a dataset from TGF-β treated MCF7 RNA-seq result, in line with our wet lab results. (Figure 3.15) This can be interpreted as abundancy of Erbin in MCF7 is parallel to TGF-β treatment, resulting in a shift from epithelial to mesenchymal features.

Triple negative breast cancer cell line MDA-MB-231 was transfected with siErbin and Erbin was efficiently downregulated. The qRT-PCR data did not show a clear MET shift in this mesenchymal-like cell line. Only Elf3 and Ovol2 was upregulated between epithelial markers, whereas the others were downregulated. (Figure 3.7) During EMT, the levels of cadherins switch from E-Cadherin to N-Cadherin, thus N-Cadherin is accepted as a mesenchymal marker. [55] Snail is a key transcriptional factor that represses E-cadherin [72] and works together with Slug [73]. Zeb2 is also another transcription factor and is critical for EMT initiation. [74] Our final choice of mesenchymal marker, Vimentin, is a widely known master regulator of EMT. [75] Between the markers above, N-Cadherin and Fibronectin was downregulated; Slug, Zeb2 and Vimentin were upregulated. (Figure 3.7) When we checked if to see if there is any transition to epithelial type by IF staining of E-cadherin, there was no signal just as the control group. (Figure 3.11) We cannot claim there is a certain shift in EMT/MET in Erbin silenced MDA-MB-231 cell line, but the loss of Erbin seems to affect the components of this transition.

Finally, MDA-66 cell line was used to knockdown Erbin. This cell line is an ER+, PR-, HER2- breast cancer cell line. After Erbin was silenced, from the epithelial markers, Grhl3, C/EBPα, and EpCam was downregulated; Ovol2 was upregulated. Among mesenchymal markers, Fibronectin, Slug and Vimentin were downregulated and Snail was upregulated. (Figure 3.9) Co-immunofluorescence imaging did not show any difference in E-cadherin. (Figure 3.12) When MDA-MB-231 and MDA-66 results are compared, the differences in the changes of EMT markers could be attributed to ERα. In breast cancer, ERα suppresses EMT and metastasis. [76, 77, 78] As informed before, Erbin initiates degradation of ERα in HCC. It can be discussed if this occurs in breast cancer as well. If it indeed does, then Erbin knockdown should result in elevated ERα levels and hence, an increase in epithelial phenotype by suppressed EMT. Compared to MDA-MB-231, in MDA-66 more of the

mesenchymal markers were downregulated. Nevertheless, to come up with this conclusion these results we present are not satisfactory and more research on this would be needed.

During the EMT process many pathways such as TGF-β pathway, MAPK pathway, PI3K/Akt pathway, Wnt pathway, and Notch pathway is in concert with each other and various transcription factors. [79] Furthermore, it has been shown that Erbin is associated with the apoptotic pathways and drug resistance. In tamoxifen resistant MCF7 and doxorubicin resistant MDA-MB-231 cell lines, Erbin expression was lower compared to the wild type cells. When Erbin was silenced in MDA-MB-231 cells, the expression of the apoptosis markers was decreased whereas anti apoptotic markers were increased. Cell survival promoter and proliferation proteins were increased in siErbin transfected MCF7 and MDA-MB-231 cells. [80] Therefore, the relationship between EMT and apoptotis is an aspect that can be further investigated through Erbin.

Erbin has roles in multiple pathways by regulating them and help the cross-talk between them. Erbin is also of importance in breast cancer because it has direct interactions with receptors that decide on the fate of the disease. This intricate relation makes it challenging to come up with a streamlined explanation about effects of the Erbin in any role, but an important puzzle to solve. It has been shown that Erbin has a role in TGF-β pathway and EMT in other cancer types by directly interacting with transcriptional proteins. Our results point out that also in breast cancer, Erbin has a role in EMT, but it might not be as simple as suppressing or promoting it. More research on different pathways related to EMT and Erbin is needed.

5. FUTURE PERSPECTIVES

For enlightening of Erbin's role in EMT in breast cancer, apart from the transcriptional regulation of the markers upon the changes made in Erbin expression more experiments are needed. More Erbin knockdown and overexpression could be performed in both the cells we used, along with other types of breast cancer cell lines to see the difference between them. The experiments we conducted can be repeated as we did not have the chance to do so. Co-immunofluorescence staining could be improved with more markers to see the changes in protein level. Also stable transfection of the vector and siRNA could be valuable to do a time course experiment.

In order to check how Erbin reacts, different EMT inducers can be utilized such as silencing E-cad, inducing with ZEB1, Snail, Twist, Slug and so on. After inducing EMT, the pathways which is shared by Erbin and EMT progress should be investigated to see what roles Erbin have in these pathways related to EMT. As examples, Raf/MEK/ERK pathway and PI3-K pathway can be given along with TGF-β pathway. In this investigation, addition to checking mRNA and protein expression changes, it would be valuable to see protein-protein interactions with experiments such as pull-down assays since it is known that in other cell types of Erbin binds to EMT-related factors and regulates them.

The possible relation between ERα/Erbin/EMT should be investigated as the state of ERα can change the trajectory of the prognosis. Erbin can be a target for therapeutic approaches.

Further in-silico analysis, especially with RNA-Seq data of these experiments should be done as the confirmation of our results.

Migration assays can be performed after silencing or overexpressing Erbin in epithelial cancer cells to check if there is any effect in the metastasis.

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