FUNCTIONAL ANALYSIS OF ERBIN GENE IN BREAST CANCER DRUG RESISTANCE

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By

Gizem Sunar

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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

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M.S. in Molecular Biology and Genetics

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Erbin is an ERBB2 interacting protein with roles in many signaling pathways. Breast cancer is one of the types of cancer that is affected by Erbin regulation. However, it is unclear how Erbin regulates the biological behavior and drug resistance of breast cancer cells. Some studies have claimed that Erbin promotes tumorigenesis and demonstrates oncogenic features in breast cancer, whereas others have indicated that it inhibits breast cancer development. The main aim of this study was to explore the role of the Erbin gene in breast cancer drug resistance. Bioinformatic analyses of breast cancer patient datasets have shown that a high level of Erbin expression predicts better survival in breast cancer patients treated with chemotherapy or targeted therapies while the Erbin level does not change the survival rates of untreated breast cancer patients. These analyses lead us to hypothesize that the Erbin expression level could alter the effect of the drug treatment and a reduced level of Erbin expression could promote resistance against doxorubicin and tamoxifen. *In vitro* studies have demonstrated that the protein expressions were apparently lower in MDA-MB-231 doxorubicin resistant (DoxR) and MCF-7 tamoxifen resistant (TamR) cells compared to non-resistant cell line counterparts. When the expression level of Erbin was downregulated by si-RNA transfection, it was observed that the protein level of the anti-apoptotic markers increased whereas apoptotic markers decreased in MDA-MB-231 cells. Proteins that promote cell survival and proliferation increased in Erbin downregulated MDA-MB-231 and MCF-7 cells. Besides, when Erbin was

reduced, the viability of the MDA-MB-231 cells against doxorubicin increased but there was no significant change for tamoxifen in MCF-7 cells. Lastly, breast cancer patients with high Erbin expression that were treated with tamoxifen, chemotherapy or trastuzumab have higher levels of DNA damage, apoptosis and cell cycle arrest-related genes. On the contrary, patients with low Erbin expression have higher levels of cyclins, CDKs and anti-apoptotic genes. In conclusion, Erbin could play an important role in the drug resistance of breast cancer cells since the reduction in Erbin expression can promote drug resistance in these cells.

Keywords: ERBIN, breast cancer, drug resistance, tamoxifen, doxorubicin, apoptosis

ÖZET

ERBİN GENİNİN MEME KANSERİ İLAÇ DİRENCİNDE FONKSİYONEL ANALİZİ

Gizem Sunar

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Işık Yuluğ

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Erbin, birçok sinyal yolağında rol alan ERBB2 etkileşimli bir proteindir. Erbin geninin hücre içindeki düzenlemelerinden etkilenen kanser türlerinden biri de meme kanseridir. Ancak, Erbin geninin meme kanseri hücrelerinin biyolojik davranışını ve ilaç direncini nasıl düzenlediği belirsizdir. Bazı araştırmalar, Erbin'in meme kanserinde tümör oluşumunu desteklediğini ve onkojenik özellik gösterdiğini iddia ederken, diğerleri ise meme kanseri gelişimini engellediğini belirtmiştir. Bu çalışmanın temel amacı, Erbin geninin meme kanseri ilaç direncindeki rolünü araştırmaktır. Meme kanseri hasta verilerinin biyoinformatik analizlerinde, yüksek seviyeli Erbin gen ifadesi, kemoterapi veya hormonel terapi ile tedavi edilen meme kanseri hastalarında ölüm oranlarının daha düşük olduğu gösterilmiştir. Bunun yanında eğer hastalar tedavi edilmediyse Erbin seviyesinin meme kanseri hastalarının hayatta kalma oranlarını değiştirmediğini gösterilmiştir. Bu analizler bizi Erbin ifade seviyesinin ilaç tedavisinin etkisini değiştirebileceğini ve Erbin ifade seviyesi azaldığında doksorubisin ve tamoksifene karşı direncin artabileceği varsayımına ulaşılmıştır. İn vitro çalışmalar, Erbin protein ifadesinin MDA-MB-231 doksorubisin dirençli (DoxR) ve MCF-7 tamoksifen dirençli (TamR) hücrelerde, dirençli olmayan hücre hatlarına kıyasla daha düşük olduğunu göstermiştir. Erbin seviyesi si-RNA transfeksiyonu ile baskılandığında, MDA-MB-231 hücrelerinde anti-apoptotik belirteçlerin protein seviyesinin arttığı, apoptotik belirteçlerin ise azaldığı görülmüştür. Erbin seviyesi düşürülmüş MDA-MB-231 ve MCF-7 hücrelerinde hücre canlılığını ve çoğalmasını destekleyen proteinler artmıştır. Ayrıca, Erbin seviyesi azaldığında, MDA-MB-231 hücrelerinin doksorubisine karşı ölüm oranları azalmış, ancak MCF-7

hücrelerinde tamoksifen tedavisi ile önemli bir değişiklik olmamıştır. Son olarak, yüksek Erbin ifadesine sahip ve tamoksifen, kemoterapi veya trastuzumab ile tedavi edilen meme kanseri hastaları, daha yüksek seviyelerde DNA hasarı ve apoptoz ile ilgili genlere sahiptir. Öte yandan, düşük Erbin ifadesi olan hastalar daha yüksek seviyelerde siklin CDK'larına ve anti-apoptotik genlere sahiptir. Sonuç olarak Erbin, meme kanseri hücrelerinin ilaç direncinde önemli bir rol oynayabilir ve Erbin seviyesinin azalması meme kanseri hücrelerinde ilaç direncini artırabilir.

Anahtar kelimeler: ERBIN, meme kanseri, ilaç direnci, tamoksifen, doksorubisin, apoptoz

To my beloved family,

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ABBREVIATIONS

AKT	V-akt murine thymoma viral oncogene homolog
APS	Ammonium peroxodisulfate
BAX	BCL2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2 protein
Bcl-xL	B-cell lymphoma-extra large
BSA	Bovine Serum Albumin
CASP	Caspase
CDK	Cyclin-dependent kinases
ddH2O	Double distilled H2O
DMEM	Dulbecco's Modified Eagle Medium
DoxR	Doxorubicin resistant
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor 1
ER	Estrogen receptor
ERBB2	Proto-oncogene erb-b2 receptor tyrosine kinase 2
ERK	Extracellular signal-regulated kinase
EMT	Epithelial-mesenchymal-transition
FBS	Fetal bovine serum
GEO	Gene Expression Omnibus
HER2	Human epidermal growth factor receptor 2
kDA	Kilo Dalton
MAPK	Mitogen-activated protein kinase

p21	Cyclin-dependent kinase inhibitor 1
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene difluoride
RB	Retinoblastoma protein
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
siRNA	Small interfering RNA
TamR	Tamoxifen resistant
TBS-T	Tris buffer saline Tween20
TGF-β	Transforming growth factor beta
Thr	Threonine
TNBC	Triple negative breast cancer

CHAPTER 1. INTRODUCTION

1.1. CANCER

Cancer is a disease that begins with aberrant proliferation of the cells and it can occur in any place in the body. There are numerous kinds of cancer types. Although these cancer types have different features and treatments [1], they have some common abilities in their development. These capabilities comprise resistance to cell death, sustainment of proliferative signaling, avoidance of growth suppressors, activating invasion and metastasis, enabled replicative immortality and inducement of angiogenesis [2,3].

Cancer causes millions of deaths every year. According to the World Health Organization, an estimated 19.3 million new cancer cases and 10 million deaths were reported worldwide in 2018. Breast, lung, colorectum and prostate cancer are the most common types of cancer in the world [4].

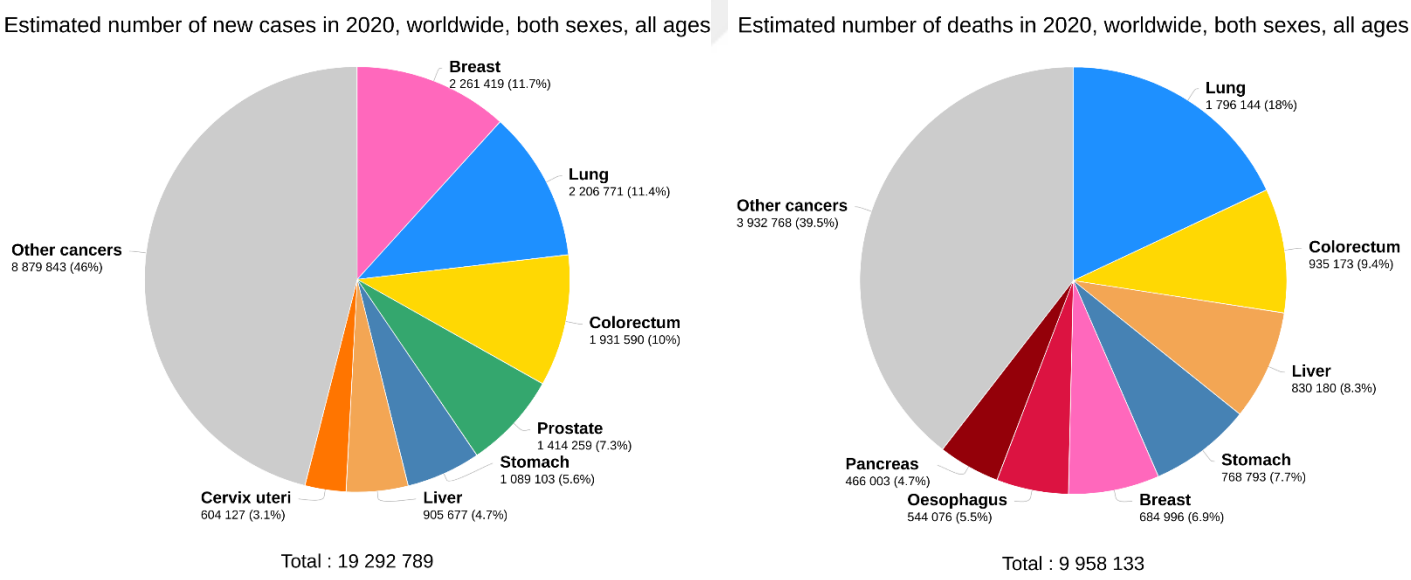


Figure 1.1. Estimated Number of New Cancer Cases and Deaths Worldwide in 2020 (Taken from *Ferlay et al., 2020* [5])

1.2. BREAST CANCER

Breast cancer is the most prevalent and mortal cancer observed among females. In 2020, 2.3 million women were diagnosed with breast cancer and 0.7 million people died due to breast cancer [3,4].

Breast cancer is initiated from mammary gland lobules. It is a heterogeneous disease [6]. For this reason, it needs to be classified efficiently for accurate diagnosis of subtypes and determination of therapeutic treatments [7]. There are three types of receptors that take a part in the development of breast cancer tumor cells. Estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) mainly have a role in the proliferation and maintenance of breast tumors [8].

1.2.1. Molecular Classification of Breast Cancer

Breast cancer is classified into at least five groups according to its receptor status. These subtypes are luminal A, luminal B, HER2, basal and normal. These subtypes are divided mainly based on the presence of the estrogen receptor. Luminal A and luminal B are ER-positive, whereas HER2, basal and normal-like breast cancers are ER-negative [8, 9]. Each class has a different diagnosis, and characteristics, and histological features controlled by distinct biological processes and responses to the therapy [10, 11].

Luminal A and luminal B-like tumors are ER-positive but they are different from each other at the level of gene expression of HER2. Luminal A subtypes do not express HER2, whereas luminal B subtypes are HER2 positive tumors. In addition, luminal A cancers have a higher level of ER-activated genes and a lower proliferation rate than luminal B cancer. Accordingly, the luminal A type has a better prognosis [12-14].

HER2 breast cancers have overexpressed HER2 and do not express ER and PR. They grow faster. HER2 breast cancers have a worse prognosis and are more aggressive than luminal type [15, 16].

Basal-like type contains triple negative breast cancers (TNBC) that lack any of the indicated three receptors. They are mostly affected by mutations of tumor suppressor genes such as p53

and BRCA1. TNBCs are larger in size, more aggressive and have higher histological grade compared to other subtypes of breast cancer, which indicates proliferative properties with high metastatic ability. Patients having TNBC have the poorest prognosis and the shortest survival times [17-19].

1.2.2. Treatment of Breast Cancer

Treatment of the different types of breast cancers varies depending on their molecular subgroups. Luminal and HER2 breast cancers enable targeted hormone therapy. Because luminal breast cancers are ER-positive, targeting ER and utilizing estrogen antagonists such as tamoxifen results in promising treatment. Similarly, HER2 breast cancers are treated with trastuzumab that is a monoclonal antibody inhibiting HER2 activity. Trastuzumab is mostly combined with chemotherapeutic drugs [12-16].

The main treatment for TNBC is chemotherapy because women having TNBC cannot take endocrine therapy or trastuzumab due to the lack of receptors. There are still no targeted therapies for TNBC. Although researchers have developed some therapeutic approaches for TNBC such as platinum salts, PARP inhibitors, anti-VEGF-A and anti-EGFR therapies, specific therapies are not available for medical treatments. Clinical trials do not show such significant results for these different approaches [17-20].

Targeted hormone therapy and chemotherapy are important for the treatment of breast cancer. Patients can develop resistance to the treatment. The understanding of the resistance mechanism is required for the benefit of the patients.

1.3. DRUG RESISTANCE IN CANCER

Cancers have been able to develop resistance to drugs and this is commonly observed against both chemotherapy and targeted therapies. Drug resistance can be *de novo* or acquired. Intrinsic or *de novo* resistance stems from some genetic mutations in the genome before any treatment, whereas acquired resistance is gained after treatment [21, 22].

Anticancer drug resistance occurs by several molecular mechanisms. Drug efflux or influx is one of the reasons that result in drug resistance. It hampers the transportation of the drug into

targeted cancer cells. Drug target alteration or inactivation also decreases the effectiveness of the drug and leads to resistance. There can be some mutations that change apoptosis and DNA damage repair pathways in cancer cells. Cancer cells can increase their ability to repair DNA damage and their evasion of the apoptosis mechanism, and inhibit cell death due to these mutations, despite anticancer drugs. [21-24]. Besides, epithelial-mesenchymal-transition (EMT) and some epigenetic alterations in cancer cells are mechanisms that cause drug resistance [22].

1.3.1. Chemotherapeutic Drugs: Doxorubicin

Triple negative breast cancer is an infrequent (15%) breast cancer type but it has a very poor prognosis due to its lack of specific targets, aggressiveness and metastatic nature. Conventional chemotherapy is the major treatment for TNBC patients. Anthracyclines that are generally combinations of doxorubicin and taxanes are the most commonly utilized drugs for chemotherapy [25].

Doxorubicin that is isolated from *Streptomyces peucetius* species is an anthracycline drug. It is used for the treatment of many cancer such as leukemia, breast, lung, soft tissue sarcoma and Hodgkin's lymphoma [26]. Doxorubicin inhibits the proliferation of cancer cells by three distinct mechanisms. First, it binds to and intercalates DNA, and it then stops DNA synthesis by inhibiting DNA polymerase. Also, doxorubicin inhibits RNA synthesis and transcription. Second, doxorubicin prevents topoisomerase II that is an enzyme relaxing the supercoils in DNA and enabling DNA replication. Both mechanisms of doxorubicin result in double breaks in DNA and trigger apoptosis. Another mechanism of doxorubicin is the production of reactive free radicals. It leads to oxidative damage and cleavage of DNA [27, 28].

Doxorubicin enables cell death by inducing apoptosis, autophagy, necrosis and early senescence [30]. Doxorubicin casuses apoptosis by initiating AMP-activated kinase (AMPK) that activates p53. The p53 protein downregulates the anti-apoptotic protein Bcl-2 and upregulates the pro-apoptotic protein Bax. This regulation initiates apoptosis in cancer cells. Upregulation of p53 also triggers cell arrest and the senescence mechanisms. Doxorubicin can also cause autophagy by oxidative stress that results in the production of ROS in the mitochondria [27].

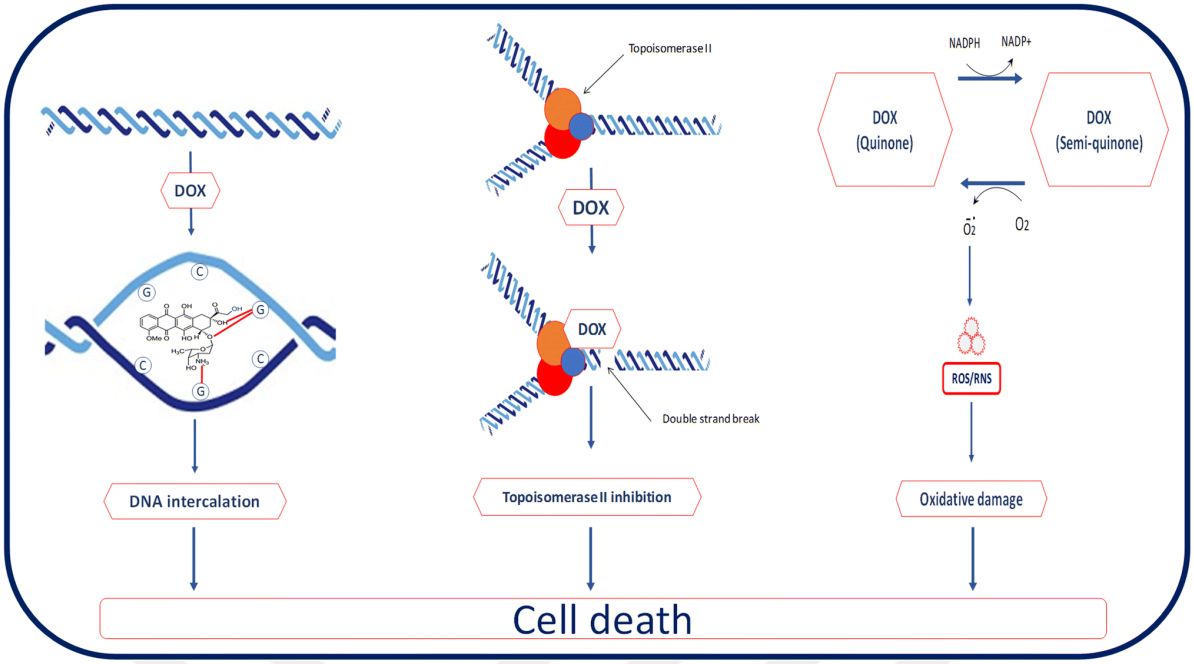


Figure 1.2. Scheme of Doxorubicin Mechanism of Action. Doxorubicin causes cell death in three ways; (I) intercalation of DNA, (II) Topoisomerase II inhibition, (III) free radical related damage. (Taken from *El-Agamy et al., 2019* [29])

Similar to other drugs, doxorubicin resistance is commonly seen in TNBC patients. As explained above; drug efflux, and mutations in apoptotic and DNA damage repair pathways resulting in inhibition of cell death can promote resistance [31]. When the level of topoisomerase II changes, it can also affect doxorubicin resistance [22]. The exact reason for the chemoresistance to doxorubicin is unknown. Further studies are required to explain the mechanism causing the development of doxorubicin resistance in cancer cells [33].

1.3.2. Endocrine Therapy: Tamoxifen

Most of the breast cancer patients are ER-positive. They are treated with endocrine therapy to inhibit ER signaling [34]. ER signaling is activated by its ligand estrogen or in an estrogen-independent manner via other transcription factors. In the classical pathway, estrogen enters the cell and binds to ER in the nucleus. After binding of the estrogen, the ER undergoes conformational alteration. It is dimerized and becomes phosphorylated. Then, the ER binds to the estrogen response element (ERE) that is upstream of estrogen dependent genes [35]. Alternatively, activation of the ER can occur outside of the nucleus. Growth factor signaling pathways can also activate the ER. The TORC1 effector p70S6K, ERK and effector AKT can

enable phosphorylation of the ER and estrogen dependent or independent ER transcriptional activity [36].

The activation of the ER pathway is blocked by multiple anti-estrogen mechanisms for the treatment of ER-positive breast cancer patients. Selective ER modulators (SERMs) prevent the activity of ER. Tamoxifen is a SERM that is commonly used in endocrine therapy. Another mechanism is that selective ER down-regulators (SERDs) such as fulvestrant which destabilize and degrade ER. Lastly, aromatase inhibitors (AIs) provide the decrease of the production of estrogen by blockage of the enzyme aromatase [37].

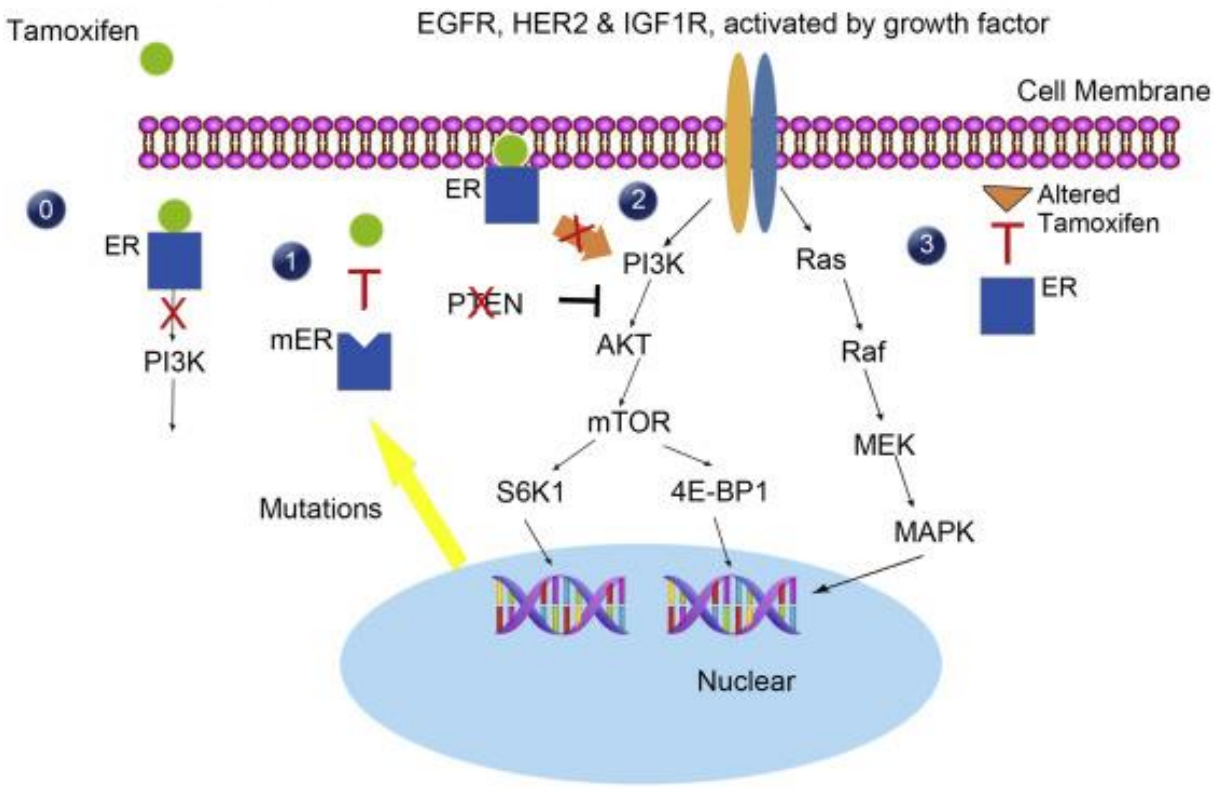


Figure 1.3. Mechanism of Tamoxifen and Tamoxifen Resistance. (0) Tamoxifen blocks ER signaling by binding to ER. (1) Mutations in the ESR1 gene, (2) bypassing inhibition of ER signaling by using alternative pathways or blockage of tumor suppressor PTEN and (3) reducing active drug concentration by altering tamoxifen can cause resistance (Taken from *Yuan et al., 2016* [38])

Although tamoxifen is the most prevalent treatment for ER-positive patients, tamoxifen resistance is developed in many breast cancers. The status of ER and its crosstalk with other growth factor pathways can promote the resistance of tamoxifen. First, mutations in the ER gene can occur and it alters phenotype the ER-positive phenotype to negative without loss of ER. Lack of ER expression promotes tamoxifen resistance [35, 38]. Second, cancer cells activate ER pathway by downregulation of tumor suppressor genes such as PTEN or upregulation of drug resistance drivers such as AKT even if there is estrogen blockage by tamoxifen [18]. Oncogenic signaling pathways including growth factor receptor tyrosine kinases (HER2 and EGFR), PI3K/AKT/mTOR pathway and Rb phosphorylation are associated with tamoxifen resistance [36, 39]. Third, tamoxifen resistance stems from the decrease of the concentration of active tamoxifen. Tamoxifen metabolism can change and it affects the efficiency of the drug [38].

Because ER signal transduction is very complex, resistance against tamoxifen is caused by many molecular mechanisms. Reasons for the resistance should be understood very well and alternative strategies should be found.

1.4. APOPTOSIS AND CANCER

Apoptosis is a mechanism that results in programmed cell death [40]. When this programmed cell death is inactivated, it can cause developmental abnormalities, tumorigenesis and other serious health problems [41]. Escape from apoptosis or resistance to cell death is one of the hallmarks of cancer. The growth control systems that manage tissue homeostasis and cell proliferation are associated with apoptosis. For this reason, the resistance of cancer cells to apoptosis is an important feature of cancer development [42].

There are two different apoptosis pathways, intrinsic and extrinsic pathways. The intrinsic or mitochondrial pathway is initiated by intracellular stress, whereas the extrinsic or death receptor pathway is activated by extracellular ligands. Though these two apoptotic pathways result in the activation of common caspases, their mechanisms differ from each other [41-44].

1.4.1. Intrinsic (Mitochondrial) Pathway

Commonly used anticancer therapies kill the cancer cells by the intrinsic apoptosis pathway [24]. The mitochondrial pathway is initiated by Bax or Bak entry into the mitochondrial membrane. It leads to the release of cytochrome c molecules from mitochondria to the cytosol. BH3-only proteins Bid and Bim help the pro-apoptotic action of Bax and Bak. Released cytochrome c binds to Apaf-1. They form a complex that is named "apoptosome" in the presence of ATP. Then, pro-caspase-9 binds to the apoptosome complex and gets activated. Activated initiator caspase which is caspase-9 cleaves executioner caspases such as caspase-3 and caspase 7. Cleavage of caspase-3 and caspase-7 provides their activation. Eventually, active executioner caspases cleave cellular substrates and it starts apoptosis [41, 45].

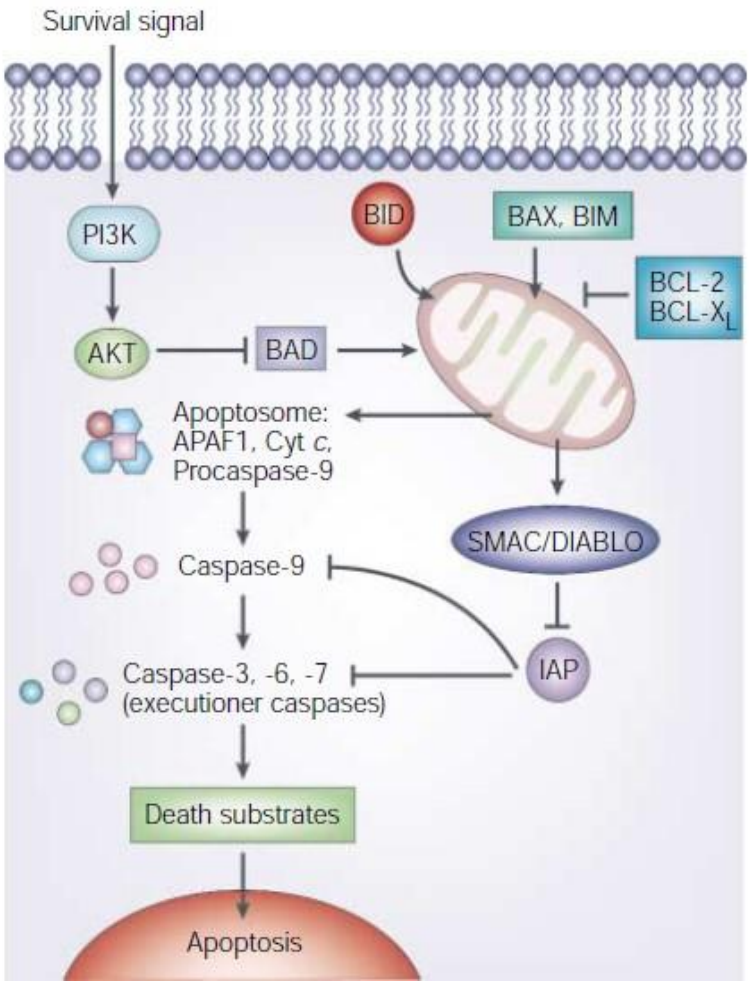


Figure 1.4. Intrinsic Apoptosis Pathway. Apoptosis through mitochondria is initiated by pro-apoptotic proteins that promote the release of cytochrome c. Activation of initiator and executioner caspases provide apoptosis. It can be prevented by anti-apoptotic proteins. (Taken from *Igney et al., 2002* [42])

Release of cytochrome c is prevented by anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL. Bcl-2 and Bcl-XL bind to Bax and Bak proteins and hinder their action [42]. Apoptosis can be blocked by inhibitors of apoptosis proteins (IAPs) that inactivate caspases.

IAPs are regulated by the Smac/DIABLO protein [46]. Another way of avoidance from apoptosis is activation of the PI3K pathway by growth factors and cytokines. The AKT protein that is activated by PI3K phosphorylates the pro-apoptotic Bad protein and inactivates it [47, 48].

Cancer treatment by chemotherapy induces apoptosis in cancer cells. Defects or mutations in the apoptotic pathway directly affect the therapy and make cancer cells resistant to anti-cancer drugs. Cancer cells can gain resistance to apoptosis and therapeutic drugs by overexpression of anti-apoptotic proteins or downregulation of pro-apoptotic proteins [48]. Some studies have demonstrated that cancers expressing a high level of the Bcl-2 and Bcl-XL anti-apoptotic proteins have resistance to chemotherapeutic drugs [49-51]. Besides, some signaling pathway kinases such as ERK, mTOR, RAS, RAF and growth factor receptors including EGFR and HER2 are linked to the apoptotic pathways. Tumor cells are dependent on these pathways. Mutations in these pathways can also inhibit apoptosis and causes resistance [48].

1.5. ERBIN

Erbin is a novel protein that is a LAP family member. Erbin has 16 leucine-rich repeats (LRR) and a single PDZ domain in its C terminus [52, 53]. It interacts specifically with ERBB2 (HER2) receptor by its PDZ domain and plays a role in the localization of ERBB2 to the basolateral domain in epithelia [52-54]. Previously, Erbin was known as an ERBB2 interacting protein. Erbin has been extensively studied for its biological function. Recently, new researches indicate its new binding partners and Erbin has become a novel type of adaptor protein. Numerous proteins have been found to be ligands of the Erbin PDZ domain [55]. It contributes to many cell signaling mechanisms such as MAPK, TGF-β and other pathways [56, 57].

1.5.1. Role of ERBIN in Different Signaling Pathways

The MAPK signaling pathway has a crucial role in cell proliferation, differentiation and migration in the cell. This pathway relies on the activation of receptor tyrosine kinase (RTK) by phosphorylation and the pathway continues by initiating a protein kinase cascade [58]. Erbin is a negative regulator of the MAPK signaling pathway. It inhibits MAPK activation by disturbing the interaction between Ras and Raf kinases. Erbin interacts with the active Ras

protein and they form a complex. As a result, the formation of this complex prevents the interaction of Raf with Ras and it becomes unable to activate Raf [59, 60].

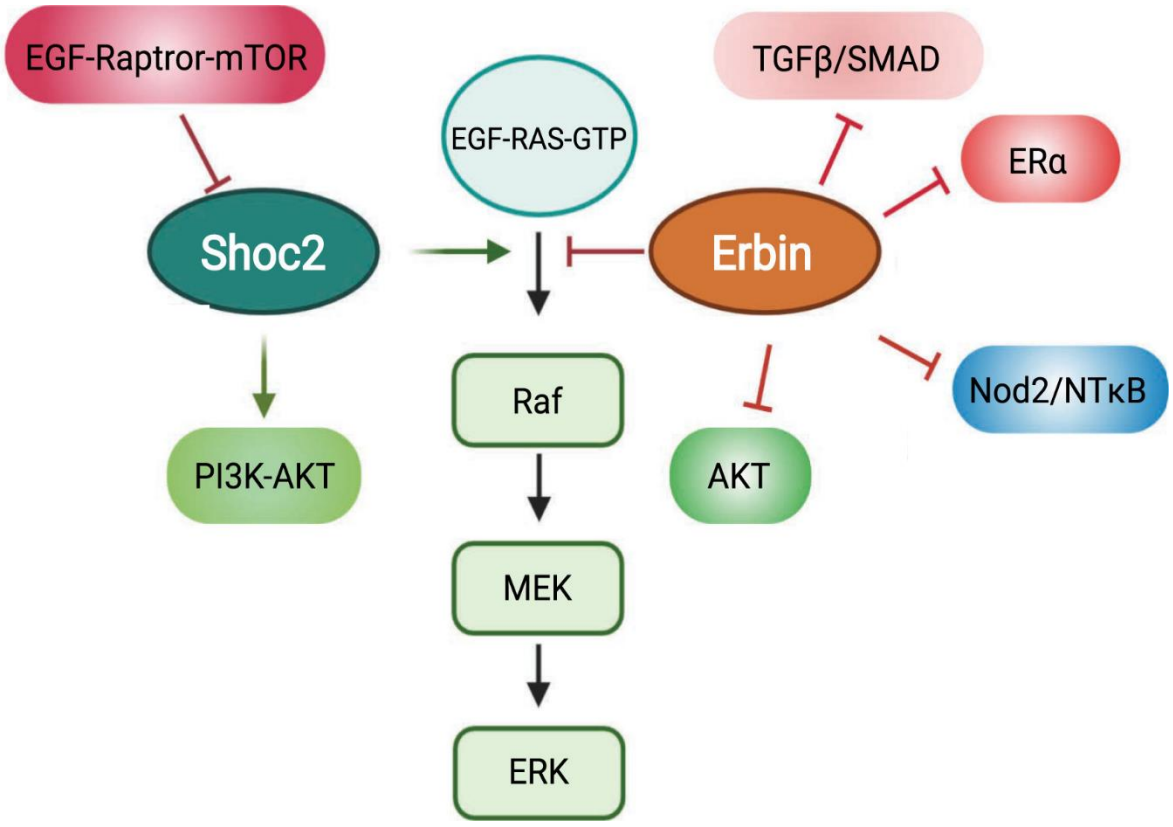


Figure 1.5. The Role of Erbin in Different Signaling Pathways (Taken from *Jang et al., 2020* [57])

TGF- β signaling has a part in cell growth, development, cell differentiation and apoptosis. Smad2 and Smad3 proteins that are intracellular mediators for TGF- β signaling are phosphorylated and oligomerize with Smad-4. This complex regulates the transcription of TGF-β upstream genes [61]. It is concluded that Erbin is a novel negative regulator of TGF- β signaling pathway. It binds to the Smad2/3 complex and inhibits the association of this β complex with Smad4. [62-64]. Therefore, Erbin has tumor suppressor properties in many cancer types by regulating the TGF-β signaling pathway. In addition, it prevents TGF-β-induced EMT by regulating the MAPK signaling pathway. Studies have demonstrated that deficiency of Erbin expression leads to the differentiation of epithelial cells into mesenchymal cells [65, 66].

1.5.2. ERBIN and Cancer

Erbin has different roles in different cancer types and diseases. It takes a part in various pathways in the cell. Studies indicate that Erbin behaves as both an oncogenic and a tumor suppressor protein. Erbin promotes tumorigenesis in hepatocellular carcinoma, gastric cancer, colon cancer and skin cancer [67-71].

In contrast, Erbin inhibits STAT3 activation in cervical cancer. The deficiency of Erbin enhances proliferation and migration of the cells, and leads to resistance of cervical cells to anoikis [72]. Another study has shown that Erbin is a negative regulator of Akt-Skp-p27 pathway. The depletion of Erbin increases S-phase entry by downregulation of p21 and p27 proteins [73]. In colorectal cancer, it also prevents migration and invasion of cancer cells. Knockdown of Erbin promotes EMT [74]. Moreover, overexpression of Erbin decreases cell proliferation and differentiation in AML cells [75].

The Role of Erbin in Breast Cancer

There are different results for the function of Erbin in breast cancer. According to *Liu et al.*, Erbin has tumor suppressor properties in breast cancer. The expression of Erbin is downregulated and protein levels are low in Erbb2 overexpressing breast cancer cells. Loss of Erbin provides the migration in these cells. It is proved that Erbin inhibits heregulin-induced AKT phosphorylation in Erbb2 overexpressing breast cancer cells. Besides, knockdown of Erbin results in trastuzumab resistance in breast cancer cells [76].
On the other hand, some studies have defended that Erbin acts as an oncogene. They show that Erbin increases the Erbb2 dependent cell proliferation and tumorigenesis in breast cancer. Erbin knockdown promotes Erbb2 degradation. It inhibits Erbb2 signaling, and decreases tumor formation. As a result, Erbb2 tumorigenesis is prevented by loss of Erbin *in vivo* in mice. These results indicate that Erbin is a positive regulator of Erbb2 dependent breast tumor formation and development. [77, 78]

The role of Erbin in human breast cancer is controversial. Neither the potential function that Erbin plays role in tumorigenesis nor the regulating mechanism is unclear. Further experiments are needed to understand the complete role of Erbin in breast cancer.

1.6. AIM OF THE STUDY

Erbin is an ERBB2 interacting protein with roles in many signaling pathways. Breast cancer is one of the types of cancer that is affected by Erbin regulation. However, it is unclear how Erbin regulates the biological behavior and drug resistance of breast cancer cells. Some studies have claimed that Erbin promotes tumorigenesis and demonstrates oncogenic features in breast cancer, whereas others have indicated that it inhibits breast cancer development. The main aim of our study was to explore the role of the Erbin gene against doxorubicin and tamoxifen resistance in breast cancer.

Bioinformatics analysis of GEO datasets has shown that a high level of ERBIN expression predicts better survival in breast cancer patients treated with chemotherapy or targeted therapies while the Erbin level does not change the survival rates of untreated breast cancer patients. We hypothesized that the Erbin expression level can alter the effect of the drug treatment and a reduced level of Erbin promotes resistance against doxorubicin and tamoxifen. First, we evaluated whether there was any difference at the protein level of Erbin between the drug resistant and sensitive cell lines. The MDA-MB-231 TNBC cell line was used for doxorubicin resistance and the MCF-7 ER+ luminal cell line was used for tamoxifen resistance. Apoptotic markers were checked in drug resistant and sensitive cells, because drug resistance can be associated with apoptotic pathways. Next, Erbin expression was downregulated by the si-RNA technology in naive MDA-MB-231 and MCF-7 cells. In this way, we evaluated whether a decreasing level of Erbin promotes resistance behavior of drug sensitive cancer cells. The genes affected by the downregulation of Erbin were also evaluated. Thus, we tried to review how Erbin regulates the doxorubicin and tamoxifen resistance.

1.7. EXPERIMENTAL DESIGN

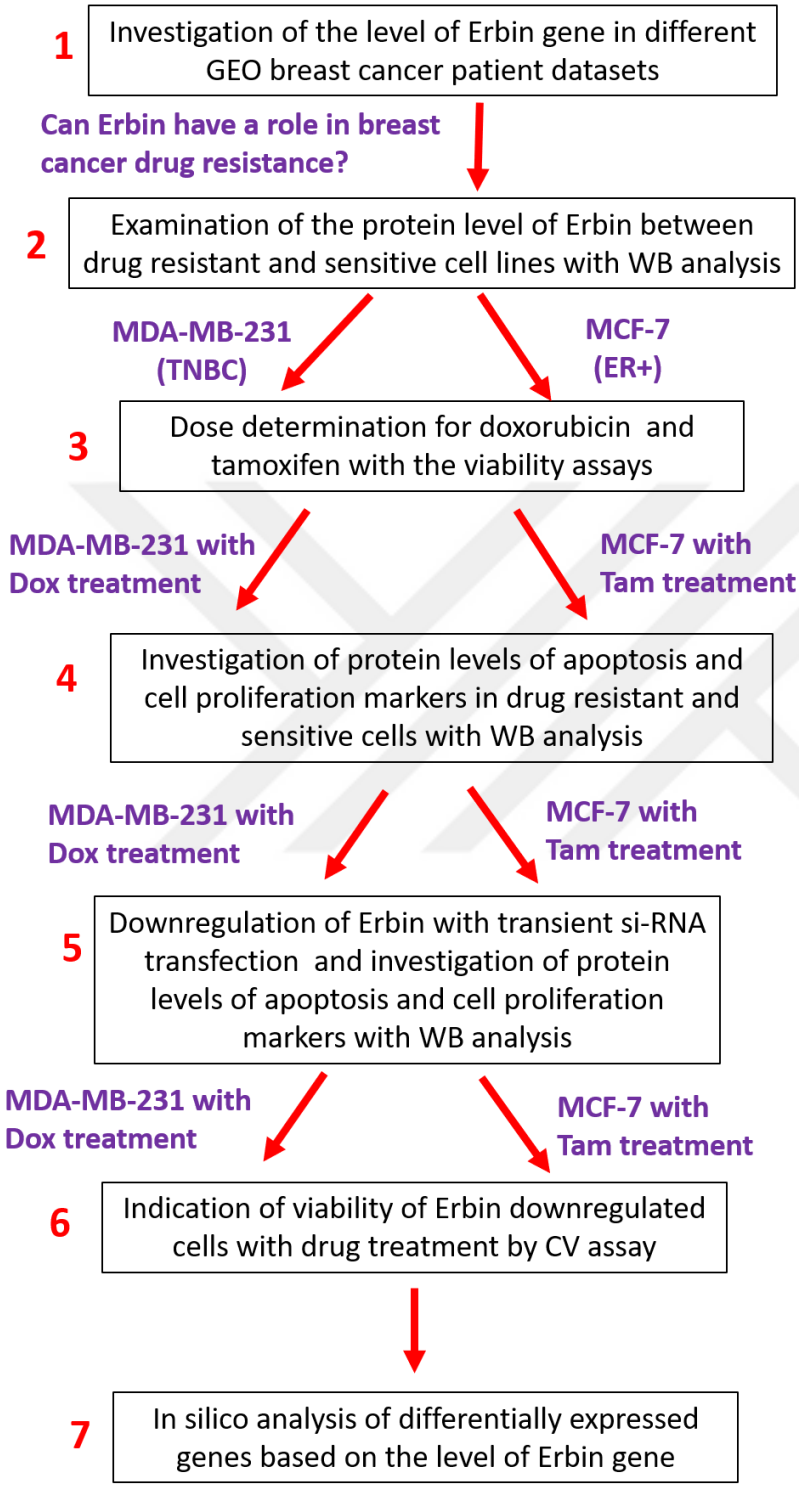


Figure 1.6. Experimental design of study ERBIN gene in breast cancer drug resistance

CHAPTER 2. MATERIAL AND METHODS

2.1. MATERIALS

2.1.1. General Laboratory Materials

General laboratory materials include routinely used substances that are chemicals, solutions, kits, equipment. Table 2.1 lists information including the catalog numbers and the companies of these materials.

2.1.1.1. Chemicals and Reagents

Table. 2.1. The list of chemicals, reagents and enzymes used for general laboratory processes.

Name	Catalog	Company (Country)
2-mercaptoethanol	M3148	Sigma Aldrich (USA)
Agarose	BHE500	Prona (Spain)
Ampicillin	A0839	Applichem (Germany)
Agar	05039	Sigma Aldrich (USA)
Acrylamide	A9099	Sigma Aldrich (USA)
Ammonium persulfate	A3678	Sigma Aldrich (USA)
Bis-acrylamide	M7279	Sigma Aldrich (USA)
Bovine Serum Albumin Fraction V (BSA)	10735078001	Roche (USA)
Bromophenol blue	B5525	Sigma Aldrich (USA)
Crystal Violet	V5265	Sigma Aldrich (USA)
DEPC	A0881	Applichem (Germany)
Ethidium Bromide	17898	Thermo Scientific (USA)
EDTA	A3562	Applichem (Germany)
ECL Prime System	RPN2232	Life Sciences (USA)
Gene Ruler DNA Ladder (1 kb)	SM0311	Thermo Scientific (USA)
Gene Ruler DNA Ladder (50 bp)	SM373	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)
PageRuler Prestained Protein Ladder, (250kDa)	26619	Thermo Scientific (USA)
Rnase A	R6513	Sigma Aldrich (USA)
Roche PVDF Membranes (0.2uM)	3010040001	Roche (USA)
SDS	71725	Sigma Aldrich (USA)
Sulforhodamine B (SRB)	230162	Sigma Aldrich (USA)
Taq DNA Polymerase	EP0402	Sigma Aldrich (USA)
TEMED	1610801	Biorad (USA)
Tris	T1503	Sigma Aldrich (USA)
Tween-20	822184	Merck (Germany)
Trichloroacetic acid (TCA)	33731	Sigma Aldrich (USA)
Yeast extract	1702	Conda (Spain)

2.1.1.2. Solutions

Table. 2.2. The list of routinely used solutions/ buffers.

Solution/Buffer	**Recipe**
RIPA buffer (1 ml)	1 µl 1 M Tris-HCL (pH=8.0); 20 µl EDTA; 300 µl 5M NaCl ; 100 µl NP-40; 40 µl 25X protease inhibitor
4x Protein Loading Buffer (5 ml)	0.02 g Bromophenol blue; 2 ml 20% SDS; 200 mM Tris-HCL (pH=6.8); 30% glycerol. 5% β-mercaptoethanol is added before loading.
5X Running Buffer (1 L)	15 g Trisma Base; 72 g Glycine; 5 g SDS in dH_2O
Wet Transfer Buffer (1 L)	6 g Trisma Base ; 28.8 g Glycine; 20 % methanol in dH_2O
10X TBS (1 L)	12.19 g Tris base; 87.79 g NaCl in dH_2O (pH = 8.0)
TBS-T (0.2%) (500ml)	1ml Tween-20 in 50 ml 10X TBS in ddH2O
Blocking solution (10 ml) (3%)	0.3 milk powder in 10 ml 1X TBS-T
Ponceau S staining solution	1g Ponceau S; 50 ml acetic acid in ddH2O
Mild stripping buffer (10 ml)	1.5 g glycine; 1g SDS; 1 ml Tween-20 in dH2O (pH = 2.2)

10X PBS (1 L)	80.0 g NaCl; 2 g KCl; 14.4 g Na2HPO4.2H2O; 2.4 g KH_2PO_4 in ddH_2O (pH=7.4)
SRB solution (0.4%) (100 ml)	400 mg SRB; 1% acetic acid in dH2O.
Fixation Solution	10% acetic acid; 10% ethanol in dH_2O
Crystal Violet Solution	0.4% crystal violet; 20% ethanol in dH_2O
Destaining Solution	10% acetic acid; 90% dH_2O
LB (100 ml)	1 g Tryptone; 1 g NaCl; 0.5 g yeast extract
50X TAE (100 ml)	24.2 g Tris-base; 5.71 ml glacial acetic acid; 10 mL 0.5M EDTA (pH=8.0)

2.1.1.3. Kits

Table 2. 3. The list of Kits utilized in the experiments

Name of the Kit	Catalog #	Company Name (Country)
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)
PureLink Quick Plasmid Miniprep Kit	K210011	Thermo Scientific (USA)
Qiaquick Gel Extraction Kit	28706	Qiagen (USA)
Pierce™ BCA Protein Assay Kit	23225	Thermo Scientific (USA)

2.1.1.4. Primers

Primers that are utilized for qRT-PCR are listed in Table 2.4. All primers were purchased from and synthesized by Iontek (Istanbul). All primers were solved in nuclease-free water and their final concentration was 100 μM.

Table 2. 4. The list of primers used in the study

Primer	PCR	Primer Sequence (5' to 3')	Tm (°C)	Size (bp)
GAPDH F	RT	GGCTGAGAACGGGAAGCTTGTCAT	60	140
GAPDH R		CAGCCTTCTCCATGGTGGTGAAGA		
ERBIN F	RT	CTAATCAGATTGAAGAGCTTCC	60	122
ERBIN R		AACTCCTGTATTCCATTCTTGC		

2.1.1.5. Antibodies

The listed antibodies were used in western blot experiments. Their working concentrations were optimized under the guidance of their manufacturers.

Table 2. 5. The list of antibodies used in the western blots

Name of the antibody	Catalog#	Company
Rabbit anti-ERBIN Antibody *	NA	NA
Mouse anti-Actin Antibody	A5441	Sigma-Aldrich
Rabbit anti-Calnexin Antibody	C4731	Sigma Aldrich
Mouse anti-Bcl-2 (C-2) Antibody	sc-7382	Santa Cruz Biotechnology
Mouse anti-Bcl-xL Antibody (H-5)	sc-8392	Santa Cruz Biotechnology
Rabbit anti-Caspase-9 Antibody	9502	Cell signaling
Mouse anti- ERα Antibody (F-10)	sc-8002	Santa Cruz Biotechnology
Anti-rabbit IgG-HRP	A6154	Sigma-Aldrich
Anti-mouse IgG-HRP	A0168	Sigma-Aldrich

* ERBIN antibody was a gift from Dr. Jean-Paul Borg (INSERM, Marseille, France). It was produced in his lab.

Table 2. 6. Antibodies kindly provided by Assoc. Prof. Dr. Özlen Konu and Assoc. Prof. Dr. Özgür Şahin

Name of the antibody	Catalog#	Company
Rabbit anti-Bax Antibody	5023P	Cell signaling
Rabbit anti- p-γH2AX Antibody	sc-517348	Santa Cruz Biotechnology
Rabbit anti-Cdk-2 Antibody	C5223	Sigma Aldrich
Rabbit anti-p-RB Antibody	8516	Cell signaling
Mouse anti-p21 Antibody	NA	NA
Rabbit anti-AKT Antibody	9272	Cell signaling
Rabbit anti- p-AKT (Ser-473) Antibody	9275	Cell signaling
Rabbit anti- p-AKT (Thr-308) Antibody	9271	Cell signaling
Anti-rabbit cleaved Caspase-3	9664	Cell signaling
Anti-mouse cleaved Caspase-7	8438	Cell signaling

2.1.1.6. Equipment

Table 2. 7. The list of equipment used in the experiments

Name of the instrument	Company
PCR Thermal cycler	Applied Biosystems (USA)
AutoFlow NU-8500 Water Jacket CO2 Incubator	NuAire (USA)
Centrifuges 5810 and 5810 R	Eppendorf (Germany)
Amersham Imager 600	Dharmacon (USA)
NanoDrop ONE	Thermo Scientific (USA)
BD Accuri™ C6 Cytometer	BD Biosciences (USA)
Cell Culture Hood	NuAire (USA)

2.1.2. Cell Culture Materials

Cell culture reagents, cell lines and their mediums that were used in the cell culture room were provided. Experiments were performed in aseptic conditions under the laminar flow hood.

2.1.2.1. Cell Culture Reagents

Table 2.8. The list of chemicals, reagents and kits used in cell culture experiments

Name	Catalog #	Company (Country)
DMEM Low Glucose w/o L-Glutamine w/ Sodium Pyruvate	L0064-500	Biowest (USA)
DMEM, low glucose, pyruvate, no glutamine, no phenol red	11880-028	Thermo-Fischer Scientific (USA)
Fetal Bovine Serum (FBS)	CH30160	GE Healthcare (UK)
PBS	L0615-500	Biowest (USA)
Trypsin/EDTA (0.25%)	SV30031	GE Healthcare (UK)
Trypsin/EDTA (10X) Solution	X0930-100 T	Biowest (USA)
Non-Essential Amino Acids	11140035	Thermo-Fischer Scientific (USA)
Sodium Pyruvate (100mM)	11360	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 RNAiMAX transfection reagent	13778075	Thermo-Fischer Scientific (USA)
4-Hydroxytamoxifen	T176	Sigma Aldrich (USA)
Doxorubicin (Adriamycin)*	NA	NA

*Doxorubicin was bought by the pharmacy.

2.1.2.2. Cell Lines and Mediums

Table 2. 9. Cell lines and their growth mediums used for our experiment

Cell Line	Medium
MDA-MB-231 WT	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids
MDA-MB-231 DOXR	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids
MCF-7 WT	DMEM w/o phenol red; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids ; 0.1% insulin
MCF-7 TAMR	DMEM w/o phenol red; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids; 0.1% insulin
BT-474 WT	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids; 0.1% insulin
BT-474 TMABR	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids; 0.1% insulin
SKBR-3 WT	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids
SKBR-3 TMABR	DMEM/ Low Glucose; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids

T47D WT	DMEM w/o phenol red; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids; 1% sodium pyruvate
T47D TAMR	DMEM w/o phenol red; 10%FBS; 1% Penicillin/Streptomycin; 1% nonessential amino acids; 1% sodium pyruvate

2.1.2.3. Nucleic Acids

Table 2.10. The list of nucleic acids that were used in transfection

Name	Catalog #	Company	Target sequence
siGENOME Non-Targeting siRNA #2	D-001210-02-20	Thermo Scientific (USA)	UAAGGCUAUGAAGAGAUAC
Hs_ERBB2IP_7 FlexiTube siRNA	SI00122766	Qiagen (USA)	TTGGGCAGCTTACTAACTTAA
Hs_ERBB2IP_12 FlexiTube siRNA	SI03063704	Qiagen (USA)	CAGACTCTATAGGAGGGTTAA

2.2. METHODS

2.2.1. Cell Culture Based Techniques

2.2.1.1. Cell Culture Maintenance and Culture Conditions

All cell lines were grown under sterile conditions at 37 °C with 5% CO_2 and 95% humidity in the incubator. Frozen cells were stored in liquid nitrogen. Cells were taken from the nitrogen and thawed in order to grow them. Growth medium was added into thawed cells and they were centrifuged to remove DMSO. After centrifuge, cell pellets were dissolved into the growth medium and moved into the T-25 flasks. When cells become 80%-90% confluent, they were passaged into T-75 flasks. Cell passage was repeated in 1:2-1:4 ratio when cells reached enough confluency. Passage time changed depending on the cell line. Generally, passage time for WT cells 3-4 days, whereas it is 5-7 days for resistant cell lines.

For cell passage, the growth medium was sucked with aspirator and cells were washed with PBS. Then, trypsin was added into cells and waited for 5 min in the incubator for detachment of the cells. Detached cells were centrifuged, the cell pellet was dissolved into a fresh growth medium. Cells were split and added into new T-75 flasks with fresh medium.

For storage of the cells, freezing medium that contains 90% FBS and 10% DMSO were prepared. After detachment of the cells from the flask, the cell pellet was dissolved into 1 ml of freezing medium and added into sterile cryo-vials. First, cells were stored at -20°C fridge for 1-2 hours. Then, they were transferred into a -80°C fridge. Cells were stored in liquid nitrogen for longer periods.

2.2.1.2. Transient Transfection

MDA-MB-231 and MCF-7 WT cells were transfected in order to silence the expression of Erbin gene. Cells were transfected with Hs_ERBB2IP_7 FlexiTube siRNA (si7-Erbin), Hs_ERBB2IP_12 FlexiTube siRNA (si12-Erbin) and siGENOME Non-Targeting siRNA #2 (scr-RNA). It was confirmed that there is no target for scr-RNA in the Erbin genome.

For the transfection, 200.000 cells/well for MDA-MB-231 cells and 250.000 cells/well for MCF-7 cells were seeded into 6-well plates. Next day, transfection procedure was performed.

First, 2.5 µl of si7-Erbin, si12-Erbin and scr-RNA were diluted in 250 µl of Opti-MEM in different tubes. Then, each diluted RNA was added into 250 µl of Opti-MEM containing 5 µl of Lipofectamine RNAiMAX transfection reagent. RNA-transfection reagent complexes in 500 µl of Opti-MEM were incubated for 20 mins at room temperature. After incubation, these complexes were added onto cells with 1.5 ml of fresh medium. After 72 hours incubation, cell pellets were collected for western blot analysis.

Also, cells were transfected for crystal violet assay. For the transfection, 50.000 cells/well for MDA-MB-231 cells and 750.000 cells/well for MCF-7 cells were seeded into 12-well plates. Next day, transfection procedure was performed. First, 1.25 µl of si7-Erbin, si12-Erbin and scr-RNA were diluted in 125 µl of Opti-MEM in different tubes. Then, each diluted RNA was added into 125 µl of Opti-MEM containing 2.5 µl of Lipofectamine RNAiMAX transfection reagent. RNA-transfection reagent complexes in 250 µl of Opti-MEM were incubated for 20 mins at room temperature. After incubation, these complexes were added onto cells with 1 ml of fresh medium. After 72 hours incubation, crystal violet assay was started.

2.2.1.3. SRB Assay

SRB assay were performed with MDA-MB-231 WT& DOXR cells and MCF-7 WT&TAMR in order to find viability of the cells with doxorubicin and tamoxifen treatment. MDA-MB-231 cells were treated with different doses of doxorubicin (0.1 uM, 0.25 uM, 0.5 uM, 0.75 uM, 1 uM and 5 uM) and MCF-7 cells were treated with various doses of tamoxifen (2.5 uM, 5 uM, 7.5 uM, 10 uM, 15 uM and 20 uM). For the SRB assay, 4000 cells/well were seeded into 96-well plates. Next day, doxorubicin and tamoxifen were mixed with growth medium and were added into each well. There were 4 replicas for each treatment. Cells were treated with drugs for 72 hours.

After 3 days, SRB assay was done. First, cells were fixed with TCA solution at +4°C for 1 hour in dark. After fixation, they were washed with ddH_2O five times and SRB solution was added into each well. SRB solution was incubated for 30 minutes in dark at room temperature. Then, cells were washed with 1% acetic acid five times and waited until drying. Lastly, 150 ul of 10 mM Tris base solution was added into each well and plate was read at 564 nm in a spectrophotometer.

2.2.1.4. Crystal Violet Assay

Crystal violet assay was performed with MDA-MB-231 WT& DOXR cells and MCF-7 WT&TAMR. MDA-MB-231 cells were treated with different doses of doxorubicin (0.1 uM, 0.5 uM and 1 uM) and MCF-7 cells were treated with different doses of tamoxifen (5 uM, 7.5 uM and 10 uM). For the crystal violet assay, 50.000 cells/well for MDA-MB-231 cells and 750.000 cells/well for MCF-7 cells were seeded into 12-well plates. Next day, doxorubicin and tamoxifen were mixed with growth medium and were added into each well or first, transfection was examined then, drugs were added in the third day. There were 3 replicas for each treatment. Cells were treated with drugs for 72 hours.

After 3 days, crystal violet assay was started. First, cells were rinsed with PBS and fixed with fixation solution overnight at room temperature. Next day, they were rinsed with PBS again and crystal violet solution was added into each well. Cells were incubated with crystal violet solution for 1 hour at room temperature in dark. After removal of crystal violet solution, cells were washed with tap water and waited until they were dried. Then, photos of the plates were taken. Destaining solution was added to each well to measure the concentration of crystal violet dye. Plates were incubated for 1 hour and plates were read at 595 nm in a spectrophotometer.

The viability of the cells was analyzed with GraphPad Prism program statistically. 2way ANOVA was performed to compare the viability of the cells. Tukey's and Sidak's multiple comparison tests were done.

2.2.2. Laboratory Techniques

2.2.2.1. Total Protein Isolation from Cell Pellets

For the protein isolation, cell pellets were collected with ice-cold PBS and centrifuged at +4°C. RIPA buffer (Table 2.2) was added into cell pellets and mixed. The amount of the RIPA buffer (30-100 ul) changed according to the size of the cell pellet. The mixtures were incubated on a shaker at +4°C for 30 minutes. Then, they were centrifuged at 13.000 rpm at +4°C for 30 minutes. After centrifugation, supernatants that contain total proteins were collected and proteins were stored at -80°C for further experiments.

2.2.2.2. Protein Quantification with BCA Protein Assay

For Western Blot, the concentration of proteins loading SDS-PAGE should be equal. For this reason, protein quantification was done with Pierce™ BCA Protein Assay Kit. The kit contained 2 mg/ml BSA standard, BCA Reagent A and BCA Reagent B. BSA standard was diluted with sterile autoclaved ddH_2O with 1:20 ratio in order to convert it to working concentration. First, different amounts of BSA standard (0, 10, 20, 30, 40, 50, 70, 100 ul) were added into 96 well plate to prepare the standard curve and wells were filled with ddH_2O to make total amount 100 ul for each well. Then, 100 ul of ddH_2O and 1 ul of different proteins were added to other wells. Last, 100 ul of BCA working solution (with 50:1 of reagent A:B ratio) was added into each well and the plate was incubated for 30 minutes at 37°C in dark. Last, the absorbance value of each well was measured at 562 nm in a spectrophotometer. According to absorbance values, a standard curve was plotted, and this was used to find protein concentrations with known absorbance values so that the concentrations of the proteins were calculated.

2.2.2.3. SDS-PAGE

Proteins were mixed with protein loading buffer (Table 2.2) according to calculated protein concentrations and the mixtures were boiled at 95°C for 5 minutes on heater block. SDS-polyacrylamide resolving and stacking gels were prepared. 8% or 10% of resolving gel (Table 2.11) was used for the proteins depending on their molecular weight.

Table 2.11. Constituents of SDS-Polyacrylamide Gel.

Resolving Gel (10 ml)			**Stacking Gel (5 ml)**	
Constituent	**8%**	**12 %**	**Constituent**	**5%**
ddH_20	4.7 ml	4.1 ml	ddH_20	3.4 ml
30% Acrylamide-bisacrylamide Mix	2.7 ml	3.3 ml	30% Acrylamide-bisacrylamide Mix	850 ul
1M Tris HCl (pH 8.8)	2.5 ml	2.5 ml	1M Tris HCl (pH 6.8)	625 ul
10% SDS	100 ul	100 ul	10% SDS	50 ul
10% APS	100 ul	100 ul	10% APS	50 ul
TEMED	8 ul	4 ul	TEMED	5 ul

SDS-PAGE was run in 1X running buffer (Table 2.2) at 80 V until proteins passed the stacking gel and voltage was increased to 120 V until the loading dye reached at the end of the gel.

2.2.2.4. Western Blot

After the proteins were run in SDS-PAGE, they were transferred to PVDF membrane by using a wet transfer system. First, the membrane was activated with methanol. The materials for the wet transfer (sponge, Wattman paper, membrane) were soaked with transfer buffer (Table 2.2). Proteins were transferred at 250 mA for 2.5 hours. After the transfer, it was checked whether proteins were successfully transferred to the membrane with Ponceau S staining solution (Table 2.2). Afterward, the membrane was incubated in blocking solution on shaker slowly for 1 hour at room temperature. After blocking, the membrane was incubated with primary antibody solutions containing 3% BSA and primary antibody in TBS-T overnight at +4^{o}C on the shaker slowly Next day, the membrane was washed three times with TBS-T solution in on shaker fast. After the wash, the membrane was incubated in secondary antibody solution containing 3% milk powder and rabbit or mouse secondary antibody (1:5000 ratio) in TBS-T on shaker slowly for 1 hour at room temperature. After the secondary antibody incubation was finished, the membrane was washed again three times with TBS-T. Finally, the membrane was developed using ECL prime system and, visualization was done in Amersham Imager 600.

Images that demonstrated protein levels were quantified with ImageJ program. Actin protein was confirmed as a housekeeping protein and other proteins were normalized according to the protein level of Actin.

CHAPTER 3. RESULTS

3.1. HIGH ERBIN EXPRESSION PREDICTS BETTER SURVIVAL IN TREATED BREAST CANCER PATIENTS

Since the function of Erbin in breast cancer is controversial, the level of the Erbin was investigated in different breast cancer datasets. Kaplan-Meier survival analyses displayed that high level of Erbin expression predicts better overall or relapse free survival in breast cancer patients treated with chemotherapy or targeted therapies while the Erbin level does not change the survival rates of untreated breast cancer patients (**Figure 3.1**).

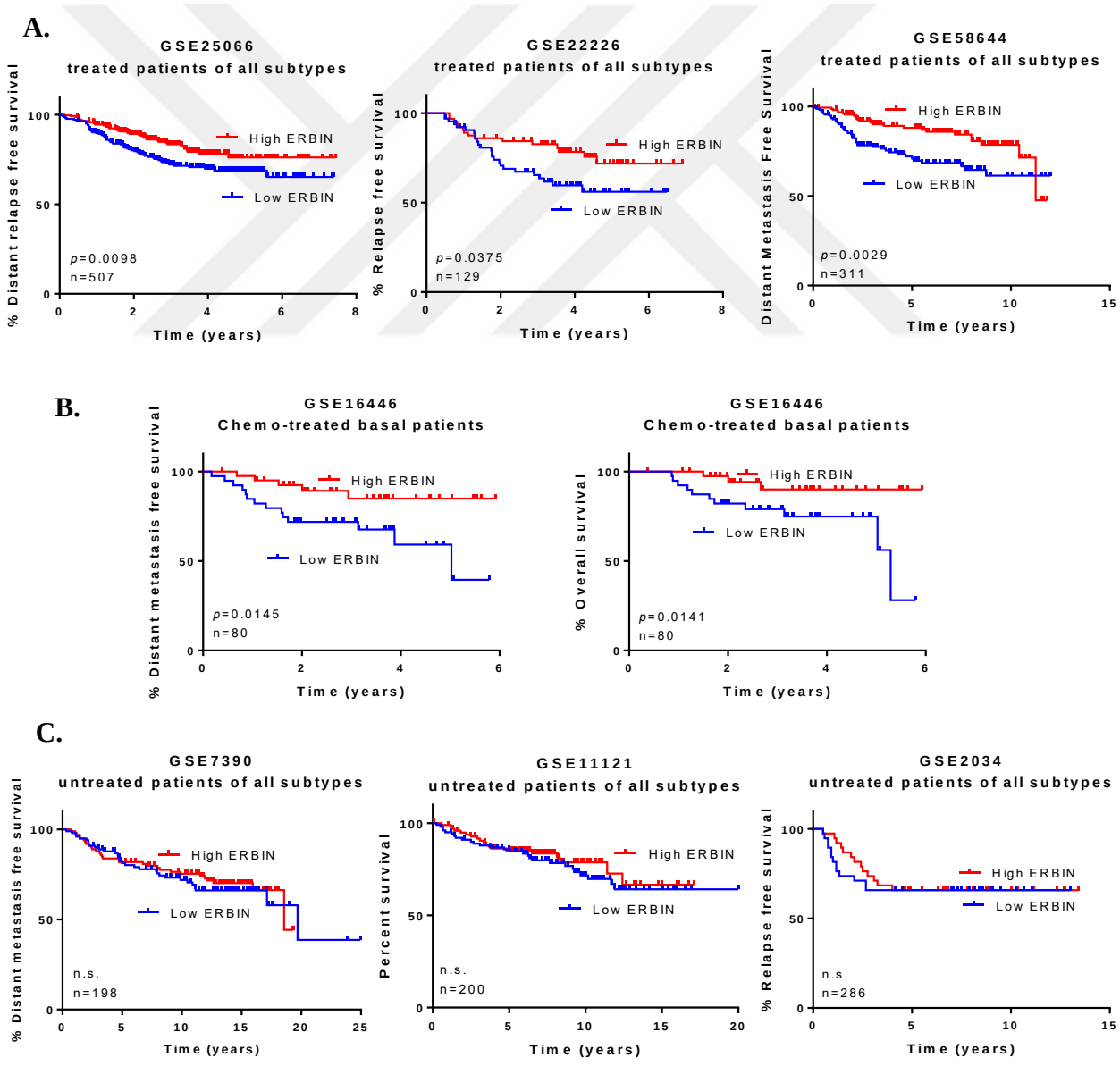


Figure 3.1. High Erbin expression predicts better survival in breast cancer patients treated with chemotherapy or targeted therapies. (A) Patients having all subtypes of breast cancer were treated with neoadjuvant taxane and anthracycline chemotherapy, tamoxifen, trastuzumab, or combined therapy. **(B)** Basal type breast cancer patients were treated with anthracyclines that is epirubicin **(C**) Breast cancer patients having all subtypes did not receive anthracycline or taxane or targeted therapy. *(This analysis was done by Özge Saatçi from Özgür Şahin's group)*

GSE25066, GSE22226 and GSE58644 datasets contain treated breast patients having basal, luminal and HER2 subtype of breast cancer. According to the GSE25066 dataset, 507 breast cancer patients received neoadjuvant chemotherapy with an anthracycline and taxane. ER-positive patients were also treated with endocrine therapy [79, 80]. GSE22226 patients (n=129) were treated with neoadjuvant chemotherapy containing doxorubicin, taxane or trastuzumab [81]. GSE58644 (n=311) patients were also treated with tamoxifen, chemotherapy, trastuzumab, or combined therapy [82]. GSE16446 (n= 80) dataset includes the basal type of breast cancer patients that had anthracycline chemotherapy that is epirubicin. Epirubicin blocks topoisomerase II and intercalates DNA [83, 84]. GSE7390, GSE11121 and GSE2034 datasets contain all subtypes of breast cancer patients and these patients did not treat with chemotherapy or targeted therapy [85-88].

The survival analyses of breast cancer patients based on the expression level of Erbin showed that Erbin can have an important role in breast cancer drug resistance and further investigation is essential.

3.2. PROTEIN LEVEL OF ERBIN IN DRUG SENSITIVE AND RESISTANT BREAST CANCER CELL LINES

After we decided that Erbin could have a role in drug resistance, Erbin levels were examined between different drug resistant and sensitive breast cancer cell lines. Cell lines were obtained from Özgür Şahin's group. Protein expression of doxorubicin resistant (DoxR) MDA-MB-231, tamoxifen resistant (TamR) MCF-7 and T47D, trastuzumab resistant (TmabR) BT-474 and SKBR-3 cells was investigated by Western Blot analysis. The protein expressions were lower in MDA-MB-231 DoxR and MCF-7 TamR cells apparently compared to the WT cells. In contrast, there was not a significant difference between T47D, BT-474 and SKBR-3 WT & resistant cells. (**Figure 3.2)**. MDA-MB-231 cells are TNBC cells and MCF-7 cells were

Luminal A type breast cancer cells [9]. According to Western Blot results, we decided to continue our study with MDA-MB-231 and MCF-7 cells.

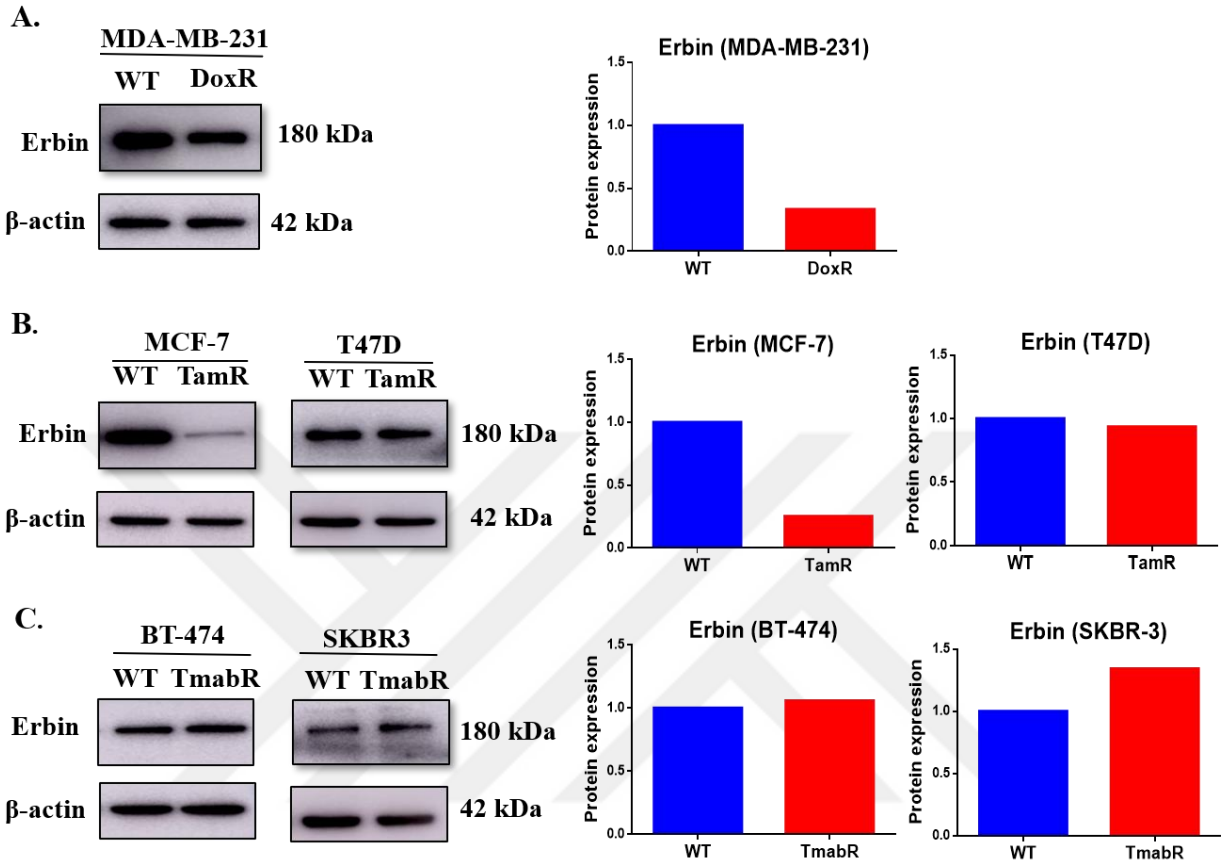


Figure 3.2. Western Blot results of different WT and resistant cell lines for Erbin expression. Protein expression of Erbin was shown in **(A)** MDA-MB-231 WT & DoxR **(B)** MCF-7 and T47D WT & TamR **(C)** BT-474 and SKBR-3 WT & TmabR cells. Western Blot quantifications were normalized based on Actin levels by ImageJ.

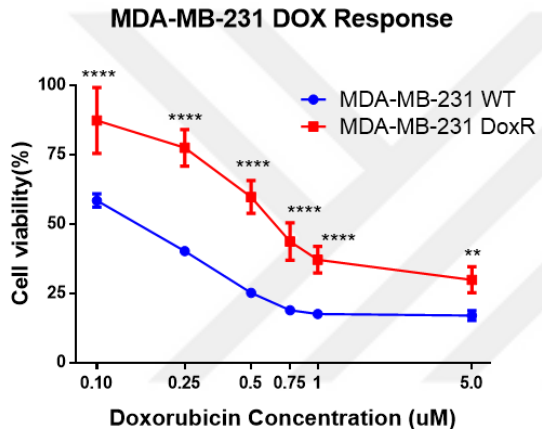
3.3. DOSE RESPONSES OF RESISTANT AND WT CELLS AGAINST ANTI-CANCER DRUGS

In order to confirm the resistance of the MDA-MB-231 DoxR and MCF-7 TamR cells, we carried out cell viability assays between WT and resistant cell lines. In addition, drug doses that result in the most different viability between WT and resistant cells were selected to use these doses for further drug treatment experiments. First, SRB assays were performed, and then results were supported with crystal violet assays.

3.3.1. Dose Response of MDA-MB-231 WT and DoxR Cells Against Doxorubicin

MDA-MB-231 doxorubicin resistant and sensitive cells were treated with increasing doses of doxorubicin in 3 days. After 3 days, SRB assay was performed. **Figure 3.3** shows SRB results of WT and DoxR cells. According to the results, DoxR cells evidently proliferated more than WT cells. The difference of the viability between WT and DoxR cells with all concentrations of doxorubicin was significant ($p<0.01$). This result demonstrates that MDA-MB-231 DoxR cell line has acquired doxorubicin resistance.

A.



B.

	Cell Viability (%)	
Dox Dose(uM)	**WT**	**DoxR**
0.1	58	87
0.25	40	77
0.5	25	60
0.75	19	44
1	18	37
5	17	30

Figure 3.3. Dose response results of doxorubicin resistant MDA-MB-231 cells and its sensitive counterpart. MDA-MB-231 WT and DoxR cells were treated with increasing doses of doxorubicin in quadruples with 96 well plates. SRB assay was performed after 3 days treatment. **(A)** Doxorubicin response was shown by the viability graph. IC50 value of MDA-MB-231 WT cells was 0.17 uM, whereas IC50 value of DoxR cells was 0.66 uM. The analysis was done by Sidak's multiple comparisons test (****$p<0.0001$, **$p<0.01$) **(B)** Viability of the cells with different dose treatments was displayed as viability percentage of the cells by the table.

After SRB assay, crystal violet assay was examined to confirm and find out doxorubicin doses that lead to different effect on DoxR and WT cells. For crystal violet assay, 0.1 uM, 0.5 uM and 1 uM of doxorubicin concentrations were selected to enable the difference of viability between wells observable. The images of crystal violet staining displayed that the number of DoxR cells was higher than WT cells with all concentrations of doxorubicin treatment (**Figure 3.4A**). For 0.1 uM and 0.5 uM doxorubicin, the distinction between DoxR and WT cells was significant ($p<0.01$) (**Figure 3.4B**).

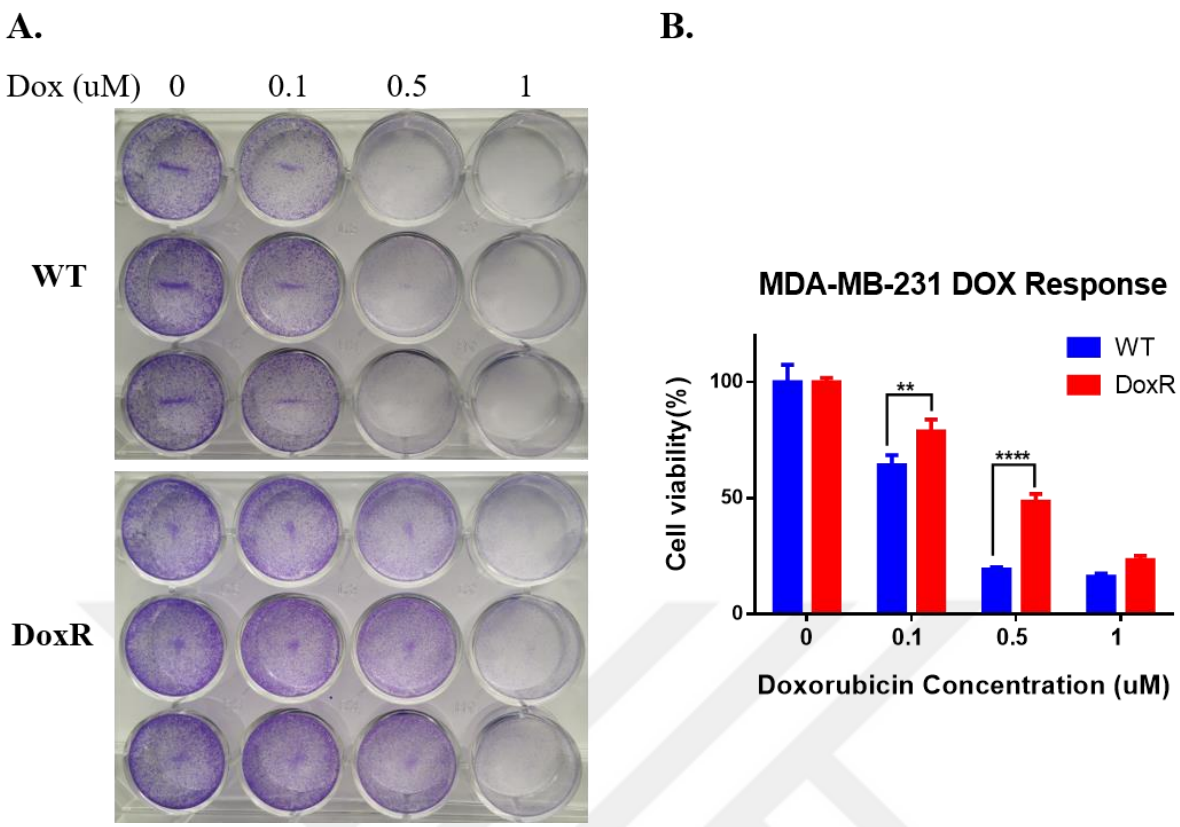


Figure 3.4. Crystal violet results of doxorubicin treated WT and DoxR cells. MDA-MB-231 WT and DoxR cells were treated with 0.1, 0.5 and 1 uM of doxorubicin in triplicates with 12 well plates. Crystal violet assay was performed after 3 days treatment. **(A)** The images of doxorubicin treated WT and DoxR cells after crystal violet staining are shown. 12 wells were triplicates vertically. **(B)** Doxorubicin response was shown by viability graph. Analysis was done by 2way ANOVA (Sidak's multiple comparisons test) (****p<0.0001, **p<0.01)

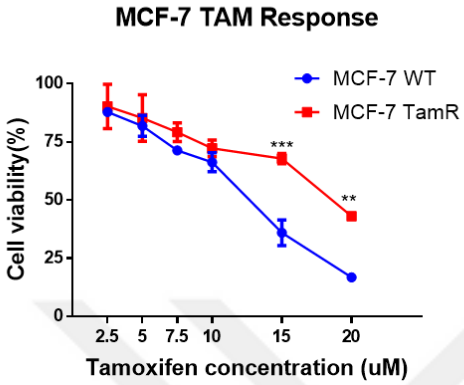
As a treatment concentration; 0.1 uM and 0.5 uM doses of doxorubicin were selected for the further western blot analyses and crystal violet assays because 0.1 uM and 0.5 uM doxorubicin affect the WT and DoxR cells differently based on their molecular mechanism and it results in significant proliferation difference between cells.

3.3.2. Dose Response of MCF-7 WT and TamR Cells Against Tamoxifen

MCF-7 tamoxifen sensitive and resistant cells were treated with increasing doses of tamoxifen. SRB assay was examined after 3 days. **Figure 3.5** demonstrates SRB results of WT and TamR cells. The effect of the all doses of tamoxifen is not observable as much as doxorubicin for MCF-7 WT and TamR cells but even so TamR cells proliferated more than WT cells. The

alteration of the viability between WT and TamR cells with 15 uM and 20 uM tamoxifen was significant. However, we did not use these doses for crystal violet assay, because 15 uM and 20 uM tamoxifen were too high for WT cells.

A.



B.

	Cell Viability (%)	
Tam Dose (uM)	**WT**	**TamR**
2.5	87.9	90.3
5	81.9	85.3
7.5	71.4	79.2
10	66.3	72.3
15	36.0	67.9
20	16.9	43.1

Figure 3.5. Dose response results of tamoxifen resistant and sensitive MCF-7 cells. MCF-7 WT and TamR cells were treated with increasing doses of tamoxifen in quadruples with 96 well plates. SRB assay was done after 3 days treatment. **(A)** Tamoxifen response was demonstrated by viability graph. IC50 value of MCF-7 TamR cells was 12.75 uM, whereas IC50 value of WT cells was 17.75 uM. Analysis was done by Sidak's multiple comparisons test (***p<0.001, **p<0.01) **(B)** Viability of the cells with different dose treatments was shown as viability percentage of the cells by table.

Crystal violet assay was performed with 5 uM, 7.5 uM and 10 uM of tamoxifen concentrations. Tamoxifen resistant MCF-7 cells grow slower than WT cells without tamoxifen treatment. As a result of tamoxifen treatment for 3 days, the images of crystal violet staining showed that the number of TamR cells were higher than WT cells. Tamoxifen treatment did not affect the viability of the TamR cells as much as WT cells (**Figure 3.6A**). For all doses of tamoxifen, difference between WT and TamR cells was significant ($p<0.01$) (**Figure 3.4B**). Moreover, difference of the number of the cells between different doses was also significant in WT cells, while this comparison was not significant for TamR cells. These results confirmed the tamoxifen resistance of MCF-7 TamR cell line.

For the further western blots and crystal violet assays, 5 uM and 7.5 uM tamoxifen concentrations were selected as a treatment dose because these two concentrations can lead to different molecular alterations in the WT and TamR cells.

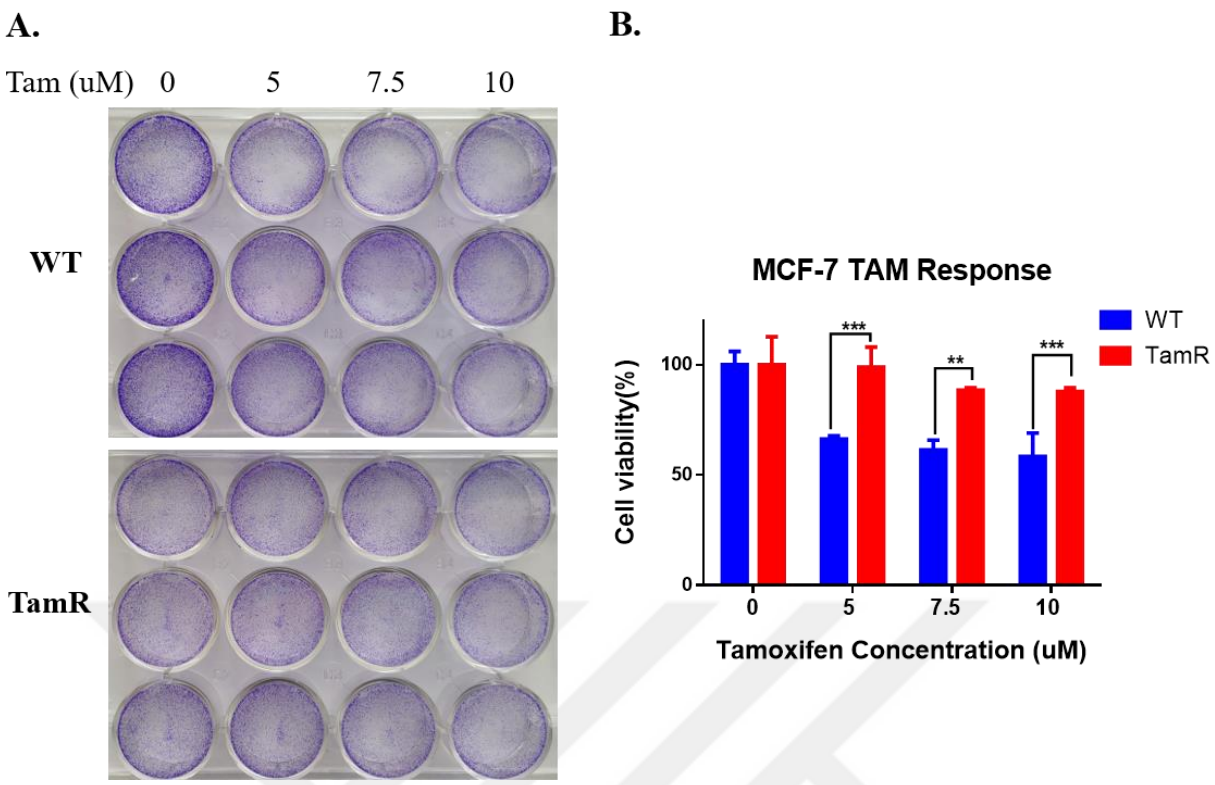


Figure 3.6. Crystal violet results of tamoxifen treated WT and TamR cells. MCF-7 WT and TamR cells were treated with 5, 7.5 and 10 uM of tamoxifen in triplicates with 12 well plates. Crystal violet assay was performed after 3 days treatment. **(A)** The images of tamoxifen treated WT and TamR cells after crystal violet staining are shown. 12 wells were triplicates vertically. **(B)** Tamoxifen response was shown by viability graph. Analysis was done by 2way ANOVA (Sidak's and Tukey's multiple comparisons tests) (***p<0.001, **p<0.01)

3.4. MOLECULAR CHARACTERIZATION OF DOXORUBICIN AND TAMOXIFEN RESISTANCE

After conformation and dose determination of doxorubicin and tamoxifen resistant cell lines, molecular characterization of these cells was done by western blot analysis. We tried to find out which mechanism that causes doxorubicin and tamoxifen resistance altered. Anti- cancer drug resistance can cause alterations in downstream proteins of apoptotic pathways [48]. For this reason, we investigated the expression level of anti- apoptotic, pro-apoptotic and cell cycle proteins in MDA-MB-231 doxorubicin resistant and MCF-7 tamoxifen resistant cells.

3.4.1. Molecular Alterations in Doxorubicin Resistant MDA-MB-231 Cells

In order to investigate the effect of doxorubicin resistance, MDA-MB-231 WT and DoxR cells were treated with doxorubicin for 72 hours. Because apoptotic markers were examined, floating

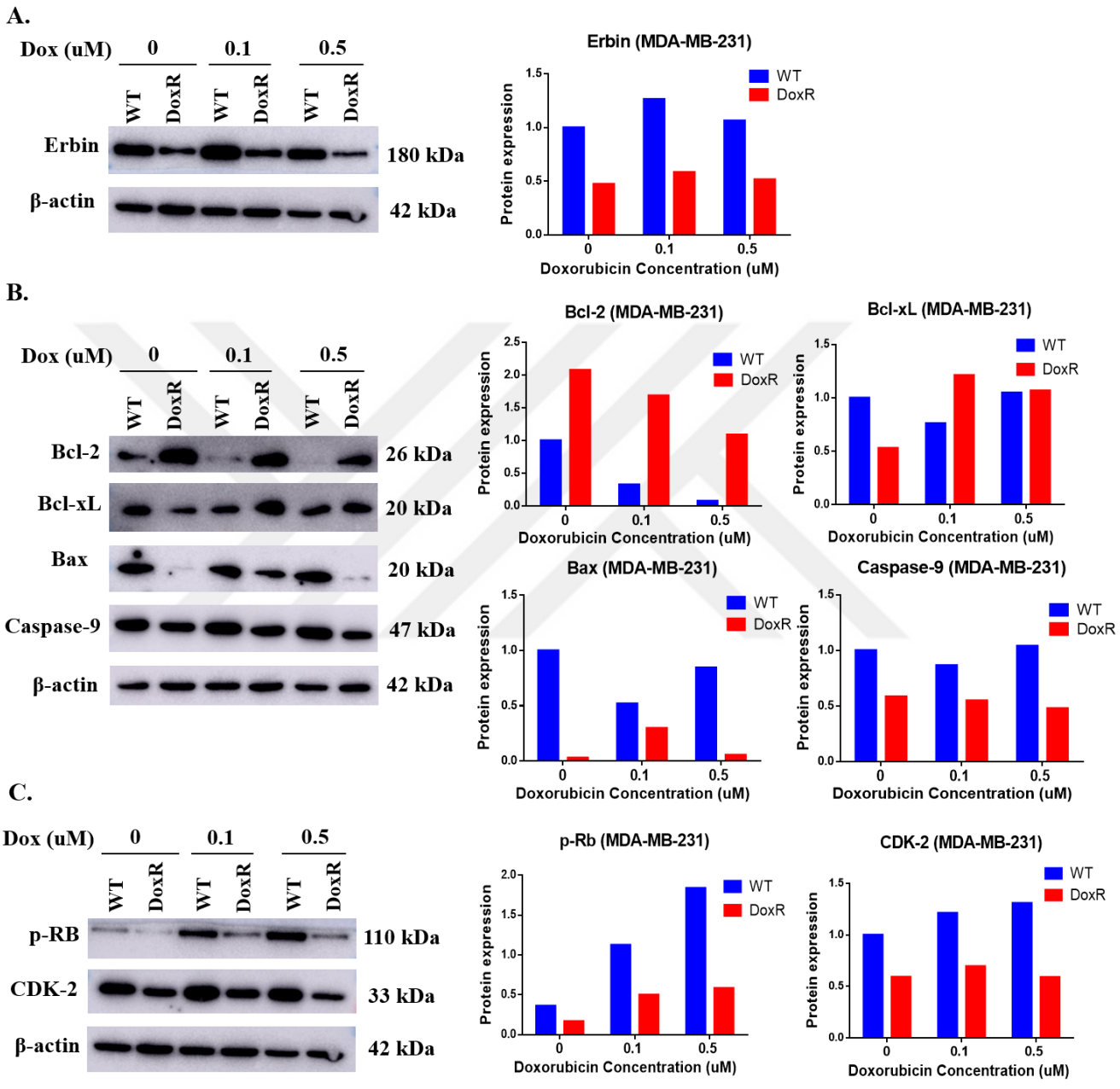


Figure 3.7. Western Blot results of doxorubicin treated WT and DoxR cells with characteristic changes in apoptosis and cell cycle proteins. MDA-MB-231 WT and DoxR cells were treated with 0.1 and 0.5 uM of doxorubicin for 72 hours. Protein expression of **(A)** Erbin, **(B)** anti-apoptotic proteins Bcl-2 and Bcl-xL, pro-apoptotic proteins Bax and Caspase-9, **(C)** p-RB and cell cycle protein CDK-2 were performed by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ.

dead cells were also taken while cell pellets were collectted for protein lysate. Protein expression of Erbin decreased in MDA-MB-231 DoxR cell with doxorubicin treated and untreated samples as expected (**Figure 3.7A**).

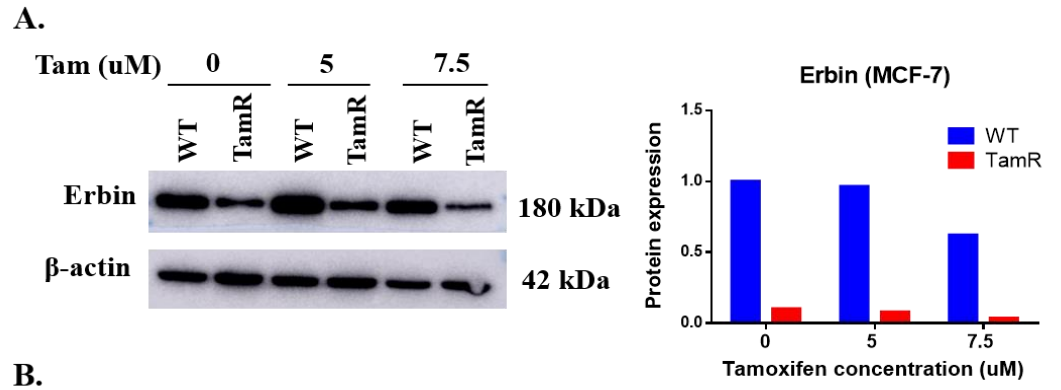
Bcl-2 and Bcl-xL are anti-apoptotic proteins, and they inhibit the cytochrome-c release into mitochondria in the intrinsic pathway and prevent apoptosis [47]. The level of Bcl-2 and Bcl-xL increased in doxorubicin treated DoxR cells. On the other hand, Caspase-9 and Bax are pro-apoptotic proteins. Bax initiates intrinsic apoptosis pathway and cleaved Caspase-9 activates executioner caspases that start apoptosis [42]. Caspase-9 and Bax protein expression decreased in DoxR cells (**Figure 3.7B**). Overexpression of anti-apoptotic proteins and down-regulation of pro-apoptotic proteins can lead to anti-cancer drug resistance [48, 50]. These results displayed that doxorubicin resistance changed the apoptosis mechanism of MDA-MB-231 DoxR cells.

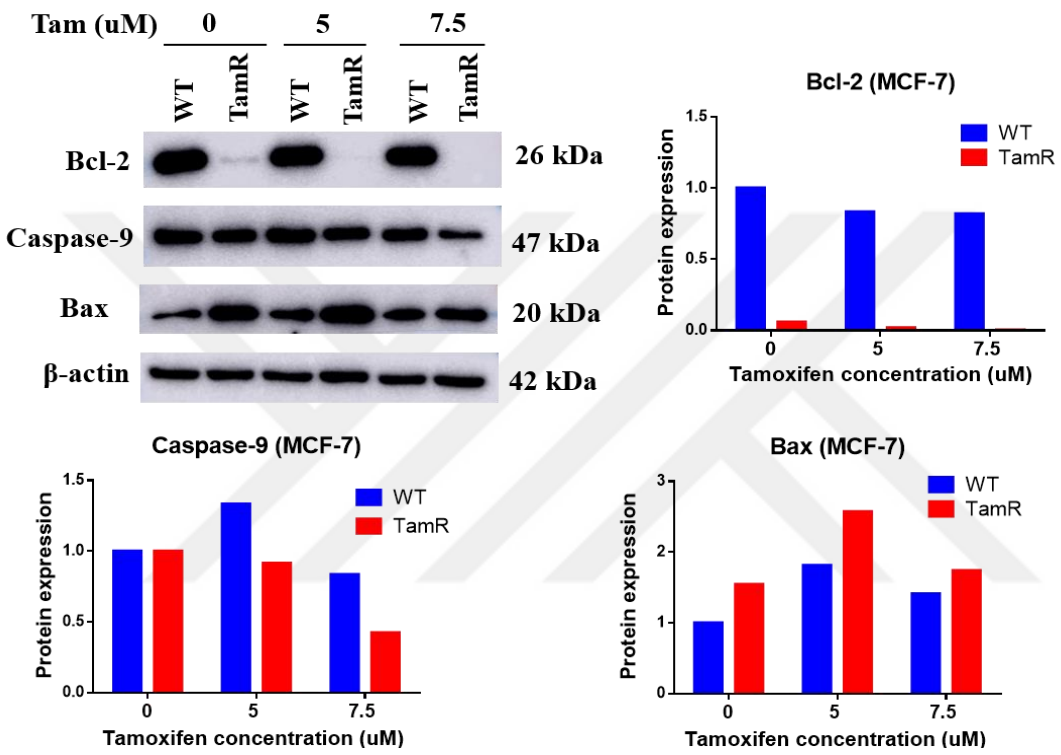
Rb is a tumor suppressor protein that binds to E2F and inhibits E2F transcription and CDKs activity. Phosphorylation of Rb protein results in its inactivation and it enables CDKs activity [89]. CDKs have an important role in cell cycle division. As a result of p21 and p27 degradation, Cdk2 increases. Cdk2 is required during G1/S phase [90]. p-Rb and Cdk2 proteins were reduced in DoxR cells compare to WT cells (**Figure 3.7C**). Because doxorubicin causes DNA damages, cell cycle arrest mechanism is recruited. Increase of p-Rb and Cdk2 proteins indicated the accumulation of the proteins in G1/S phase with doxorubicin treatment. The level of p-Rb and Cdk2 was reduced in DoxR cells because they was not affected by doxorubicin as WT cells.

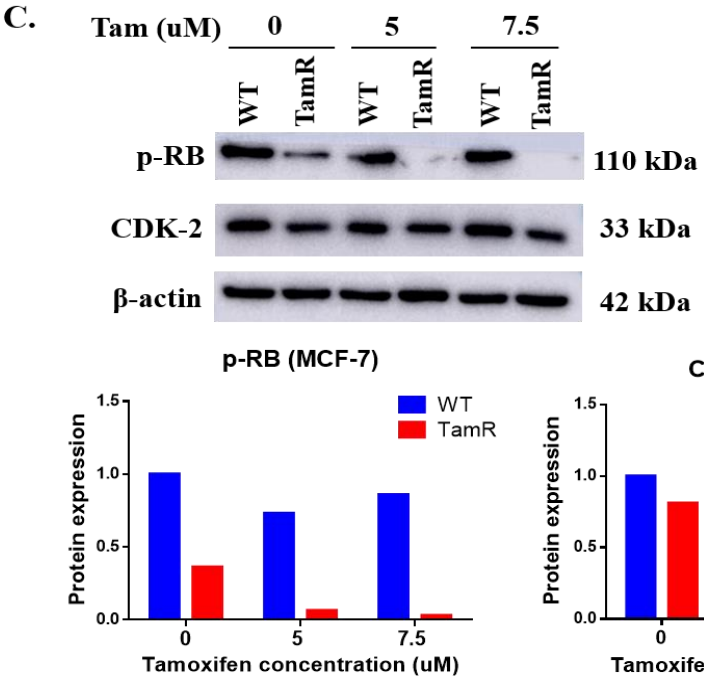
3.4.2. Molecular Alterations in Tamoxifen Resistant MCF-7 Cells

MCF-7 TamR and WT cells were treated with tamoxifen for 72 hours to identify altered cellular mechanism due to tamoxifen resistance. The experimental process was the same as MDA-MB-231 cells. As a result of western blot analysis of these cells, the level of Erbin protein was reduced in MCF-7 TamR cells with tamoxifen treated and untreated samples as expected (**Figure 3.8A**).

In MCF-7 TamR cells, Caspase-9 expression decreased because apoptosis is reduced. Normally, we expected that Bcl-2 would have higher expression and Bax protein would decrease in TamR cells compared to tamoxifen sensitive cells. In contrast, anti-apoptotic Bcl-2


A.
Tam (uM)
0
5
7.5
WT
TamR
Erbin
β-actin
180 kDa
42 kDa
Erbin (MCF-7)
Protein expression
Tamoxifen concentration (uM)


B.
Tam (uM)
0
5
7.5
WT
TamR
Bcl-2
Caspase-9
Bax
β-actin
26 kDa
47 kDa
20 kDa
42 kDa
Bcl-2 (MCF-7)
Caspase-9 (MCF-7)
Bax (MCF-7)
Protein expression
Tamoxifen concentration (uM)


C.
Tam (uM)
0
5
7.5
WT
TamR
p-RB
CDK-2
β-actin
110 kDa
33 kDa
42 kDa
p-RB (MCF-7)
CDK-2 (MCF-7)
Protein expression
Tamoxifen concentration (uM)

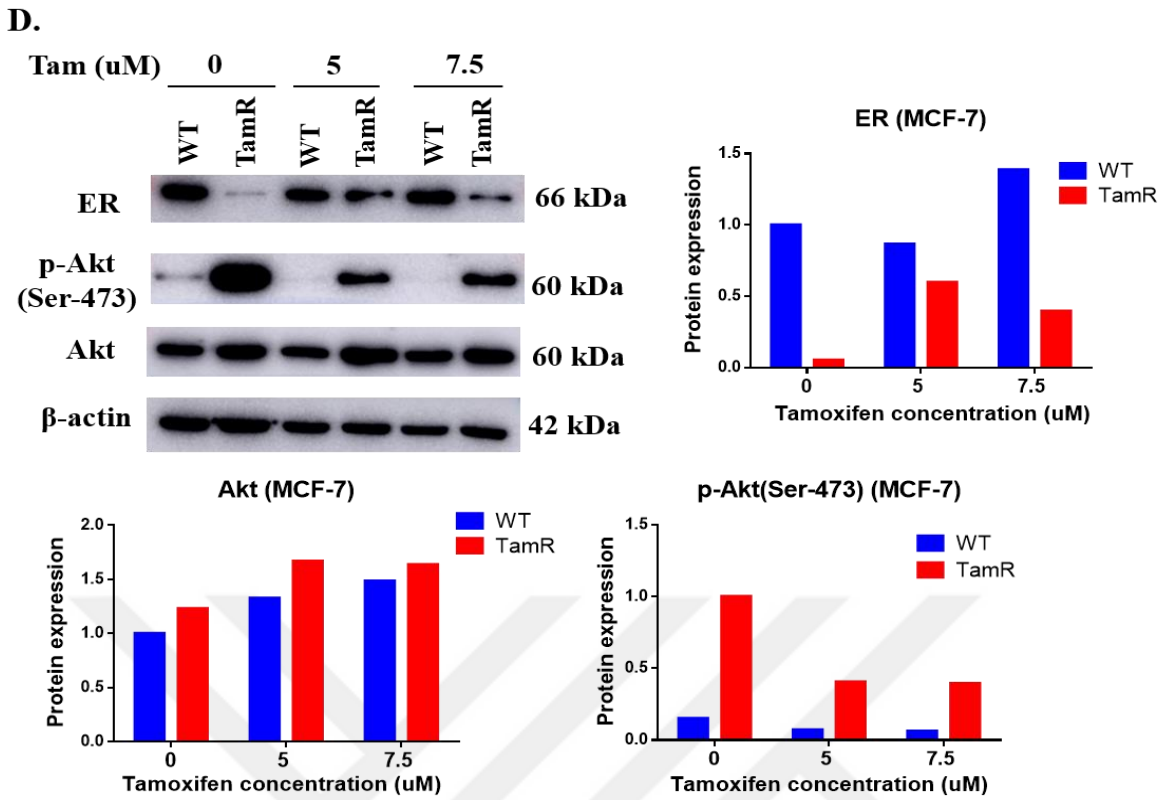


Figure 3.8. Western Blot results of tamoxifen treated WT and TamR cells with characteristic changes in different pathways. MCF-7 WT and TamR cells were treated with 5 and 7.5 uM of tamoxifen for 72 hours. Protein expression of **(A)** Erbin, **(B)** anti-apoptotic protein Bcl-2, pro-apoptotic proteins Bax and Caspase-9, **(C)** p-RB and cell cycle protein CDK-2, **(D)** ER and PI3K pathway downstream protein Akt and its phosphorylated version were performed by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ.

protein expression was almost disappeared and pro-apoptotic Bax protein increased (**Figure 3.8B**). On the other hand, there are some studies that Bcl-2 overexpression sensitizes cancer cells to anti-cancer drugs and is related to improved survival [91-93]. In addition, cell cycle proteins Cdk2 and p-Rb decreased in MCF-7 TamR cells as DoxR cells (**Figure 3.8C**).

ER is the key regulator of tamoxifen mechanism and loss of ER expression is one of the reasons for gaining tamoxifen resistance [35,38]. **Figure 3.8D** shows that ER expression was reduced dramatically in MCF-7 TamR cells. Moreover, overactivation of the PI3K/AKT pathway is frequently associated with acquired tamoxifen resistance [39]. As can be seen in **Figure 3.8**, Akt expression increased in TamR cells and phosphorylation at Ser-473 site of Akt was overactivated compare to sensitive MCF-7 cells.

3.5. DOWNREGULATION OF ERBIN CHANGES REGULATION OF DIFFERENT PATHWAYS

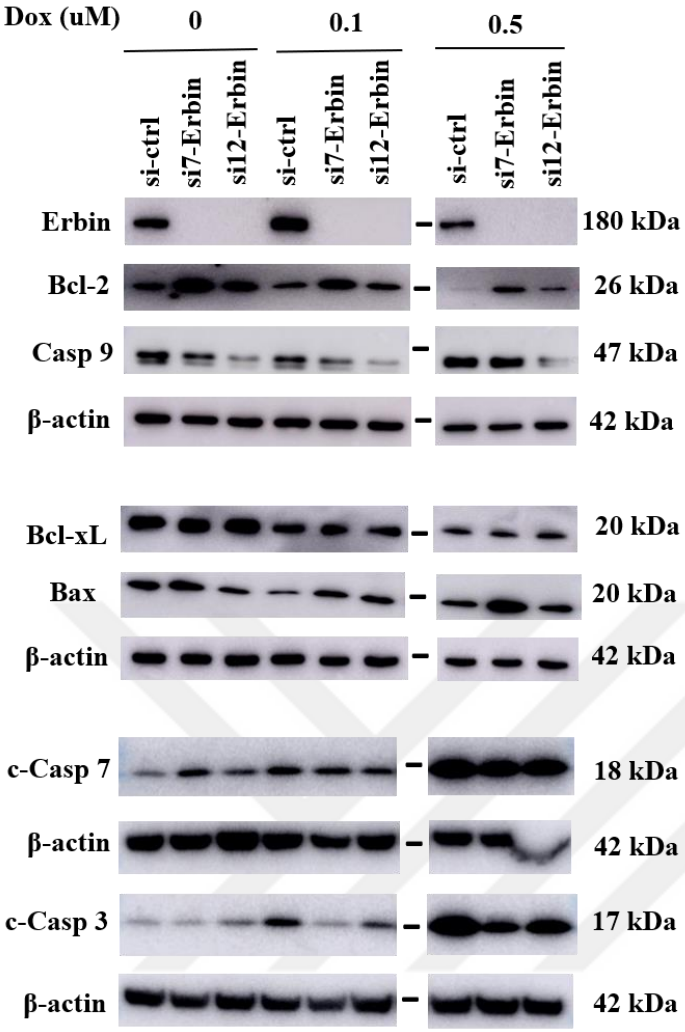
After we demonstrated that Erbin expression was lower in MDA-MB-231 DoxR and MCF-7 TamR cells compare to sensitive cells, we supposed that if Erbin expression is downregulated in WT cells, they can behave as resistant cells. Also, we tried to investigate how Erbin downregulation affects the cells and different pathway mechanisms.

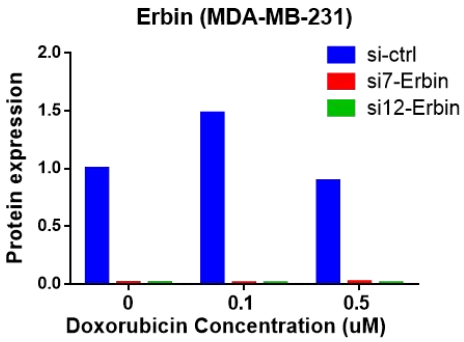
The Erbin gene was downregulated with two different si-RNAs that are si7-Erbin and si12-Erbin by transient transfection. These si-RNAs target different parts of the Erbin in the genome. Nontargeting scramble RNA was used as a si-control RNA (si-ctrl).

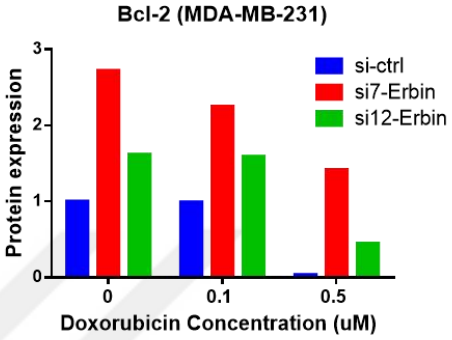
3.5.1. Erbin Silencing and Doxorubicin Resistance in MDA-MB-231 Cells

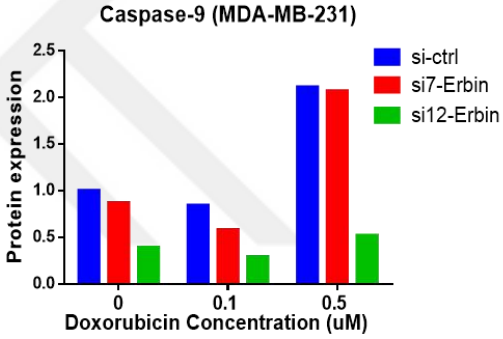
Erbin was silenced in MDA-MB-231 WT cells by transient transfection with either si-Erbin RNAs or si-ctrl RNA. After 24 hours, they were treated with 0.1 uM and 0.5 uM doxorubicin for additional 48 hours. Then, western analysis was done. It was found that there were some alterations in the downstream elements of apoptotic, cell cycle and PI3K pathways by silencing of Erbin gene. According to **Figure 3.9**, Erbin was silenced successfully with both si-RNAs and doxorubicin doses.

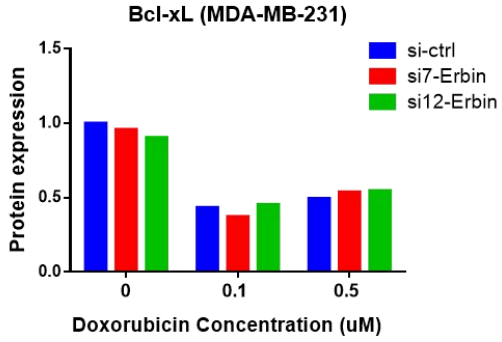
Proteins that take part in intrinsic apoptotic pathway were examined. In MDA-MB-231 DoxR cells, protein expression of anti-apoptotic proteins increased and pro-apoptotic proteins decreased (**Figure 3.7**). The result was similar in Erbin silenced and doxorubicin treated MDA-MB-231 WT cells. Bcl-2 increased and Caspase-9 decreased in these cells. Protein expression of Bcl-xL was almost the same in all samples. Interestingly, pro-apoptotic Bax protein expression increased in Erbin silenced cells. Caspase-3 and Caspase-7 are effector caspases. They are activated when they are cleaved by Caspase-9. Cleaved Caspase-3 and Caspase-7 start apoptosis in the cell [41]. Silencing of Erbin resulted in decrease of Caspase-3 level in doxorubicin treated cells apparently but Caspase-7 expression was almost the same. We can conclude that Erbin silencing alters intrinsic apoptosis mechanism and reduces apoptosis in MDA-MB-231 cells as in DoxR cells (**Figure 3.9)**.

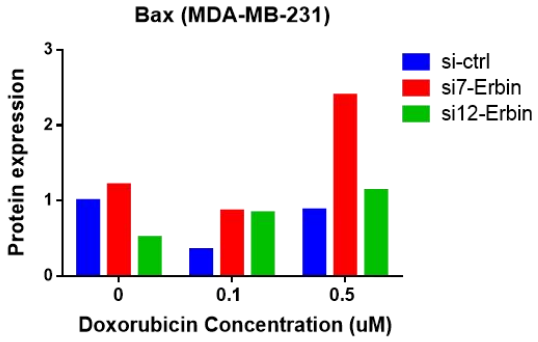

Dox (uM)
0
0.1
0.5
si-ctrl
si7-Erbin
si12-Erbin
Erbin
180 kDa
Bcl-2
26 kDa
Casp 9
47 kDa
β-actin
42 kDa
Bcl-xL
20 kDa
Bax
20 kDa
β-actin
42 kDa
c-Casp 7
18 kDa
β-actin
42 kDa
c-Casp 3
17 kDa
β-actin
42 kDa

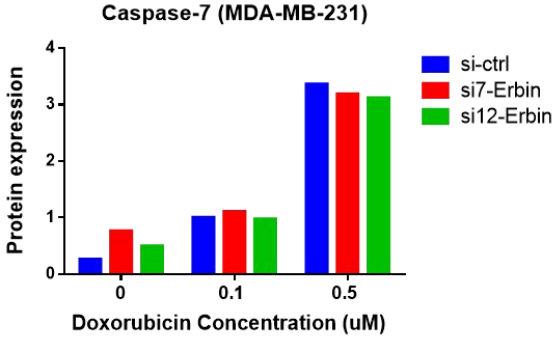

Erbin (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)


Bcl-2 (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)


Caspase-9 (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)


Bcl-xL (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)


Bax (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)


Caspase-7 (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)

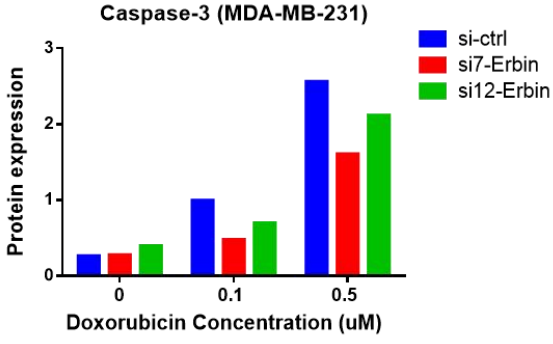

Caspase-3 (MDA-MB-231)
si-ctrl
si7-Erbin
si12-Erbin
Protein expression
Doxorubicin Concentration (uM)

Figure 3.9. Silencing of the Erbin gene affects the intrinsic apoptotic pathway as a result of doxorubicin treatment. Protein expression of Erbin, anti-apoptotic proteins, pro-apoptotic proteins and caspases were performed in Erbin silenced and doxorubicin treated MDA-MB-231 WT cells by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

Rb and Cdk2 proteins were analyzed in order to explore whether Erbin silencing has an effect on cell proliferation. p-Rb and Cdk2 increased in Erbin silenced cells. Downregulation of Erbin expression increases cell proliferation and viability against doxorubicin treatment. In addition, some DNA damage response pathway proteins were checked. Activation of this pathway causes cell cycle arrest and initiation of DNA repair. Phosphorylation of Chk1 results in cell cycle arrest in G1/S phase. If Chk1 is inhibited by cytotoxic chemotherapy, it leads inhibition of DNA repair and result in cell death [94]. Results showed that there was no p-Chk1 expression in si-ctrl cells, whereas it was expressed in Erbin silenced cells with doxorubicin treatment. p21 protein is one of the regulators of DNA repair. It was reported that p21 causes cell cycles arrest and prevents doxorubicin induced apoptosis by p53 mechanism [95]. p21 expression increase with 0.1 uM doxorubicin treatment compare to control. However, p21 expression increased with si7-Erbin silencing but it decreased with si12-Erbin when cells were treated with 0.5 uM doxorubicin. Phosphorylation of gamma-H2AX on serine 139 site indicates DNA damage and DNA double strand breaks [96]. Interestingly, the expression of p-gamma-H2AX on the serine 139 increased in Erbin silenced cells with 0.1 uM doxorubicin treatment but was reduced with 0.5 uM doxorubicin treatment. However, DNA damage does not mean induction of apoptosis every time. Even if some results were inconsistent, it was demonstrated that silencing of Erbin in MDA-MB-231 cells leads to increase of cell proliferation and inhibition of apoptosis through the DNA damage response mechanism against doxorubicin treatment (**Figure 3.10**).

Because the PI3K pathway is associated with cell survival and proliferation, the downstream protein Akt was also explored. Akt is phosphorylated on two different sites that are Thr-308 and Ser-473. These two sites indicate activation of different elements. The Ser-473 site is specifically phosphorylated by the mTORC2 complex specifically. In contrast, phosphorylation of Akt at Thr-308 and at Ser-473 activates the mTORC1 complex that triggers cell growth and proliferation [97, 98]. **Figure 3.11** showed that p-Akt on Ser-473 site was reduced but

phosphorylation of Akt on the Thr-308 site increased in Erbin silenced cells. It was concluded that Erbin silencing also affects some regulations in PI3K pathway in MDA-MB-231 cells.

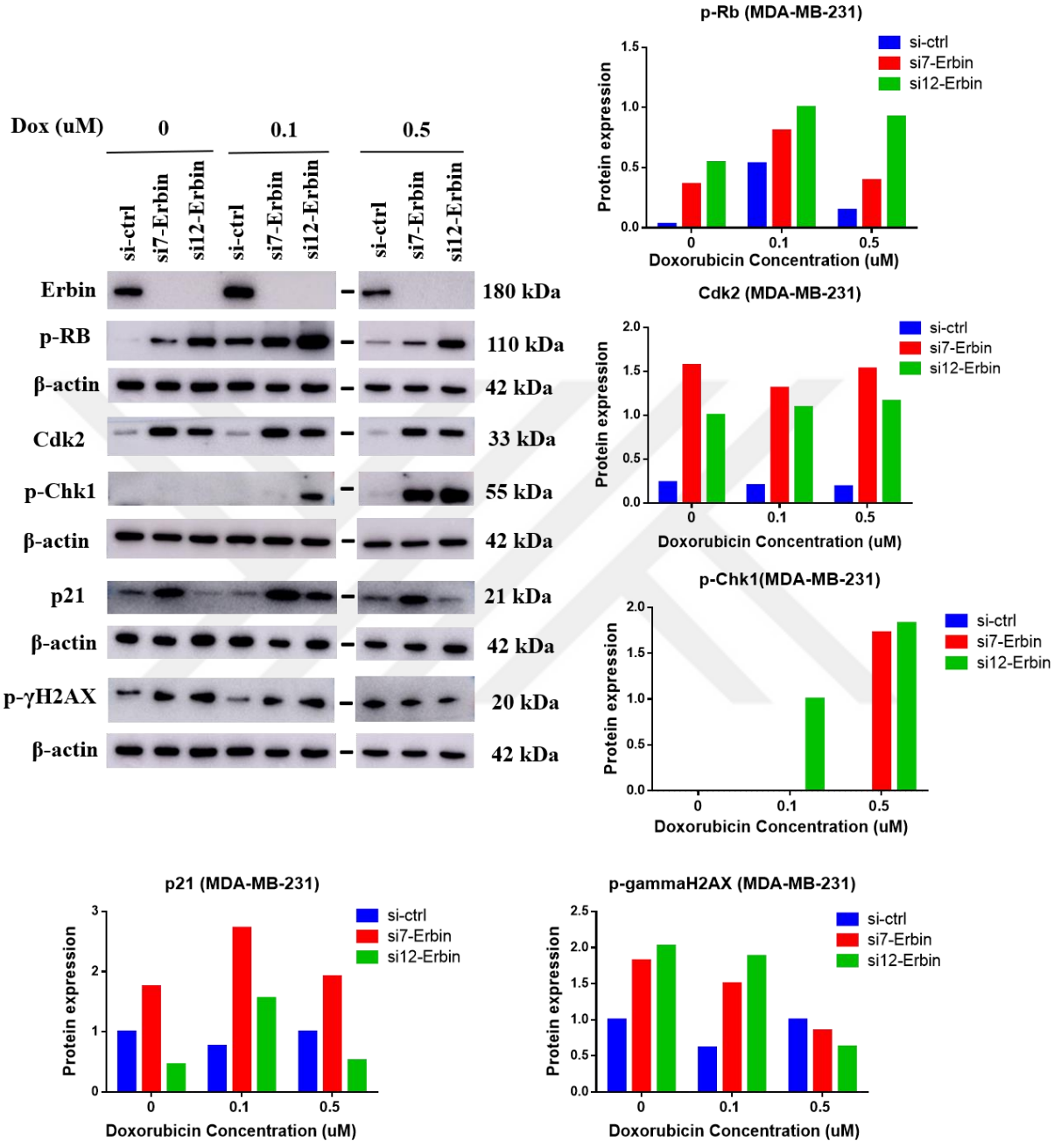


Figure 3.10. Silencing of the Erbin gene affects the downstream proteins of cell cycle and DNA damage response pathways as a result of doxorubicin treatment. MDA-MB-231 WT cells were transfected with si-Erbin and treated with 0.1 and 0.5 uM of doxorubicin for an additional 48 hours. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

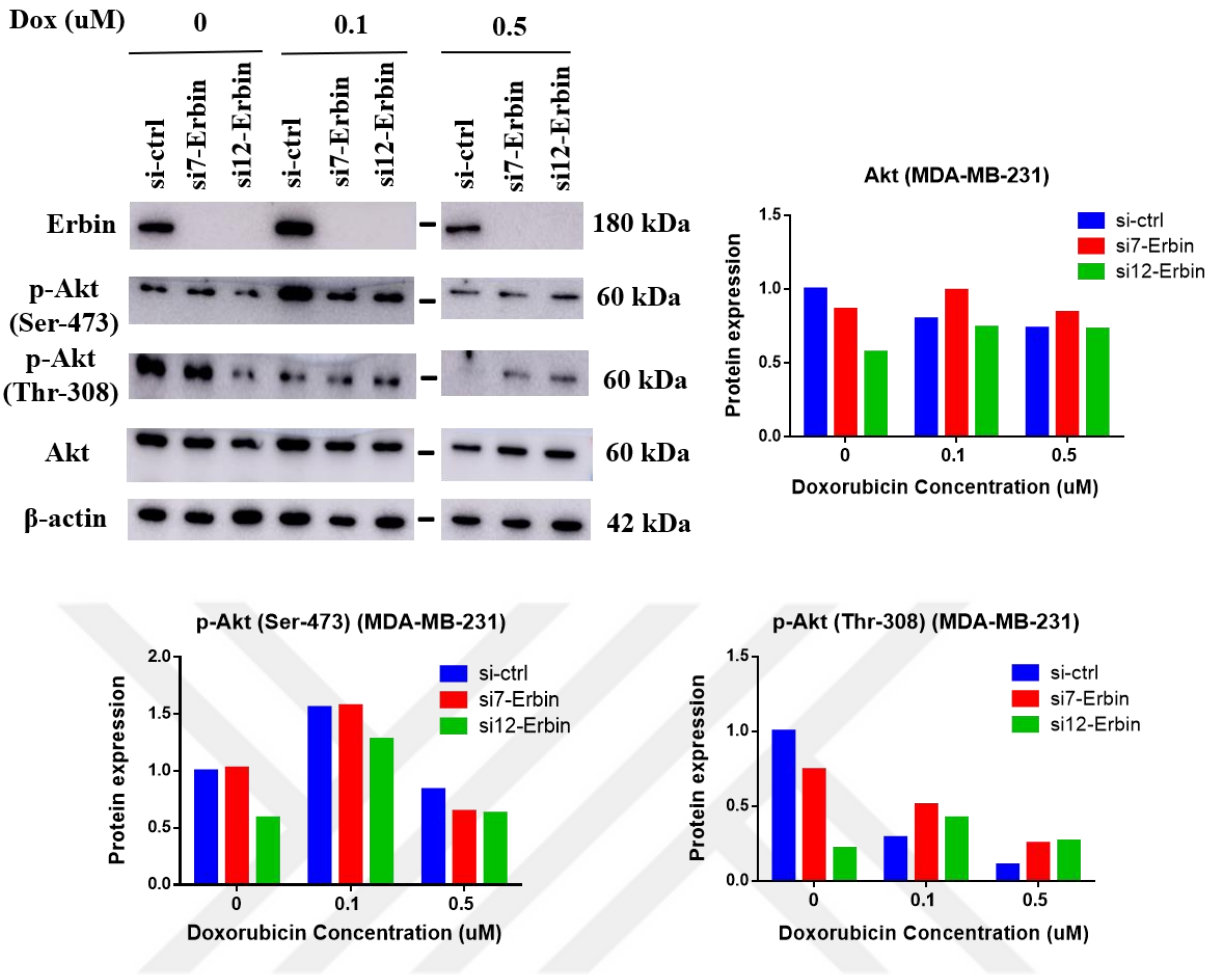


Figure 3.11. Silencing of the Erbin gene affects PI3K pathway. Protein expression of Akt proteins were performed in Erbin silenced and doxorubicin treated MDA-MB-231 WT cells by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

3.5.2. Erbin Downregulation and Tamoxifen Resistance in MCF-7 Cells

Erbin was downregulated in MCF-7 WT cells by transient transfection with either si-Erbin RNAs or si-ctrl RNA. After 24 hours, cells were treated with 5 uM and 7.5 uM tamoxifen for an additional 72 hours. Then, protein expression of downstream elements of the apoptotic, cell cycle and PI3K pathways was analyzed by western blot. **Figure 3.12** displayed that Erbin was downregulated with two different si-RNAs and tamoxifen doses successfully.

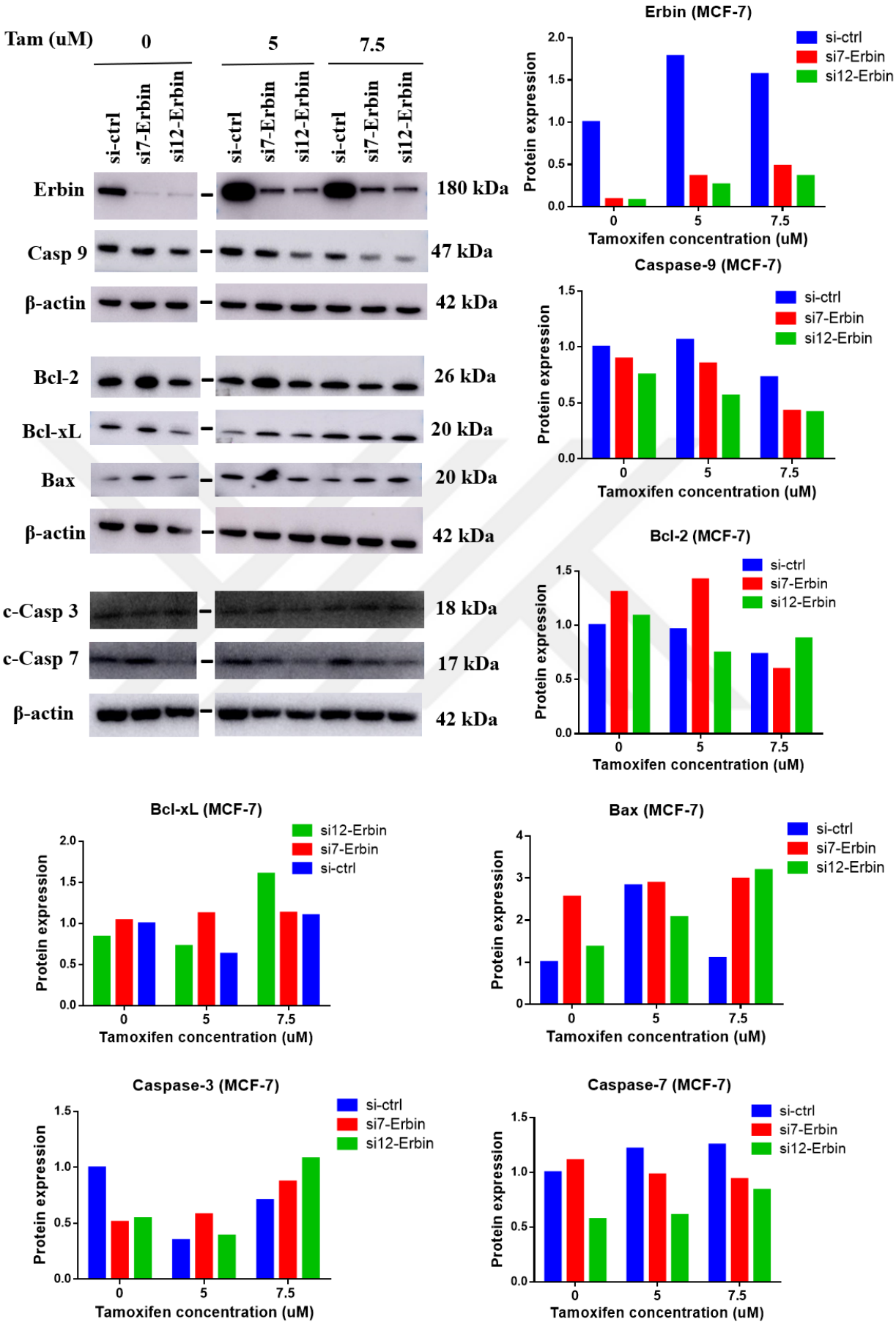

Tam (uM)
0
5
7.5
si-ctrl
si7-Erbin
si12-Erbin
Erbin
180 kDa
Casp 9
47 kDa
β-actin
42 kDa
Bcl-2
26 kDa
Bcl-xL
20 kDa
Bax
20 kDa
β-actin
42 kDa
c-Casp 3
18 kDa
c-Casp 7
17 kDa
β-actin
42 kDa
Erbin (MCF-7)
Protein expression
Tamoxifen concentration (uM)
Caspase-9 (MCF-7)
Bcl-2 (MCF-7)
Bcl-xL (MCF-7)
Bax (MCF-7)
Caspase-3 (MCF-7)
Caspase-7 (MCF-7)

Figure 3.12. Downregulation of the Erbin gene affects intrinsic apoptotic pathway as a result of tamoxifen treatment. Protein expression of Erbin, anti-apoptotic proteins, pro-apoptotic proteins and caspases were performed in Erbin downregulated and tamoxifen treated MCF-7 WT cells by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

Regulators of the apoptosis pathway were examined. Caspase-9 expression was reduced when Erbin gene was downregulated. In contrast, Bcl-2, Bcl-xL and Bax expression were inconsistent. Protein expression of Bcl-2 increased in Erbin downregulated untreated cells but the effects of si7-Erbin and si12-Erbin were different with 5 uM and 7.5 uM tamoxifen treatment. Bcl-xL increased when Erbin was downregulated in untreated and 5 uM tamoxifen treated cells but it decreased in Erbin downregulated cells with 7.5 uM tamoxifen treatment. In addition, Bax expression increased in Erbin downregulated cells except 5 uM tamoxifen treated cells. Lastly, the expression of cleaved Caspase-7 was reduced but cleaved Caspase-3 increased when Erbin was downregulated in MCF-7 cells (**Figure 3.12**). The result of the regulators of the intrinsic apoptosis pathway is controversial. We cannot exactly elucidate that apoptosis decreased when Erbin expression was downregulated in MCF-7 cells.

According to **Figure 3.13**, cell proliferation regulators p-Rb and Cdk2 increased when Erbin was downregulated in MCF-7 cells as doxorubicin treated MDA-MB-231 cells. The expression of p-gamma-H2AX on serine 139 decreased in Erbin downregulated cells. It means that Erbin downregulation can inhibit DNA damage and DNA double strand breaks. These results showed that downregulation of Erbin expression increases proliferation in tamoxifen treatment. Moreover, p21 expression decreased with tamoxifen treatment and Erbin downregulation. Some studies claimed that loss of p21 function causes tamoxifen stimulated growth and hyperphosphorylation of ER in breast epithelial cancer cells [99]. When Erbin was downregulated, p21 was also reduced and it enhanced cell growth in tamoxifen treatment based on the study and our results.

Because ER is most important regulator of tamoxifen treatment and its expression is a sign of tamoxifen resistance, the level of ER protein was checked in Erbin downregulated MCF-7 cells. Interestingly, the expression of ER decreased dramatically in Erbin downregulated cells with 5 uM tamoxifen treatment as TamR cells but it increased when tamoxifen concentration became 7.5 uM (**Figure 3.14**).

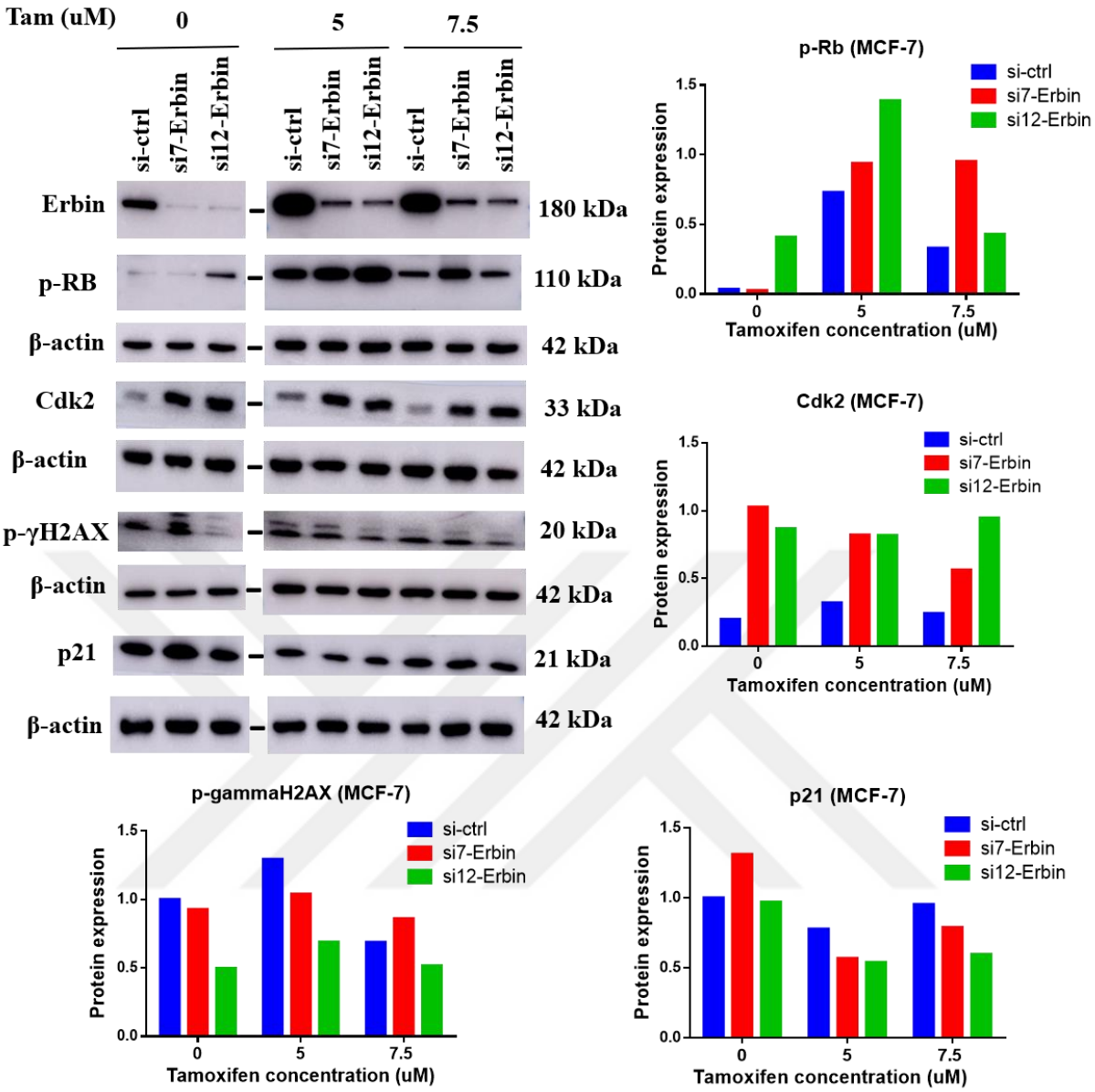


Figure 3.13. Downregulation of the Erbin gene affects downstream proteins of cell cycle and DNA damage response pathways as a result of tamoxifen treatment. MCF-7 WT cells were transfected with si-Erbin and treated with 5 and 7.5 uM of tamoxifen for an additional 72 hours. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

The expression of Akt was also examined. In TamR cells, Akt expression increased and phosphorylation at Ser-473 site of Akt was overactivated compared to the sensitive MCF-7 cell (**Figure 3.8D)**. However, when Erbin was downregulated, phosphorylation of Akt was observed at the Thr-308 site. p-Akt increased in Erbin downregulated cells with 7.5 uM tamoxifen treatment but it was almost gone with 5 uM tamoxifen (**Figure 3.14**).

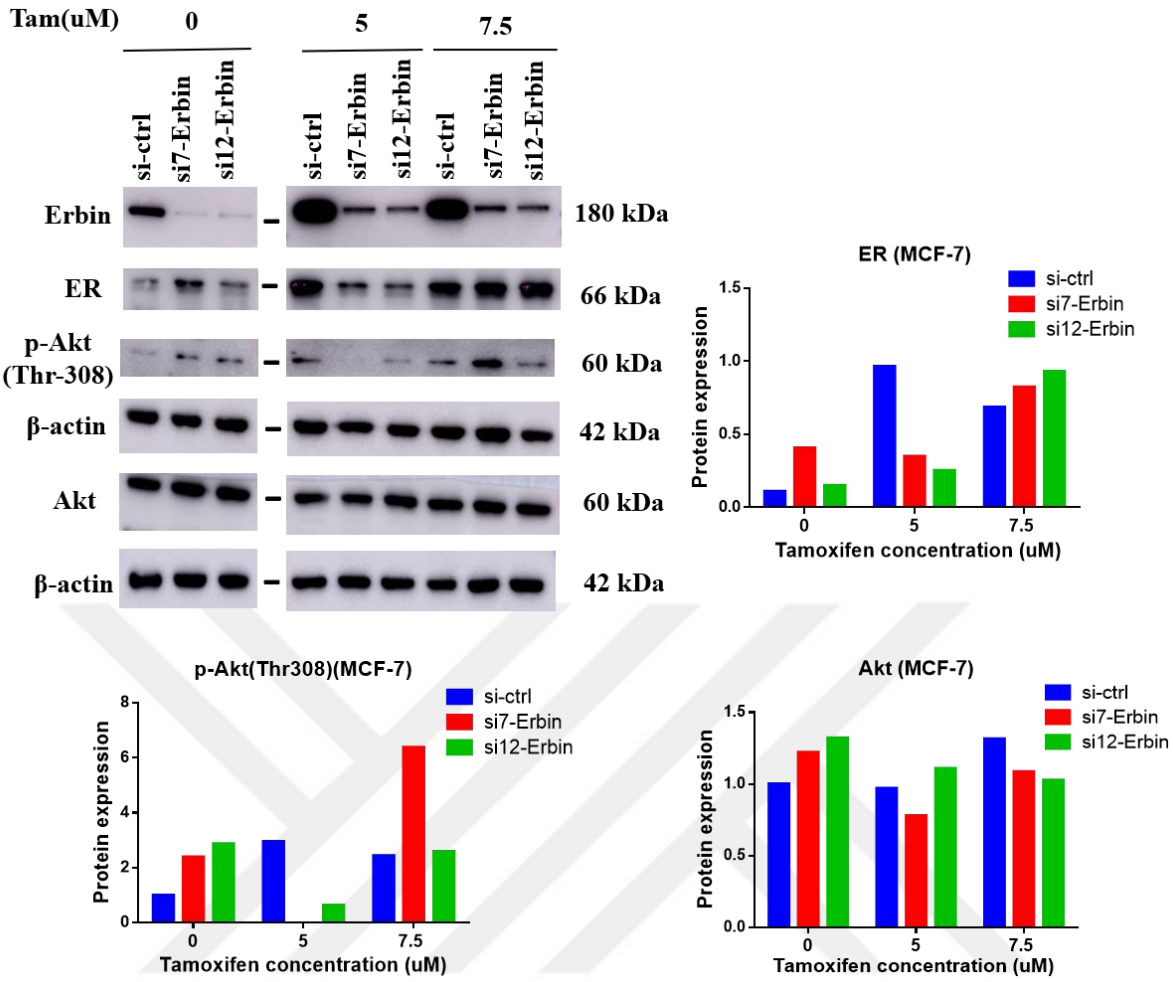


Figure 3.14. Downregulation of Erbin affects ER expression and the PI3K pathway. Protein expression of Akt proteins was performed in Erbin downregulated and tamoxifen treated MCF-7 WT cells by Western blot analysis. Western blot quantifications were normalized based on Actin levels by ImageJ. Dashes shows the different gels that were performed with the same system at the same time.

3.6. REDUCED LEVEL OF ERBIN AFFECTS CELL VIABILITY AGAINST DRUG TREATMENT

We elucidated that reduced level of Erbin altered regulators of apoptosis, cell cycle, DNA damage response and PI3K pathways that are responsible for cell survival and proliferation. As a next step, we performed crystal violet assays in order to demonstrate whether the downregulation of Erbin affects viability of the cells when they are treated with doxorubicin or tamoxifen. Erbin was downregulated with transient transfection in MDA-MB-231 and MCF-7 cells as previously. After 24 hours, MDA-MB-231 cells were treated with doxorubicin and MCF-7 cells were treated with tamoxifen for 72 hours. Then, crystal violet assay was examined.

3.6.1. Silencing of Erbin Increases Cell Viability Against Doxorubicin Treatment

MDA-MB-231 cells were treated with 0.1 uM and 0.5 uM doxorubicin for 72 hours. In order to check whether si-RNA transfection worked, protein expression of Erbin was demonstrated with western blot. Erbin was silenced both doxorubicin treated and untreated cells (**Figure 3.15B**). As a result of the crystal violet assay, the cell viability of Erbin silenced MDA-MB-231 cells was higher than control cells with doxorubicin treatment (**Figure 3.15A**). The difference between Erbin silenced and si-ctrl cells was significant for si7-Erbin and it was close to significant for si12-Erbin ($p<0.05$) (**Figure 3.15C**).

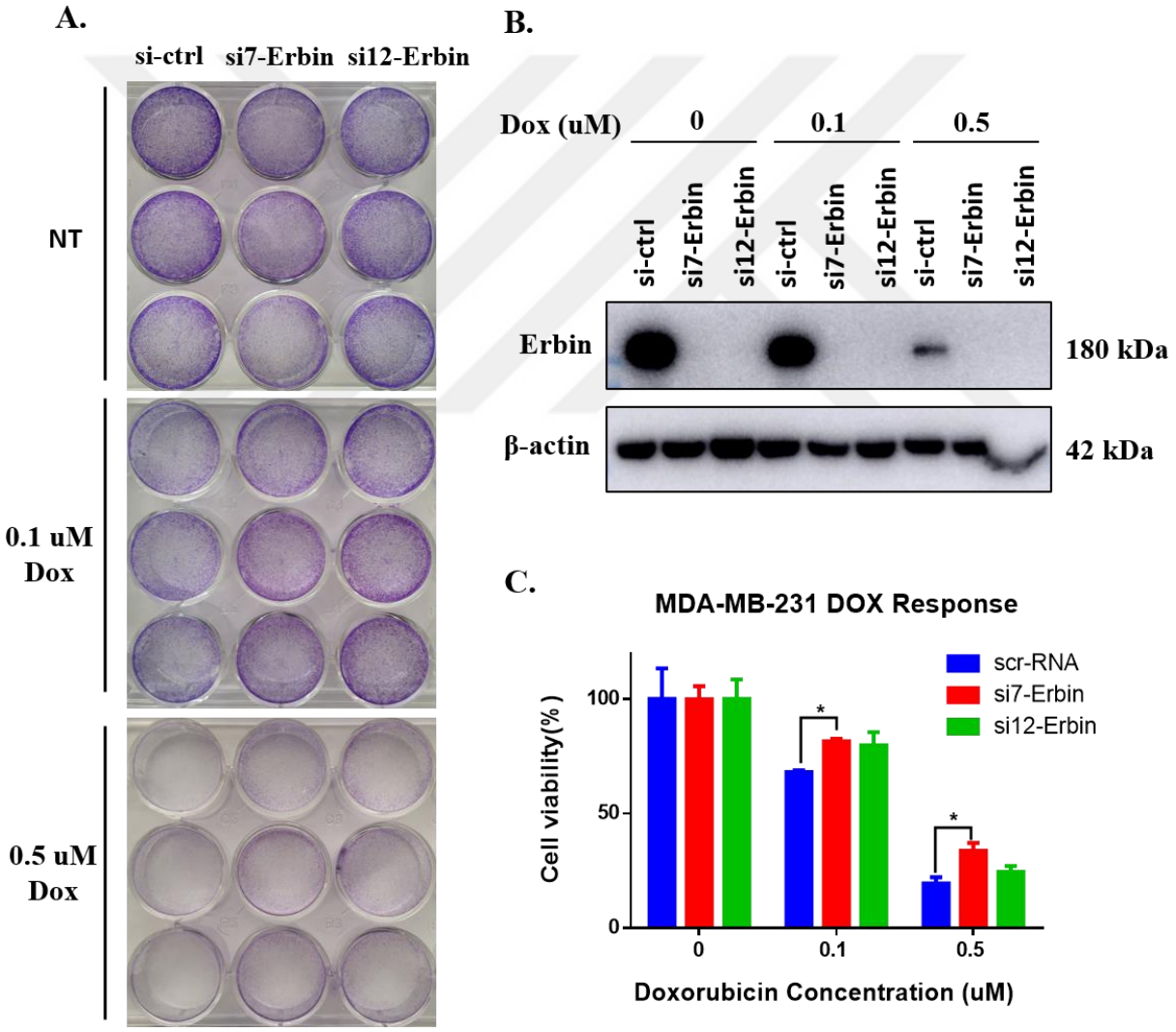


Figure 3.15. Crystal violet results of Erbin silenced and doxorubicin treated cells. MDA-MB-231 WT cells were transfected with either si-ctrl or si-RNAs. Then, they were treated with 0.1 uM and 0.5 uM of doxorubicin in triplicates with 12 well plates. Crystal violet assay was performed after 3 days of doxorubicin treatment. **(A)** The images of doxorubicin treated cells after crystal violet staining are

shown. 12 wells were triplicates vertically. **(B)**In order to show silencing of Erbin**,** western blot analysis was done**. (C)** Doxorubicin response was shown by viability graph. Analysis was done by 2way ANOVA (Sidak's multiple comparisons test) (*p<0.05)

We can conclude that reduced Erbin expression caused alteration of pathways that regulate cell survival and cell proliferation. As a result of these alterations, when Erbin expression was reduced, MDA-MB-231 cells that are TNBC did not respond to doxorubicin treatment as much as control cells. They attempted to behave like doxorubicin resistant cells.

3.6.2. Downregulation of Erbin Does Not Affect Cell Viability Against Tamoxifen

MCF-7 cells were treated with 5 uM and 7.5 uM tamoxifen for 72 hours. Protein expression of Erbin was shown with western blot to check whether si-RNA transfection was done successfully. Protein expression of Erbin was downregulated in both tamoxifen treated and untreated cells (**Figure 3.16B**).

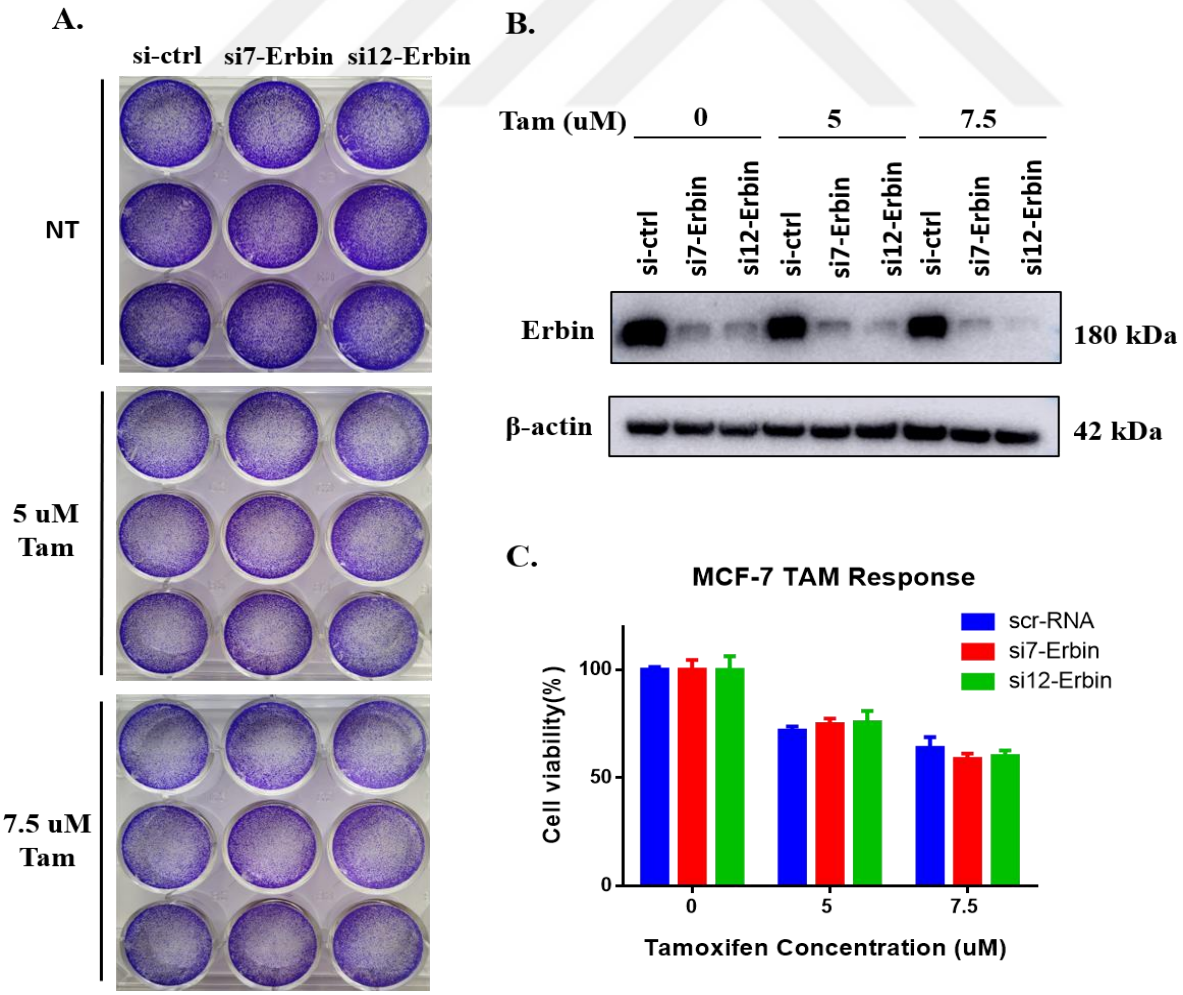


Figure 3.16. Crystal violet results of Erbin downregulated and tamoxifen treated cells. MCF-7 WT cells were transfected with either si-ctrl or si-RNAs. Then, they were treated with 5 uM and 7.5 uM of tamoxifen in triplicates with 12 well plates. Crystal violet assay was performed after 3 days tamoxifen of treatment. **(A)** The images of tamoxifen treated cells after crystal violet staining are shown. 12 wells were triplicates vertically. **(B)** In order to show silencing of Erbin**,** western blot analysis was done**. (C)** Tamoxifen response was shown by viability graph. Analysis was done by 2way ANOVA (Sidak's multiple comparisons test)

As a result of crystal violet assay, cell viability of MCF-7 cells was almost the same with tamoxifen treatment (**Figure 3.16A**). The difference between Erbin silenced and si-ctrl cells was not significant (**Figure 3.16 C**). Previously, we demonstrated that the downregulation of Erbin in MCF-7 cells changed the protein levels of downstream elements such as caspases, Cdk2 and p-Rb that trigger cell proliferation and survival but did not directly affect cell viability with tamoxifen treatment.

3.7. *IN SILICO* ANALYSES OF DIFFERENTIALLY EXPRESSED GENES BASED ON LOW AND HIGH ERBIN EXPRESSION

After the effect of Erbin on doxorubicin and tamoxifen resistance was investigated with *in vitro* studies, we supported the results with *in silico* analysis. Differential expression analyses of datasets including breast cancer patients that treated with chemotherapy or targeted therapies were performed. GSE16446 and GSE58644 datasets were analyzed. Analyses were done by Özgür Şahin's group. Previously, we found that high level of Erbin expression predicts better survival in GSE16446 and GSE58644 datasets including treated breast cancer patients (**Figure 3.1**).

GSE16446 dataset includes basal type of breast cancer patients that had anthracycline chemotherapy. The differentially expressed genes were grouped based on high and low Erbin expression. A Heatmap of these genes was generated. Heatmap analysis displayed that chemo-treated basal patients with high Erbin expression have higher levels of DNA damage, apoptosis and cell cycle arrest-related genes that are ATM, ATR, CASP7, BIM (BCL2L11), p27 (CDKN1B). In contrary, patients with low Erbin expression have higher levels of cyclins, CDKs, anti-apoptotic genes that are CDK2, CDK4, CDK6, CCND2, CCNE1, BCL2L1 (**Figure 3.17A**)

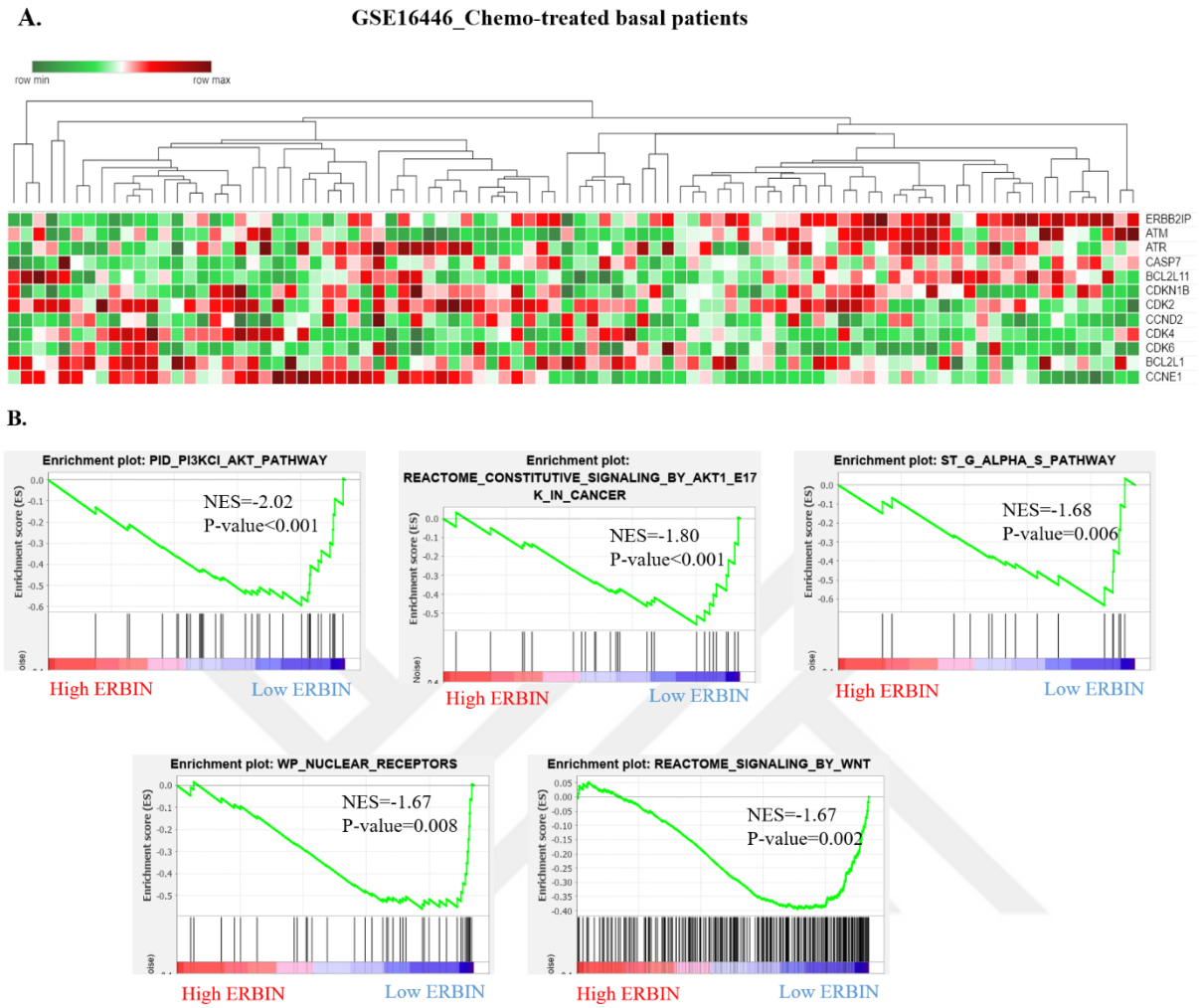


Figure 3.17. Differential expression analyses of GSE16446 dataset. This dataset including basal type breast cancer patients were treated with anthracyclines. **(A)** Heatmap analyses of differentially expressed genes according to the expression level of Erbin were done. **(B)** Enrichment plots of pathways associated with differential Erbin expression are shown. *(This analysis was done by Özgür Şahin's group)*

In addition to the heatmap analysis, enrichment plots of the pathways associated with differential Erbin expression were also created. Results showed that genes related to PI3K/AKT, WNT and G1/S transition are enriched in patients with low Erbin expression (**Figure 3.17B**). These analyses supported our findings about the effect of low Erbin expression against doxorubicin resistance.

GSE58644 dataset contains patients having all subtypes of breast cancer. These patients were treated with tamoxifen, chemotherapy, trastuzumab or combined therapy. According to high

and low Erbin expression, the differentially expressed genes were grouped. Heatmap analysis of these genes was done. Results demonstrated that treated patients with high Erbin expression have higher levels of DNA damage and apoptosis-related genes that are ATM, ATR, BIM (BCL2L11) and ER. In contrast, breast cancer patients with low Erbin expression have higher levels of cyclins (**Figure 3.18**). In conclusion, these findings further corroborate the function of Erbin in the development of anti-cancer drug resistance in breast cancer.

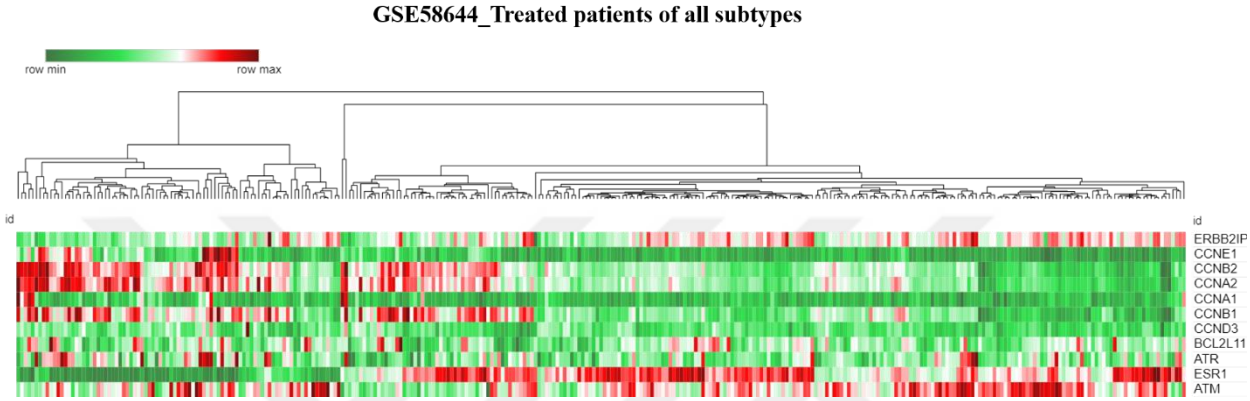


Figure 3.18 Differential expression analyses of GSE58644 dataset. Patients having all subtypes of breast cancer were treated with chemotherapy, tamoxifen, trastuzumab or combined therapy. Heatmap analyses of differentially expressed genes according to the expression level of Erbin was done. *(This analysis was done by Özgür Şahin's group)*

3.8. OVEREXPRESSION OF ERBIN IN DRUG RESISTANT CELL LINES

Erbin gene was overexpressed in MDA-MB-231 DoxR and MCF-7 TamR cells with transient transfection of pRK5-myc-ERBIN plasmid. After transfection, half of the cells were died. Because drug resistant cells were too fragile, we tried the same procedure with MDA-MB-231 and MCF-7 WT cells but the death of the cells were too much again. However, the ratio of alive cells with pRK5-myc-ERBIN plasmid transfection was higher in WT cells compared to resistant cells. We checked the protein level of Erbin of alive cells with western blot analysis and observed that there is no significant between Erbin overexpressed and control cells. Also, transfection efficiency of drug resistant and WT cells were measured with using GFP plasmid and the efficiency of these cells was lower than 10%. For this reason, the protocole of pRK5-myc-ERBIN plasmid transfection should be optimized. The reason of unsuccessful transfection is caused by plasmid or the transfection method should be found.

CHAPTER 4. DISCUSSION

Erbin is known as ERBB2 interacting protein. It is a novel protein that interacts with numerous different proteins and regulates many signaling pathways such as MAPK and TGF-β [55-57]. Erbin has different roles in different cancer types. It behaves as both an oncogenic and a tumor suppressor protein. Also, the role of Erbin in human breast cancer is controversial. Some studies stated that Erbin increases the ERBB2 dependent cell proliferation and tumorigenesis in breast cancer [77, 78]. In contrast, Liu et al claimed that Erbin is downregulated in ERBB2 overexpressing breast cancer cells and it inhibits heregulin-induced AKT phosphorylation in these cells. They proved that knockdown of Erbin leads to trastuzumab resistance in breast cancer cells [76]. As a result, neither the function of Erbin plays role in tumorigenesis nor the regulating mechanism is unclear. Further experiments are needed in order to understand the complete role of Erbin in breast cancer.

We started our research with investigating the level of Erbin in different breast cancer patient datasets. These datasets consist of patients treated with chemotherapy or endocrine therapy and untreated patients that had all subtypes of breast cancer. Survival analyses showed that a high level of Erbin expression predicts better survival in treated patients, whereas the level of Erbin does not affect the survival rates of untreated patients (**Figure 3.1**). As a result of these survival analyses of breast cancer patients based on expression the level of Erbin, we hypothesized that Erbin can have an important role in drug resistance in breast cancer. Besides, there are some studies about the association of Erbin with drug resistance in different cancer types. Erbin promotes sensitization of gemcitabine that is a chemotherapeutic drug in pancreatic ductal carcinoma by inhibiting MAPK signals [100]. Also, the deficiency of Erbin enhances proliferation of the cervical cancer cells and causes resistance of the cells to anoikis [72]. One study revealed that the knockdown of Erbin results in trastuzumab resistance in breast cancer. However, another study reported that Erbin provides tamoxifen resistance by causing degradation of ER in breast cancer [69].

After we hypothesized that Erbin can play a role in breast cancer, we examined the level of Erbin between various drug resistant and sensitive breast cancer cell lines. Drug resistant cells lines includes doxorubicin, tamoxifen or trastuzumab resistant cells. According to the results, protein expression of Erbin was lower in doxorubicin resistant MDA-MB-231 and tamoxifen

resistant MCF-7 cells compared to their sensitive counterparts (**Figure 3.2**). This finding indicated that reduced Erbin expression might cause doxorubicin resistance in MDA-MB-231 cells and tamoxifen resistance in MCF-7 cells.

There are studies about trastuzumab resistance and downregulation of Erbin gene. According to our *in silico* analysis, trastuzumab treated breast cancer patients were also affected by Erbin levels. However, we could not observe the protein level difference of Erbin between drug resistant and sensitive cells. Resistance of these cells should be checked with viability assays. Further analyses are needed for understand relation of Erbin gene and trastuzumab resistance.

4.1. ERBIN AND DOXORUBICIN RESISTANCE

TNBC has poor prognosis due to its lack of specific targets, aggressiveness and metastatic nature. TNBC patients are commonly treated with chemotherapy. Anthracyclines that are generally combinations of doxorubicin and taxanes are the most commonly utilized drugs for chemotherapy [25]. However, resistance to the chemotherapy is frequently seen in breast cancer patients.

Doxorubicin blocks cancer cell proliferation by creating double strand breaks in the DNA and preventing DNA synthesis [27-29]. Changes in the DNA damage repair and apoptosis pathway can promote chemotherapeutic resistance but the exact reason of doxorubicin resistance is unknown [31]. Downregulation of Erbin in breast cancer might be one of the reasons for doxorubicin resistance. As a next step, we investigated altered mechanisms with doxorubicin resistance by comparing protein levels of apoptosis and cell cycle proteins between doxorubicin resistant and sensitive triple negative MDA-MB-231 cells. Downregulation of pro-apoptotic Bax and Caspase-9 as well as upregulation of anti-apoptotic Bcl-2 and Bcl-xL proteins were examined in doxorubicin resistant cells (**Figure 3.7**). Studies have claimed that doxorubicin leads to a reverse effect, it downregulates Bcl-2 and upregulates Caspase-9 and Caspase-3 [28]. It is a sign of doxorubicin resistance. Also, decrease of Cdk2 and p-Rb proteins in DoxR cells indicates that accumulation of these proteins in G1/S phase with doxorubicin treatment was reduced and DoxR cells continued their cell proliferation mechanism unlike WT cells. These results displayed that doxorubicin resistance alters the apoptosis mechanism of MDA-MB-231 doxorubicin resistant cells.

Because we claimed that Erbin downregulation could cause doxorubicin resistance, the Erbin gene was silenced with transient si-RNA transfection. It was investigated how Erbin downregulation affects the cells and different pathway mechanisms with doxorubicin treatment. We indicated that doxorubicin resistance changed the apoptosis pathway. Therefore, downstream elements of intrinsic pathway were examined when Erbin was silenced in MDA-MB-231 cells. The protein expression of Bcl-2 increased and Caspase-9 decreased in Erbin silenced cells with doxorubicin treatment as in doxorubicin resistant cells. Besides, Caspase-3 was reduced in these cells (**Figure 3.9**). This finding elucidates how Erbin silencing leads to a reduction of apoptosis through the intrinsic pathway against doxorubicin treatment.

Apoptosis is also associated with cell proliferation. Hence, the level of Rb and Cdk2 were checked to find out whether Erbin silencing has an effect on cell proliferation. p-Rb and Cdk2 proteins increased in Erbin silenced cells (**Figure 3.10**). Phosphorylation of Rb causes its inactivation and promotes CDK activity [89]. It was also reported that inactivation of the Rb pathway resuls in resistance to anthracycline therapy in breast cancer patients [101]. Cdk2 has a role in G1-S transition and initiation of DNA synthesis [90]. This demonstrates that reduced Erbin expression increases cell proliferation and viability against doxorubicin treatment.

It is known that doxorubicin leads to dsDNA breaks and it results in DNA damage. When DNA is damaged, cell cycle checkpoint proteins and DNA repair regulators are activated. Resistance to topoisomerase inhibitors causes alterations in DNA repair machinery [31]. Phosphorylation of Chk1 results in cell cycle arrest in the G1/S phase. If Chk1 is inhibited by cytotoxic chemotherapy, it leads to inhibition of DNA repair and results in cell death [94]. Also, phosphorylation of Chk1 protein promotes radio-resistance in breast cancer [102,103]. Increase of p-Chk level in Erbin silenced cells with doxorubicin treatment has shown that DNA repair was achieved in cancer cells and they did not enter apoptosis. p21 protein also has a role in the DNA damage response pathway. Our results showed that p21 expression increased in Erbin silenced cells (**Figure 3.10**). It was shown that p21 leads to cell cycle arrest and prevents p-53 dependent apoptosis induced by doxorubicin [95]. We demonstrated that the silence of Erbin results in cell cycle arrest with doxorubicin treatment but it inhibits apoptosis.

The PI3K pathway promotes cell survival and proliferation. Akt is one of the downstream elements of these pathways. Activation of Akt affects intrinsic apoptosis pathway by activity of Bcl-2 and inhibition Bad and Bax [104]. Akt is activated by its phosphorylation on Thr-308

and Ser-473. The Ser-473 site is specifically phosphorylated by the mTORC2 complex specifically. Phosphorylation of Akt at Thr-308 and at Ser-473 activates the mTORC1 complex that promotes cell growth and proliferation [97, 98]. Phosphorylation of Akt on the Ser-473 site decreased but phosphorylation of Akt on the Thr-308 site increased in Erbin silenced cells (**Figure 3.11**). This means that the level of Erbin affects PI3K pathway upon mTORC1 activation. The MAPK pathway enhances Akt activity. Other studies have shown that Erbin inhibits MAPK activation by disrupting interaction between Ras and Raf kinases [59,60]. Silencing of Erbin can eliminate inhibiting the activation of Erbin on the MAPK pathway and it can result in overactivation of Akt despite doxorubicin treatment.

We showed that reduced Erbin level promotes regulators of cell proliferation and survival. As a next step, it was evaluated that whether the silencing of Erbin directly affects viability of the cells when they are treated with doxorubicin. Silencing of Erbin significantly increased the viability of MDA-MB-231 cells against doxorubicin treatment (**Figure 3.15**). It was revealed that silencing of Erbin leads to alteration in the intrinsic pathway, cell cycle regulators, DNA damage response pathway and PI3K pathway. These alterations promote cell survival and proliferation, and inhibit apoptosis. When Erbin is reduced, doxorubicin did not kill MDA-MB-231 cells effectively as much as control cells.

In addition to *in vitro* studies, the effect of downregulation of Erbin on doxorubicin resistance was supported with *in silico* analysis. Differential expression analyses of anthracycline chemotherapy treated basal type of breast cancer patients demonstrated that chemo-treated basal patients with high Erbin expression have higher levels of DNA damage, apoptosis and cell cycle arrest-related genes. However, patients with low Erbin expression have higher levels of cyclins, CDKs, anti-apoptotic genes (**Figure 3.17**). Besides, genes having a role in PI3K/AKT and G1/S transition are enriched in patients with low Erbin expression.

In conclusion, the expression level of Erbin has an essential role against doxorubicin resistance in TNBC. When Erbin expression was reduced, TNBC cells did not respond doxorubicin treatment and behaved like doxorubicin resistant cells. It is obvious that the alterations in the intrinsic pathway, cell cycle regulators, DNA damage response pathway and PI3K pathway through Erbin silencing trigger the doxorubicin resistance mechanism and inhibit apoptosis of TNBC cells against doxorubicin treatment. However, by which mechanism Erbin regulates the doxorubicin resistance is still unclear because Erbin regulates many signaling pathways and

Erbin silencing affects four different pathways. Further experiments are needed in order to explore the mechanism that Erbin is directly involved in and by which controls doxorubicin resistance.

4.2. ERBIN AND TAMOXIFEN RESISTANCE

ER+ patients compose the majority of the breast cancer patients. Although tamoxifen is the most prevalent treatment for the ER positive patients, tamoxifen resistance developes many breast cancers. The status of ER and its crosstalk with other growth factor pathways can promote the resistance of tamoxifen [38]. Besides, oncogenic signaling pathways including growth factor receptor tyrosine kinases, PI3K/AKT/mTOR pathway and Rb phosphorylation are associated with tamoxifen resistance [36, 39]. Because ER signaling is very complex, tamoxifen resistance is caused by many molecular mechanisms. Reasons of the resistance should be understood very well and alternative strategies should be found.

Downregulation of Erbin can have an effect on tamoxifen resistance in ER+ breast cancer. After we indicated that Erbin level was lower in tamoxifen resistant MCF-7 cells, we examined which mechanisms change with tamoxifen resistance by comparing protein levels of the downstream elements of apoptosis, cell cycle and PI3K pathways between tamoxifen resistant and sensitive MCF-7 cells. Tamoxifen enhances cytochrome c release. Apoptosis by tamoxifen is mediated by the intrinsic apoptosis pathway. It results in the downregulation of Bcl-2 and the upregulation of Bax and caspases [105, 106]. Downregulation of Caspase-9 protein with tamoxifen treatment was demonstrated in tamoxifen resistant cells because apoptosis is reduced. However, disappearance of protein expression of Bcl-2 and increase of Bax protein in tamoxifen resistant cells was unexpected (**Figure 3.8**). Interestingly, it was reported that Bcl-2 overexpression can sensitize cancer cells to anti-cancer drugs and related to improved survival in breast cancer [91, 92]. Also, cell cycle proteins p-Rb and Cdk2 were reduced in tamoxifen resistant cells as doxorubicin resistant cells. Because tamoxifen mainly affects ER expression and signaling, loss of ER is one of the reasons of tamoxifen resistance. It was displayed that ER expression decreased with tamoxifen treatment in tamoxifen resistant cells as expected. There is an important association between the PI3K/AKT/mTOR pathway and the ER pathway. PI3K signaling activates estrogen dependent ER signaling. ER also promotes the activation of downstream elements of the PI3K pathway [107]. Upregulation of Akt results in a poor

prognosis in breast cancer patients. In addition, gain of function of PI3K/AKT pathway causes tamoxifen resistance [39]. We showed that Akt increased and phosphorylation at Ser-473 site of Akt was overexpressed in tamoxifen resistant cells. Even if some results are inconsistent, it was indicated that tamoxifen resistance results in loss of ER expression and Akt overactivation, and alters the apoptosis mechanism in MCF-7 tamoxifen resistant cells.

After trying to explore the mechanism in tamoxifen resistant MCF-7 cells, the Erbin gene was downregulated with transient si-RNA transfection. We considered that if Erbin expression is downregulated in WT cells, they might behave as resistant cells. We displayed that the downregulation of Erbin affects different pathway mechanisms in ER+ MCF-7 cells with tamoxifen treatment. The changes in protein expressions were not expected results for the mitochondrial apoptosis pathway. Caspase-9 expression was reduced in Erbin downregulated cells but Bcl-2, Bcl-xL and Bax expression were inconsistent. We used two different si-RNAs for transfection and the expressions of the Bcl-2, Bcl-xL and Bax were different for these si-RNAs. Also, we showed that the expression of cleaved Caspase-7 was reduced but cleaved Caspase-3 was increased when Erbin was downregulated in MCF-7 cells (**Figure 3.12**). As a result, we could not apparently claim that that apoptosis through intrinsic pathway decreases in MCF-7 cells when Erbin expression was downregulated.

The expression of cell proliferation proteins Cdk2 and p-Rb was increased in Erbin downregulated cells with tamoxifen as with doxorubicin treatment (**Figure 3.13**). The function of Cdk2 and Rb proteins in the cell cycle mechanism was described previously. Also, it was reported that tamoxifen treatment results in dephosphorylation of Rb protein [105]. The increase of phosphorylation of Rb protein might be interpreted as Erbin downregulated cells not responding to tamoxifen as much as MCF-7 WT cells and with an increase of proliferation in these cells. Also, p-gamma-H2AX on serine 139 was reduced in Erbin downregulated cells. It means that DNA damage after tamoxifen treatment was decreased in these cells.

We obtained a dramatic decrease of the ER level in tamoxifen resistant cells. It was also observed that ER expression was reduced in Erbin downregulated cells with 5 uM tamoxifen treatment but the expression increased with 7.5 uM tamoxifen (**Figure 3.14**). The reason can be that a high concentration of tamoxifen activates non-ER pathways to initiate apoptosis [106]. For this reason, 7.5 uM tamoxifen concentration might not affect ER expression directly. Because the PI3K-Akt pathway is one of the key pathways that influences the tamoxifen mechanism, the expression of Akt was demonstrated. The phosphorylation of Akt was observed

at the Thr-308 site. p-Akt increased in Erbin downregulated cells with 7.5 uM tamoxifen treatment. Interestingly, phosphorylation at Ser-473 site of Akt was overexpressed in tamoxifen resistant cells. The phosphorylation site was altered with Erbin downregulation. *Huang et al.* stated that depletion of Erbin enhances PI3K signaling and results in reduced p21 expression. It promotes cell proliferation of cervical cancer cells [73]. We also showed that p21 expression decreased in Erbin downregulated cells. It can be inferred that the downregulation of Erbin over activate PI3K and ER signaling somehow. It results in inhibition of apoptosis and increase of cell proliferation against tamoxifen treatment.

For the last part of our *in vitro* studies, a viability assay was conducted to understand whether the downregulation of Erbin directly increases the viability of the cells when they are treated with tamoxifen. However, we could not observe a significant difference between Erbin downregulated and control cells for cell viability with tamoxifen treatment (**Figure 3.16**). Downregulation of Erbin does not directly affect cell viability against tamoxifen treatment. On the contrary, it is apparent that downregulation of Erbin affect the cell cycle and PI3K pathway promoting cell proliferation. This might stem from the transient effect of Erbin downregulation. If we downregulate Erbin permanently, we might observe direct increase of cell viability because drug resistance occurs over time.

As a last step, differential expression analyses of breast cancer patients were performed *in silico* to corroborate our *in vitro* studies. Differential expression analyses of breast cancer patients of all subtypes that were treated with tamoxifen, chemotherapy, trastuzumab or combined therapy indicated that patients with high Erbin expression have higher levels of DNA damage and apoptosis-related genes, whereas patients with low Erbin expression have higher levels of cyclins (**Figure 3.18**). *In silico* analyses support the function of Erbin in the development of anti-cancer drug resistance in breast cancer. According to the result, the effect of Erbin on anti-drug resistance might not be drug specific because the dataset also includes trastuzumab treated patients. Ultimately, the mechanism of these drugs causes inhibition of cell proliferation and apoptosis of cancer cells, Erbin might directly affect one of the pathways that we investigated. In order to find the exact mechanism, further experiments are required.

CHAPTER 5. FURTHER PERSPECTIVES

Our studies revealed that the Erbin gene could have a role against doxorubicin and tamoxifen resistance. Further analysis with Annexin V/PI staining could be performed to provide more insightful information for intact cells, early and late apoptotic cells upon ERBIN downregulation. The flow cytometry analysis can be performed in order to understand in what stage of the cell cycle will be affected by ERBIN downregulation or upregulation in doxorubicin or tamoxifen treated cells.

Regulators of apoptosis, cell cycle, DNA repair and PI3K pathways that are related with cell survival were examined. Downregulation of Erbin resulted in increase of cell proliferation regulators and reduced expression of apoptotic markers. In order to understand the possible roles of Erbin, other elements of these pathways can be investigated in ERBIN downregulated and also upregulated cells. The expression of cell cycle regulators such as cyclins, p53 and ATM/ATR for DNA damage response and mTORCs for PI3K pathway can also be analyzed . ERBIN localization could be examined with ERBIN specific antibody by immunofluorescence in control, ERBIN up- or -downregulated cells with and without drug treatment. In addition, apoptotic and antipoptotic markers might also be examined with immunofluorescence microscopy in Erbin down- or upregualted cells. Besides, co-immunoprecipitation assays can be performed to find out the exact protein interactions for Erbin with doxorubicin and tamoxifen treatment. In this way, it can be explored by which mechanism Erbin affects the drug resistance.

If the stable overexpression of Erbin gene in doxorubicin and tamoxifen resistant cells can be achieved, the same experiments could be performed to understand the possible roles of ERBIN in drug resistance. ERBIN overexpression could effect the cell growth in a very early stage before the cell analysis experiments are performed. The best way to overcome this problem is to express ERBIN in a controlable manner, such as using tetracyclin regulated gene expression vectors. Then its effect on the downstream elements of apoptosis, cell cycle, DNA damage and PI3K pathways could be examined more efficiently. It could be expected that the expressions of apoptotic markers will increase and proteins promoting cell survival and proliferation will decrease with overexpression of Erbin in drug resistant cells. Also, cell viability assays can be performed with these cells. If resistant cells are sensitized against doxorubicin and tamoxifen with overexpression of Erbin, this can also corroborate the notion that decrease of Erbin expression promotes doxorubicin and tamoxifen resistance in breast cancer.

REFERENCES

1. Michor, F., Iwasa, Y., & Nowak, M. A. (2004). Dynamics of cancer progression. *Nature reviews. Cancer*, *4*(3), 197–205. https://doi.org/10.1038/nrc1295
2. Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70. https://doi.org/10.1016/s0092-8674(00)81683-9
3. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi:10.1016/j.cell.2011.02.013
4. Mathur, P., Sathishkumar, K., Chaturvedi, M., Das, P., Sudarshan, K. L., Santhappan, S., Nallasamy, V., John, A., Narasimhan, S., Roselind, F. S., & ICMR-NCDIR-NCRP Investigator Group (2020). Cancer Statistics, 2020: Report From National Cancer Registry Programme, India. *JCO global oncology*, *6*, 1063–1075. https://doi.org/10.1200/GO.20.00122 (2020).
5. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, 10.3322/caac.21660. Advance online publication. https://doi.org/10.3322/caac.21660
6. Polyak K. (2011). Heterogeneity in breast cancer. *The Journal of clinical investigation*, *121*(10), 3786–3788. https://doi.org/10.1172/JCI60534
7. Harbeck, N., & Gnant, M. (2017). Breast cancer. *Lancet (London, England)*, *389*(10074), 1134–1150. https://doi.org/10.1016/S0140-6736(16)31891-8
8. Colombo, P. E., Milanezi, F., Weigelt, B., & Reis-Filho, J. S. (2011). Microarrays in the 2010s: the contribution of microarray-based gene expression profiling to breast cancer classification, prognostication and prediction. *Breast cancer research: BCR*, *13*(3), 212. https://doi.org/10.1186/bcr2890
9. Holliday, D. L., & Speirs, V. (2011). Choosing the right cell line for breast cancer research. *Breast cancer research: BCR*, *13*(4), 215. https://doi.org/10.1186/bcr2889
10. Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A. L., Brown, P. O., & Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752. https://doi.org/10.1038/35021093

11. Blows, F. M., Driver, K. E., Schmidt, M. K., Broeks, A., van Leeuwen, F. E., Wesseling, J., Cheang, M. C., Gelmon, K., Nielsen, T. O., Blomqvist, C., Heikkilä, P., Heikkinen, T., Nevanlinna, H., Akslen, L. A., Bégin, L. R., Foulkes, W. D., Couch, F. J., Wang, X., Cafourek, V., Olson, J. E., … Huntsman, D. (2010). Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLoS medicine*, *7*(5), e1000279. https://doi.org/10.1371/journal.pmed.1000279
12. Loi, S., Haibe-Kains, B., Desmedt, C., Wirapati, P., Lallemand, F., Tutt, A. M., Gillet, C., Ellis, P., Ryder, K., Reid, J. F., Daidone, M. G., Pierotti, M. A., Berns, E. M., Jansen, M. P., Foekens, J. A., Delorenzi, M., Bontempi, G., Piccart, M. J., & Sotiriou, C. (2008). Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. *BMC genomics*, *9*, 239. https://doi.org/10.1186/1471-2164-9-239
13. Oh, D. S., Troester, M. A., Usary, J., Hu, Z., He, X., Fan, C., Wu, J., Carey, L. A., & Perou, C. M. (2006). Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. *Journal of clinical oncolog: official journal of the American Society of Clinical Oncology*, *24*(11), 1656–1664. https://doi.org/10.1200/JCO.2005.03.2755
14. Symmans, W. F., Hatzis, C., Sotiriou, C., Andre, F., Peintinger, F., Regitnig, P., Daxenbichler, G., Desmedt, C., Domont, J., Marth, C., Delaloge, S., Bauernhofer, T., Valero, V., Booser, D. J., Hortobagyi, G. N., & Pusztai, L. (2010). Genomic index of sensitivity to endocrine therapy for breast cancer. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, *28*(27), 4111–4119. https://doi.org/10.1200/JCO.2010.28.4273
15. Mitri, Z., Constantine, T., & O'Regan, R. (2012). The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemotherapy research and practice*, *2012*, 743193. https://doi.org/10.1155/2012/743193
16. Li, H., Shao, B., Yan, Y., Song, G., Liu, X., Wang, J., & Liang, X. (2016). Efficacy and safety of trastuzumab combined with chemotherapy for first-line treatment and beyond progression of HER2-overexpressing advanced breast cancer. *Chinese journal of cancer research = Chung-kuo yen cheng yen chiu*, *28*(3), 330–338. https://doi.org/10.21147/j.issn.1000-9604.2016.03.07
17. Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., Lickley, L. A., Rawlinson, E., Sun, P., & Narod, S. A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical cancer research: an official journal of the*

American Association for Cancer Research, *13*(15 Pt 1), 4429–4434. https://doi.org/10.1158/1078-0432.CCR-06-3045

18. Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation*, *121*(7), 2750–2767. https://doi.org/10.1172/JCI45014
19. Foulkes, W. D., Smith, I. E., & Reis-Filho, J. S. (2010). Triple-negative breast cancer. *The New England journal of medicine*, *363*(20), 1938–1948. https://doi.org/10.1056/NEJMra1001389
20. Marmé, F., & Schneeweiss, A. (2015). Targeted Therapies in Triple-Negative Breast Cancer. *Breast care (Basel, Switzerland)*, *10*(3), 159–166. https://doi.org/10.1159/000433622
21. Longley, D. B., & Johnston, P. G. (2005). Molecular mechanisms of drug resistance. *The Journal of pathology*, *205*(2), 275–292. https://doi.org/10.1002/path.1706
22. Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., & Sarkar, S. (2014). Drug resistance in cancer: an overview. *Cancers*, *6*(3), 1769–1792. https://doi.org/10.3390/cancers6031769
23. Cree, I. A., & Charlton, P. (2017). Molecular chess? Hallmarks of anti-cancer drug resistance. *BMC cancer*, *17*(1), 10. https://doi.org/10.1186/s12885-016-2999-1
24. Gilbert, L. A., & Hemann, M. T. (2011). Chemotherapeutic resistance: surviving stressful situations. *Cancer research*, *71*(15), 5062–5066. https://doi.org/10.1158/0008-5472.CAN-11-0277
25. Tormo, E., Ballester, S., Adam-Artigues, A., Burgués, O., Alonso, E., Bermejo, B., Menéndez, S., Zazo, S., Madoz-Gúrpide, J., Rovira, A., Albanell, J., Rojo, F., Lluch, A., & Eroles, P. (2019). The miRNA-449 family mediates doxorubicin resistance in triple-negative breast cancer by regulating cell cycle factors. *Scientific reports*, *9*(1), 5316. https://doi.org/10.1038/s41598-019-41472-y
26. Rivankar S. (2014). An overview of doxorubicin formulations in cancer therapy. *Journal of cancer research and therapeutics*, *10*(4), 853–858. https://doi.org/10.4103/0973-1482.139267
27. Meredith, A. M., & Dass, C. R. (2016). Increasing role of the cancer chemotherapeutic doxorubicin in cellular metabolism. *The Journal of pharmacy and pharmacology*, *68*(6), 729–741. https://doi.org/10.1111/jphp.12539

28. Tacar, O., Sriamornsak, P., & Dass, C. R. (2013). Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *The Journal of pharmacy and pharmacology*, *65*(2), 157–170. https://doi.org/10.1111/j.2042-7158.2012.01567.x
29. El-Agamy, S. E., Abdel-Aziz, A. K., Esmat, A., & Azab, S. S. (2019). Chemotherapy and cognition: comprehensive review on doxorubicin-induced chemobrain. *Cancer chemotherapy and pharmacology*, *84*(1), 1–14. https://doi.org/10.1007/s00280-019-03827-0
30. Di, X., Shiu, R. P., Newsham, I. F., & Gewirtz, D. A. (2009). Apoptosis, autophagy, accelerated senescence and reactive oxygen in the response of human breast tumor cells to adriamycin. *Biochemical pharmacology*, *77*(7), 1139–1150. https://doi.org/10.1016/j.bcp.2008.12.016
31. Rebucci, M., & Michiels, C. (2013). Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical pharmacology*, *85*(9), 1219–1226. https://doi.org/10.1016/j.bcp.2013.02.017
32. Son, Y. S., Suh, J. M., Ahn, S. H., Kim, J. C., Yi, J. Y., Hur, K. C., Hong, W. S., Muller, M. T., & Chung, I. K. (1998). Reduced activity of topoisomerase II in an Adriamycin-resistant human stomach-adenocarcinoma cell line. *Cancer chemotherapy and pharmacology*, *41*(5), 353–360. https://doi.org/10.1007/s002800050751
33. Ponnusamy, L., Mahalingaiah, P., & Singh, K. P. (2017). Treatment schedule and estrogen receptor-status influence acquisition of doxorubicin resistance in breast cancer cells. *European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences*, *104*, 424–433. https://doi.org/10.1016/j.ejps.2017.04.020
34. Schiff, R., Massarweh, S. A., Shou, J., Bharwani, L., Arpino, G., Rimawi, M., & Osborne, C. K. (2005). Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. *Cancer chemotherapy and pharmacology*, *56 Suppl 1*, 10–20. https://doi.org/10.1007/s00280-005-0108-2
35. Ring, A., & Dowsett, M. (2004). Mechanisms of tamoxifen resistance. *Endocrine-related cancer*, *11*(4), 643–658. https://doi.org/10.1677/erc.1.00776
36. Miller T. W. (2013). Endocrine resistance: what do we know?. *American Society of Clinical Oncology educational book. American Society of Clinical Oncology. Annual Meeting*, 10.1200/EdBook_AM.2013.33.e37. https://doi.org/10.1200/EdBook_AM.2013.33.e37

37. Fan, W., Chang, J., & Fu, P. (2015). Endocrine therapy resistance in breast cancer: current status, possible mechanisms and overcoming strategies. *Future medicinal chemistry*, *7*(12), 1511–1519. https://doi.org/10.4155/fmc.15.93
38. Tang, Y., Wang, Y., Kiani, M. F., & Wang, B. (2016). Classification, Treatment Strategy, and Associated Drug Resistance in Breast Cancer. *Clinical breast cancer*, *16*(5), 335–343. https://doi.org/10.1016/j.clbc.2016.05.012
39. Araki, K., & Miyoshi, Y. (2018). Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer. *Breast cancer (Tokyo, Japan)*, *25*(4), 392–401. https://doi.org/10.1007/s12282-017-0812-x
40. Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell*, *116*(2), 205–219. https://doi.org/10.1016/s0092-8674(04)00046-7
41. Ghavami, S., Hashemi, M., Ande, S. R., Yeganeh, B., Xiao, W., Eshraghi, M., Bus, C. J., Kadkhoda, K., Wiechec, E., Halayko, A. J., & Los, M. (2009). Apoptosis and cancer: mutations within caspase genes. *Journal of medical genetics*, *46*(8), 497–510. https://doi.org/10.1136/jmg.2009.066944
42. Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature reviews. Cancer*, *2*(4), 277–288. https://doi.org/10.1038/nrc776
43. Letai, A. (2017). Apoptosis and Cancer. *Annual Review of Cancer Biology, 1*(1), 275-294. doi:10.1146/annurev-cancerbio-050216-121933
44. Pfeffer, C. M., & Singh, A. (2018). Apoptosis: A Target for Anticancer Therapy. *International journal of molecular sciences*, *19*(2), 448. https://doi.org/10.3390/ijms19020448
45. Lowe, S. W., & Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis, 21*(3), 485-495. doi:10.1093/carcin/21.3.485
46. Adrain, C., Creagh, E. M., & Martin, S. J. (2001). Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *The EMBO journal*, *20*(23), 6627–6636. https://doi.org/10.1093/emboj/20.23.6627
47. Huang, D. C., & Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. *Cell*, *103*(6), 839–842. https://doi.org/10.1016/s0092-8674(00)00187-2
48. Carneiro, B. A., & El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. *Nature reviews. Clinical oncology*, *17*(7), 395–417. https://doi.org/10.1038/s41571-020-0341-y
49. Campos, L., Rouault, J. P., Sabido, O., Oriol, P., Roubi, N., Vasselon, C., Archimbaud, E., Magaud, J. P., & Guyotat, D. (1993). High expression of bcl-2 protein in acute myeloid

leukemia cells is associated with poor response to chemotherapy. *Blood*, *81*(11), 3091–3096.

50. Findley, H. W., Gu, L., Yeager, A. M., & Zhou, M. (1997). Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. *Blood*, *89*(8), 2986–2993.
51. Hermine, O., Haioun, C., Lepage, E., d'Agay, M. F., Briere, J., Lavignac, C., Fillet, G., Salles, G., Marolleau, J. P., Diebold, J., Reyas, F., & Gaulard, P. (1996). Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Etude des Lymphomes de l'Adulte (GELA). *Blood*, *87*(1), 265–272.
52. Huang, Y. Z., Wang, Q., Xiong, W. C., & Mei, L. (2001). Erbin is a protein concentrated at postsynaptic membranes that interacts with PSD-95. *The Journal of biological chemistry*, *276*(22), 19318–19326. https://doi.org/10.1074/jbc.M100494200
53. Jaulin-Bastard, F., Saito, H., Le Bivic, A., Ollendorff, V., Marchetto, S., Birnbaum, D., & Borg, J. P. (2001). The ERBB2/HER2 receptor differentially interacts with ERBIN and PICK1 PSD-95/DLG/ZO-1 domain proteins. *The Journal of biological chemistry*, *276*(18), 15256–15263. https://doi.org/10.1074/jbc.M010032200
54. Borg, J. P., Marchetto, S., Le Bivic, A., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adélaïde, J., Margolis, B., & Birnbaum, D. (2000). ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nature cell biology*, *2*(7), 407–414. https://doi.org/10.1038/35017038
55. Ress, A., & Moelling, K. (2006). Interaction partners of the PDZ domain of erbin. *Protein and peptide letters*, *13*(9), 877–881. https://doi.org/10.2174/092986606778256126
56. Kolch W. (2003). Erbin: sorting out ErbB2 receptors or giving Ras a break?. *Science's STKE: signal transduction knowledge environment*, *2003*(199), pe37. https://doi.org/10.1126/stke.2003.199.pe37
57. Jang, H., Stevens, P., Gao, T., & Galperin, E. (2021). The leucine-rich repeat signaling scaffolds Shoc2 and Erbin: cellular mechanism and role in disease. *The FEBS Journal, 288*(3), 721-739. doi:https://doi.org/10.1111/febs.15450
58. Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research, 12*(1), 9-18. doi:10.1038/sj.cr.7290105
59. Huang, Y. Z., Zang, M., Xiong, W. C., Luo, Z., & Mei, L. (2003). Erbin suppresses the MAP kinase pathway. *The Journal of biological chemistry*, *278*(2), 1108–1114. https://doi.org/10.1074/jbc.M205413200

60. Dai, P., Xiong, W. C., & Mei, L. (2006). Erbin inhibits RAF activation by disrupting the sur-8-Ras-Raf complex. *The Journal of biological chemistry*, *281*(2), 927–933. https://doi.org/10.1074/jbc.M507360200
61. Weiss, A., & Attisano, L. (2013). The TGFbeta superfamily signaling pathway. *Wiley interdisciplinary reviews. Developmental biology*, *2*(1), 47–63. https://doi.org/10.1002/wdev.86
62. Dai, F., Chang, C., Lin, X., Dai, P., Mei, L., & Feng, X. H. (2007). Erbin inhibits transforming growth factor beta signaling through a novel Smad-interacting domain. *Molecular and cellular biology*, *27*(17), 6183–6194. https://doi.org/10.1128/MCB.00132-07
63. Déliot, N., Chavent, M., Nourry, C., Lécine, P., Arnaud, C., Hermant, A., . . . Borg, J.-P. (2009). Biochemical studies and molecular dynamics simulations of Smad3–Erbin interaction identify a non-classical Erbin PDZ binding. *Biochemical and Biophysical Research Communications, 378*(3), 360-365. doi:https://doi.org/10.1016/j.bbrc.2008.10.175
64. Sflomos, G., Kostaras, E., Panopoulou, E., Pappas, N., Kyrkou, A., Politou, A. S., Fotsis, T., & Murphy, C. (2011). ERBIN is a new SARA-interacting protein: competition between SARA and SMAD2 and SMAD3 for binding to ERBIN. *Journal of cell science, 124*(Pt 19), 3209–3222. https://doi.org/10.1242/jcs.062307
65. Zhou, Q., Zeng, R., Xu, C., Liu, L., Chen, L., Kou, P., Pei, G., Bai, S., Zhang, Y., Li, C., Rong, S., Han, M., & Xu, G. (2012). Erbin inhibits TGF-β1-induced EMT in renal tubular epithelial cells through an ERK-dependent pathway. *Journal of molecular medicine (Berlin, Germany)*, *90*(5), 563–574. https://doi.org/10.1007/s00109-011-0833-4
66. Zhou, Q. D., Ning, Y., Zeng, R., Chen, L., Kou, P., Xu, C. O., Pei, G. C., Han, M., & Xu, G. (2013). Erbin interacts with Sema4C and inhibits Sema4C-induced epithelial-mesenchymal transition in HK2 cells. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban*, *33*(5), 672–679. https://doi.org/10.1007/s11596-013-1179-7
67. Zhang, L., Wang, Y., Wang, L., Yin, G., Li, W., Xian, Y., . . . Liu, Q. (2018). miR-23c suppresses tumor growth of human hepatocellular carcinoma by attenuating ERBB2IP. *Biomedicine & Pharmacotherapy, 107*, 424-432. doi:https://doi.org/10.1016/j.biopha.2018.07.155

68. Murakami, H., Ito, S., Tanaka, H., Kondo, E., Kodera, Y., & Nakanishi, H. (2013). Establishment of new intraperitoneal paclitaxel-resistant gastric cancer cell lines and comprehensive gene expression analysis. *Anticancer research*, *33*(10), 4299–4307.
69. Wu, H., Yao, S., Zhang, S., Wang, J. R., Guo, P. D., Li, X. M., Gan, W. J., Mei, L., Gao, T. M., & Li, J. M. (2017). Elevated expression of Erbin destabilizes ERα protein and promotes tumorigenesis in hepatocellular carcinoma. *Journal of hepatology*, *66*(6), 1193–1204. https://doi.org/10.1016/j.jhep.2017.01.030
70. Xie, C. M., Wei, D., Zhao, L., Marchetto, S., Mei, L., Borg, J. P., & Sun, Y. (2015). Erbin is a novel substrate of the Sag-βTrCP E3 ligase that regulates KrasG12D-induced skin tumorigenesis. *The Journal of cell biology*, *209*(5), 721–737. https://doi.org/10.1083/jcb.201411104
71. Yao, S., Zheng, P., Wu, H., Song, L. M., Ying, X. F., Xing, C., Li, Y., Xiao, Z. Q., Zhou, X. N., Shen, T., Chen, L., Liu, Y. H., Lai, M. D., Mei, L., Gao, T. M., & Li, J. M. (2015). Erbin interacts with c-Cbl and promotes tumourigenesis and tumour growth in colorectal cancer by preventing c-Cbl-mediated ubiquitination and down-regulation of EGFR. *The Journal of pathology*, *236*(1), 65–77. https://doi.org/10.1002/path.4502
72. Hu, Y., Chen, H., Duan, C., Liu, D., Qian, L., Yang, Z., Guo, L., Song, L., Yu, M., Hu, M., Shi, M., & Guo, N. (2013). Deficiency of Erbin induces resistance of cervical cancer cells to anoikis in a STAT3-dependent manner. *Oncogenesis*, *2*(6), e52. https://doi.org/10.1038/oncsis.2013.18
73. Huang, H., Song, Y., Wu, Y., Guo, N., Ma, Y., & Qian, L. (2015). Erbin loss promotes cancer cell proliferation through feedback activation of Akt-Skp2-p27 signaling. *Biochemical and biophysical research communications*, *463*(3), 370–376. https://doi.org/10.1016/j.bbrc.2015.05.071
74. Stevens, P. D., Wen, Y. A., Xiong, X., Zaytseva, Y. Y., Li, A. T., Wang, C., Stevens, A. T., Farmer, T. N., Gan, T., Weiss, H. L., Inagaki, M., Marchetto, S., Borg, J. P., & Gao, T. (2018). Erbin Suppresses KSR1-Mediated RAS/RAF Signaling and Tumorigenesis in Colorectal Cancer. *Cancer research*, *78*(17), 4839–4852. https://doi.org/10.1158/0008-5472.CAN-17-3629
75. Zheng, Z., Zheng, X., Zhu, Y., Gu, X., Gu, W., Xie, X., Hu, W., & Jiang, J. (2019). miR-183-5p Inhibits Occurrence and Progression of Acute Myeloid Leukemia via Targeting Erbin. *Molecular therapy : the journal of the American Society of Gene Therapy*, *27*(3), 542–558. https://doi.org/10.1016/j.ymthe.2019.01.016

76. Liu, D., Shi, M., Duan, C., Chen, H., Hu, Y., Yang, Z., Duan, H., & Guo, N. (2013). Downregulation of Erbin in Her2-overexpressing breast cancer cells promotes cell migration and induces trastuzumab resistance. *Molecular immunology*, *56*(1-2), 104–112. https://doi.org/10.1016/j.molimm.2013.04.007
77. Tao, Y., Shen, C., Luo, S., Traoré, W., Marchetto, S., Santoni, M. J., Xu, L., Wu, B., Shi, C., Mei, J., Bates, R., Liu, X., Zhao, K., Xiong, W. C., Borg, J. P., & Mei, L. (2014). Role of Erbin in ErbB2-dependent breast tumor growth. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(42), E4429–E4438. https://doi.org/10.1073/pnas.1407139111
78. Mei, L., & Borg, J. P. (2015). ERBB2 oncogenicity: ERBIN helps to perform the job. *Molecular & cellular oncology*, *2*(3), e995033. https://doi.org/10.4161/23723556.2014.995033
79. Hatzis, C., Pusztai, L., Valero, V., Booser, D. J., Esserman, L., Lluch, A., Vidaurre, T., Holmes, F., Souchon, E., Wang, H., Martin, M., Cotrina, J., Gomez, H., Hubbard, R., Chacón, J. I., Ferrer-Lozano, J., Dyer, R., Buxton, M., Gong, Y., Wu, Y., … Symmans, W. F. (2011). A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *JAMA*, *305*(18), 1873–1881. https://doi.org/10.1001/jama.2011.593
80. Itoh M, Iwamoto T, Matsuoka J, Nogami T, Motoki T, Shien T, Taira N, Niikura N, Hayashi N, Ohtani S, Higaki K, Fujiwara T, Doihara H, Symmans WF, Pusztai L. Estrogen receptor (ER) mRNA expression and molecular subtype distribution in ER-negative/progesterone receptor-positive breast cancers. Breast Cancer Res Treat. 2014 Jan;143(2):403-9. doi: 10.1007/s10549-013-2763-z. Epub 2013 Dec 15. PMID: 24337596.
81. Esserman, L. J., Berry, D. A., Cheang, M. C., Yau, C., Perou, C. M., Carey, L., DeMichele, A., Gray, J. W., Conway-Dorsey, K., Lenburg, M. E., Buxton, M. B., Davis, S. E., van't Veer, L. J., Hudis, C., Chin, K., Wolf, D., Krontiras, H., Montgomery, L., Tripathy, D., Lehman, C., … I-SPY 1 TRIAL Investigators (2012). Chemotherapy response and recurrence-free survival in neoadjuvant breast cancer depends on biomarker profiles: results from the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657). *Breast cancer research and treatment*, *132*(3), 1049–1062. https://doi.org/10.1007/s10549-011-1895-2
82. Tofigh, A., Suderman, M., Paquet, E. R., Livingstone, J., Bertos, N., Saleh, S. M., Zhao, H., Souleimanova, M., Cory, S., Lesurf, R., Shahalizadeh, S., Garcia Lopez, N., Riazalhosseini, Y., Omeroglu, A., Ursini-Siegel, J., Park, M., Dumeaux, V., & Hallett, M.

(2014). The prognostic ease and difficulty of invasive breast carcinoma. *Cell reports*, *9*(1), 129–142. https://doi.org/10.1016/j.celrep.2014.08.073

83. Desmedt, C., Di Leo, A., de Azambuja, E., Larsimont, D., Haibe-Kains, B., Selleslags, J., Delaloge, S., Duhem, C., Kains, J. P., Carly, B., Maerevoet, M., Vindevoghel, A., Rouas, G., Lallemand, F., Durbecq, V., Cardoso, F., Salgado, R., Rovere, R., Bontempi, G., Michiels, S., … Sotiriou, C. (2011). Multifactorial approach to predicting resistance to anthracyclines. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, *29*(12), 1578–1586. https://doi.org/10.1200/JCO.2010.31.2231
84. Haibe-Kains, B., Desmedt, C., Di Leo, A., Azambuja, E., Larsimont, D., Selleslags, J., Delaloge, S., Duhem, C., Kains, J. P., Carly, B., Maerevoet, M., Vindevoghel, A., Rouas, G., Lallemand, F., Durbecq, V., Cardoso, F., Salgado, R., Rovere, R., Bontempi, G., Michiels, S., … Sotiriou, C. (2013). Genome-wide gene expression profiling to predict resistance to anthracyclines in breast cancer patients. *Genomics data*, *1*, 7–10. https://doi.org/10.1016/j.gdata.2013.09.001
85. Desmedt, C., Piette, F., Loi, S., Wang, Y., Lallemand, F., Haibe-Kains, B., Viale, G., Delorenzi, M., Zhang, Y., d'Assignies, M. S., Bergh, J., Lidereau, R., Ellis, P., Harris, A. L., Klijn, J. G., Foekens, J. A., Cardoso, F., Piccart, M. J., Buyse, M., Sotiriou, C., … TRANSBIG Consortium (2007). Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. *Clinical cancer research: an official journal of the American Association for Cancer Research*, *13*(11), 3207–3214. https://doi.org/10.1158/1078-0432.CCR-06-2765
86. Patil, P., Bachant-Winner, P. O., Haibe-Kains, B., & Leek, J. T. (2015). Test set bias affects reproducibility of gene signatures. *Bioinformatics (Oxford, England)*, *31*(14), 2318–2323. https://doi.org/10.1093/bioinformatics/btv157
87. Schmidt, M., Böhm, D., von Törne, C., Steiner, E., Puhl, A., Pilch, H., Lehr, H. A., Hengstler, J. G., Kölbl, H., & Gehrmann, M. (2008). The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer research*, *68*(13), 5405–5413. https://doi.org/10.1158/0008-5472.CAN-07-5206
88. Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., Jatkoe, T., Berns, E. M., Atkins, D., & Foekens, J. A. (2005). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet (London, England)*, *365*(9460), 671–679. https://doi.org/10.1016/S0140-6736(05)17947-1

89. Giacinti, C., & Giordano, A. (2006). RB and cell cycle progression. *Oncogene*, *25*(38), 5220–5227. https://doi.org/10.1038/sj.onc.1209615
90. Golsteyn R. M. (2005). Cdk1 and Cdk2 complexes (cyclin dependent kinases) in apoptosis: a role beyond the cell cycle. *Cancer letters*, *217*(2), 129–138. https://doi.org/10.1016/j.canlet.2004.08.005
91. Tophkhane, C., Yang, S., Bales, W., Archer, L., Osunkoya, A., Thor, A. D., & Yang, X. (2007). Bcl-2 overexpression sensitizes MCF-7 cells to genistein by multiple mechanisms. *International journal of oncology*, *31*(4), 867–874.
92. Dawson, S. J., Makretsov, N., Blows, F. M., Driver, K. E., Provenzano, E., Le Quesne, J., Baglietto, L., Severi, G., Giles, G. G., McLean, C. A., Callagy, G., Green, A. R., Ellis, I., Gelmon, K., Turashvili, G., Leung, S., Aparicio, S., Huntsman, D., Caldas, C., & Pharoah, P. (2010). BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. *British journal of cancer*, *103*(5), 668–675. https://doi.org/10.1038/sj.bjc.6605736
93. Mérino, D., Khaw, S. L., Glaser, S. P., Anderson, D. J., Belmont, L. D., Wong, C., Yue, P., Robati, M., Phipson, B., Fairlie, W. D., Lee, E. F., Campbell, K. J., Vandenberg, C. J., Cory, S., Roberts, A. W., Ludlam, M. J., Huang, D. C., & Bouillet, P. (2012). Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood*, *119*(24), 5807–5816. https://doi.org/10.1182/blood-2011-12-400929
94. Bryant, C., Rawlinson, R., & Massey, A. J. (2014). Chk1 inhibition as a novel therapeutic strategy for treating triple-negative breast and ovarian cancers. *BMC cancer*, *14*, 570. https://doi.org/10.1186/1471-2407-14-570
95. Gartel, A. L., & Tyner, A. L. (2002). The Role of the Cyclin-dependent Kinase Inhibitor p21 in Apoptosis 1 Supported in part by NIH Grant R01 DK56283 (to A. L. T.) for the p21 research and Campus Research Board and Illinois Department of Public Health Penny Severns Breast and Cervical Cancer grants (to A. L. G.).1. *Molecular Cancer Therapeutics*, *1*(8), 639-649.
96. Solier, S., & Pommier, Y. (2014). The nuclear γ-H2AX apoptotic ring: implications for cancers and autoimmune diseases. *Cellular and molecular life sciences: CMLS*, *71*(12), 2289–2297. https://doi.org/10.1007/s00018-013-1555-2
97. Laplante, M., & Sabatini, D. M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *Journal of cell science*, *126*(Pt 8), 1713–1719. https://doi.org/10.1242/jcs.125773

98. Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R., & Tavaré, J. M. (2011). Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *British journal of cancer*, *104*(11), 1755–1761. https://doi.org/10.1038/bjc.2011.132

99. Abukhdeir, A. M., Vitolo, M. I., Argani, P., De Marzo, A. M., Karakas, B., Konishi, H., Gustin, J. P., Lauring, J., Garay, J. P., Pendleton, C., Konishi, Y., Blair, B. G., Brenner, K., Garrett-Mayer, E., Carraway, H., Bachman, K. E., & Park, B. H. (2008). Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(1), 288–293. https://doi.org/10.1073/pnas.0710887105

100. Li, H., Zhou, W., Li, L., Wu, J., Liu, X., Zhao, L., Jia, L., & Sun, Y. (2017). Inhibition of Neddylation Modification Sensitizes Pancreatic Cancer Cells to Gemcitabine. *Neoplasia (New York, N.Y.)*, *19*(6), 509–518. https://doi.org/10.1016/j.neo.2017.04.003

101. Knappskog, S., Berge, E. O., Chrisanthar, R., Geisler, S., Staalesen, V., Leirvaag, B., Yndestad, S., de Faveri, E., Karlsen, B. O., Wedge, D. C., Akslen, L. A., Lilleng, P. K., Løkkevik, E., Lundgren, S., Østenstad, B., Risberg, T., Mjaaland, I., Aas, T., & Lønning, P. E. (2015). Concomitant inactivation of the p53- and pRB- functional pathways predicts resistance to DNA damaging drugs in breast cancer *in vivo*. *Molecular oncology*, *9*(8), 1553–1564. https://doi.org/10.1016/j.molonc.2015.04.008

102. Alsubhi, N., Middleton, F., Abdel-Fatah, T. M. A., Stephens, P., Doherty, R., Arora, A., Madhusudan, S. (2016). Chk1 phosphorylated at serine345 is a predictor of early local recurrence and radio-resistance in breast cancer. *Molecular Oncology, 10*(2), 213-223. https://doi.org/10.1016/j.molonc.2015.09.009

103. Choi, C., Cho, W. K., Park, S., Shin, S. W., Park, W., Kim, H., & Choi, D. H. (2020). Checkpoint Kinase 1 (CHK1) Inhibition Enhances the Sensitivity of Triple-Negative Breast Cancer Cells to Proton Irradiation via Rad51 Downregulation. *International journal of molecular sciences, 21*(8), 2691. https://doi.org/10.3390/ijms21082691

104. Liu, R., Chen, Y., Liu, G., Li, C., Song, Y., Cao, Z., Liu, Y. (2020). PI3K/AKT pathway as a key link modulates the multidrug resistance of cancers. *Cell Death & Disease, 11*(9), 797. doi:10.1038/s41419-020-02998-6

105. Mandlekar, S., & Kong, A. N. (2001). Mechanisms of tamoxifen-induced apoptosis. *Apoptosis : an international journal on programmed cell death*, *6*(6), 469–477. https://doi.org/10.1023/a:1012437607881

106. Li, W., Shi, X., Xu, Y., Wan, J., Wei, S., & Zhu, R. (2017). Tamoxifen promotes apoptosis and inhibits invasion in estrogen-positive breast cancer MCF-7 cells. *Molecular medicine reports*, *16*(1), 478–484. https://doi.org/10.3892/mmr.2017.6603

107. Ciruelos Gil E. M. (2014). Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. *Cancer treatment reviews*, *40*(7), 862–871. https://doi.org/10.1016/j.ctrv.2014.03.004

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