UNDERSTANDING THE TUMOR SUPPRESSIVE FUNCTIONS OF ESTROGEN RECEPTOR β IN BREAST CANCER

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Doctor of Philosophy

By

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I dedicate this dissertation to my dear parents, brothers, sisters, to my pulchritudinous wife and to my entire family for their unconditional support and love.

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ABSTRACT

Breast cancer is a heterogeneous disease with regard to clinical outcome and molecular characteristics. Some breast cancers are more aggressive because they express mutant proteins with potent oncogenic functions including mutant p53 proteins with gain of functions. Such tumors are often resistant to therapies. Accumulating data show positive correlations between ERβ1 and better survival, indicating more important roles of the receptor in breast cancer.

First, we investigated the role of ERβ in triple negative breast cancer cells (TNBCs) that are mutant for p53. These cells are derived from very aggressive cancers with highly metastatic and chemoresistant properties. TNBC are negative for ERα, PR, and Her2, and there is no effective targeted therapy. Despite the lack of ERα, a significant number of TNBCs express ERβ. ERβ1 displays anti-migratory, anti-invasive, and anti-metastatic properties. However, the mechanism underlying its function is not well understood. Using molecular technics, we found that ERβ1 opposes mutant p53 gain-of-function through direct interaction. Most importantly, we found that ERβ1 can use mutant p53 as a co-factor to alter gene transcription. Further, the ERβ1-induced epithelial transformation was p63-dependent, suggesting an association between ERβ1, mutant p53 and p63.

Second, we investigated the role of ERβ in ERα-positive breast cancer cells. These cells are derived from less aggressive tumors that respond better to hormonal therapy. ERα-positive breast cancers often express wild-type p53, yet p53 tumor suppressive function is inactivated. Several factors including ERα have been found to inhibit wild-type p53 activity. Previous studies have shown anti-proliferative responses following upregulation of ERβ1 in ERα-positive breast cancer cells. ERβ1 was also shown to correlate with better overall and disease-free survival in patients with ERα-positive breast cancer especially after hormonal therapy. Still, the mechanism through which ERβ1 associates with improved prognosis had yet to be investigated. We show that ERβ1 enhances wild-type p53 activity in ERα-positive cells. Further, we found that ERβ1-specific ligands can affect p53 transcriptional activity. Overall, we were able to suggest some novel mechanisms through which ERβ1 elicits its tumor suppressive function and propose ERβ1 as a potential good marker and target for breast cancer treatment.

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ABBREVIATIONS

TNBC: Triple Negative Breast Cancer

ERβ: Estrogen Receptor beta

ERα: Estrogen Receptor alpha

TGFβ: Transforming Growth Factor beta

FST: Follistatin

GRP87: G protein-coupled Receptor 87

ADAMTS9: A Disintegrin and Metalloproteinase with Thrombospondin Motifs 9

SHARP1/ BHLHE41: Basic Helix-Loop-Helix Family, Member

CCNG2: Cyclin G2

GOF: Gain of Function

Mutp53: Mutant p53

WT p53: Wild-type p53

LBD: Ligand Binding Domain

DBD: DNA Binding Domain

TAD: Transactivation Domain

OD: Oligomerization Domain

TA: N-terminal Transactivation Domain

ΔN: Truncated N-terminus

BMI: Body Mass Index

PAI-1: Plasminogen Activator Inhibitor-1

PML: Promyelocytic Leukemia

GADD45A: Growth Arrest and DNA-Damage-Inducible, Alpha

PTEN: Phosphatase and Tensin Homolog

p21 (WAF1): Cyclin-Dependent Kinase Inhibitor 1A

PUMA: p53 Upregulated Modulator of Apoptosis

BAX: BCL2 Associated X Protein

BCL2: B-Cell CLL/Lymphoma 2

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3-related Protein

MDM2: Mouse Double Minute 2 Homolog

MDM4: Mdm2-Like p53-Binding Protein

Chapter I

General Introduction

1.1. Breast cancer

1.1.1. Epidemiology of breast cancer

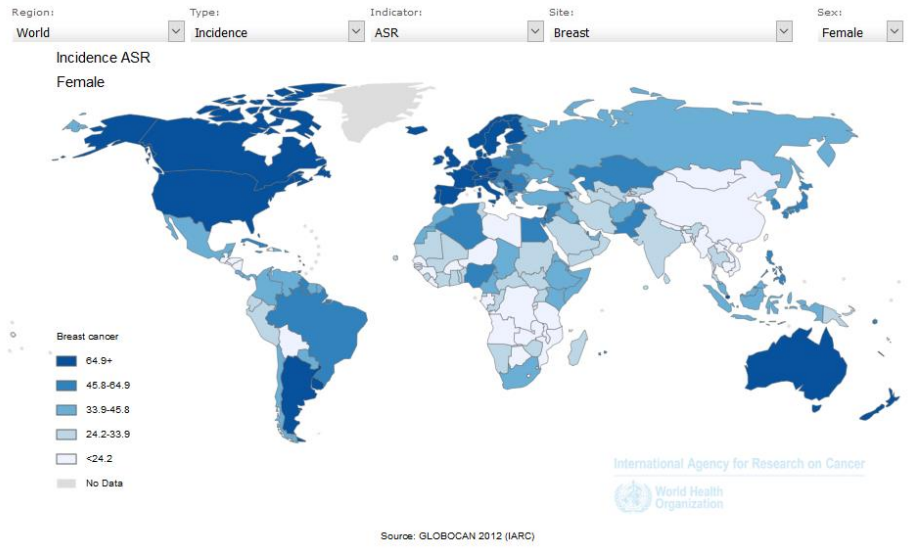
Considering both genders, breast cancer is the second most common cause of cancer worldwide and the fifth leading cause of cancer mortality (after lung, liver, stomach, and colorectal cancers). Males have a low lifetime risk (1/1000) of breast cancer which accounts for less than 1% of cancers occurring in males [1]. In contrast, over 1.67 million new cases of breast cancer were diagnosed among women in 2012 accounting for 25.2% of all cancer incidences and driving 14.7% of cancer-related deaths [2-4]; thus, breast cancer is the most common cancer type and the leading cause of cancer mortality among women worldwide. In developed countries, however, breast cancer has become the second cause of cancer related-death among women because of better clinical management.

High incidence rates of breast cancer are observed in developed countries with a peak of 92 per 100,000 women in North America. In developing countries, these

rates are much lower and fluctuate between 15 and 45 per 100,000 in Africa, 15 and 30 per 100,000 in Asia and between 20 and 90 per 100,000 in Latin America [5]. Nonetheless, in less industrialized countries, breast cancer mortality rate is often comparable to breast cancer-related deaths in more advanced countries as illustrated by Figure 1.1 [2, 3]. The poorer survival rate is due to late detection and diagnosis of cancers and also because of inaccessibility to appropriate medical care (lack of facilities and unaffordable cost of treatment options).

A

Worldwide breast cancer incidence rate



B

Worldwide breast cancer mortality rate

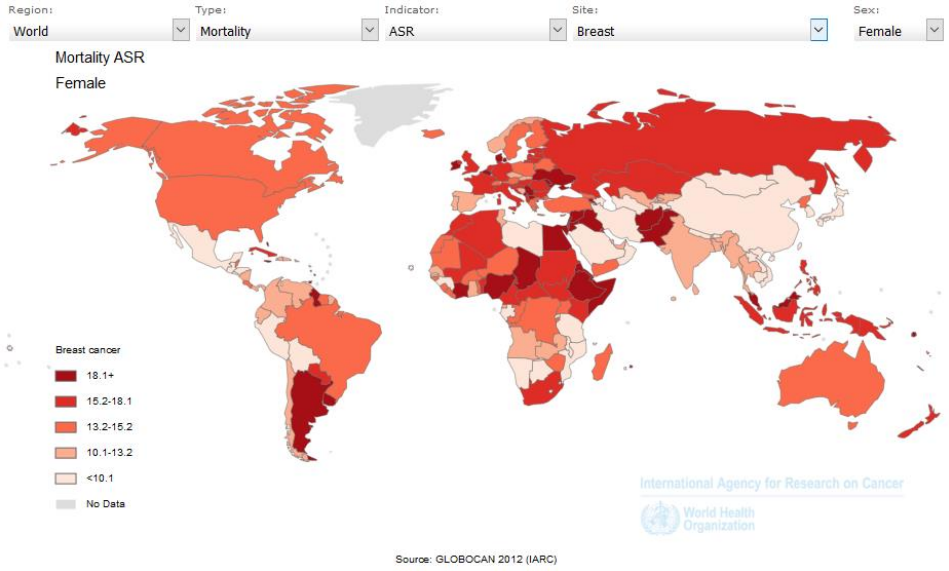


Figure 1.1: Worldwide breast cancer incidence and mortality rate. (A) Representative map of breast cancer incidence in the world. (B) Representative map of breast cancer mortality in the world. This figure has been obtained from the International Agency for research on cancer (Globacon 2012).

1.1.2. Risk factors of breast cancer

Multiple factors including prenatal conditions, diet, lack of physical activities, estrogen exposure, body mass index, depression, and quality of life can increase the risk of breast cancer. Although some risk factors have been scientifically demonstrated, many of them are still based on speculations. First of all, gender, age, and family history play an important role in breast cancer development. In fact, breast cancer incidence is very low in males with less than 1/1000 compared to the very high life time incidence of 1/8 worldwide observed in females [1, 5]. Age is also a major factor that significantly increases breast cancer risk. Recent data from the NIH show a higher risk of breast cancer in aging women. While a 30 year old woman has 0.44% risk to develop cancer within the next 10 years, this probability increases to 3.82% for a 70 year old woman. Further, breast cancer was shown to be the leading cause of death in women aged 45 to 64 in the United States (http://www.cdc.gov/nchs/deaths.htm).

People with family history of breast cancer have higher risks for cancer development. In fact, several genetic mutations increase the risk of breast cancer development (50-90%). BRCA1/2 mutations, Li-Fraumeni syndrome, and Cowden syndrome correlate with early development of breast cancer due to lack of DNA

repair mechanism, loss of wild-type p53 function and PTEN mutation, respectively [7, 10, 18].

Other important factors are obesity, life style, ethnicity, and maternal age. People with a BMI greater than 25 were reported to present higher risks for breast cancer especially in postmenopausal women [20]. A healthy life style including diet and exercising can significantly reduce breast cancer risks [19]. Ethnic groups were also shown as risk factors. For instance, African Americans were found to predominantly develop triple negative breast cancer compared to other ethnic groups [41, 42]. Early age menarche and late age first-term pregnancy as well as hormone replacement therapy (HRT) are associated with increased risks of breast-cancer development [8-10].

Finally, risks related to radiation, smoking, and alcoholism exist. Ionizing radiation and sun exposure increase cancer risk mostly in the developing breast while no significant risk was found in postmenopausal women [11]. Tobacco smoking exposes the body to several carcinogens such as nicotine, cyanide, benzene, ammonia, and arsenic, which affect breast development and increase the risks of breast cancer [12, 44, 45]. Alcohol consumption was reported to also increase breast-cancer risk especially in women drinking during pregnancy or

having started drinking at early age [13]. In addition to environmental toxicants, simultaneous exposure to several of the risk factors might critically increase the chances for breast-cancer initiation and development.

1.1.3 Pathogenesis of breast cancer

Breast is the one organ that keeps growing after adulthood. The lactiferous ducts exist at birth, but lobules start developing only at puberty. At this point, estrogen and progesterone promote branching of the ductal system. During pregnancy, progesterone and prolactin complete the maturation of breast by increasing the number and size of lobules. At the same time, myoepithelial proliferation and differentiation is induced by oxytocin. After lactation, the breast returns to it pre-pregnancy state due to lobular atrophy. Much later in life, especially after menopause, the breast atrophies considerably, due to loss of connective tissue and shrinkage of lobules and ducts [14].

Anatomically, the adult breast is formed by 4 types of tissues (Figure 1.2). The lobules, often called glandular tissues, produce milk during pregnancy. The ducts connect the lobules and converge at the nipple. The fat and fibrous connective tissues provide the breast with a specific shape and size and keep all tissues interconnected [46].

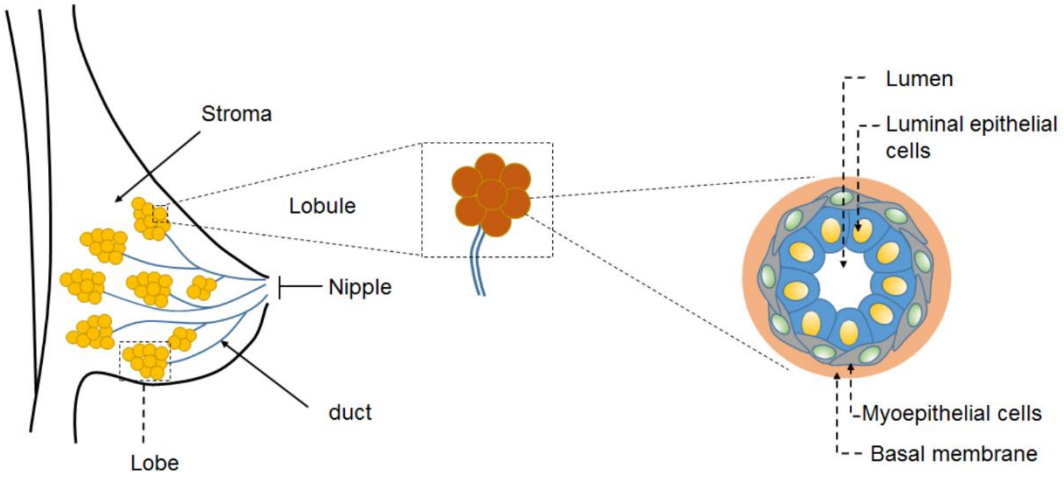


Figure 1.2: Anatomy and histology of the breast.

Under normal conditions, cells follow the regular cycle of the breast development. They differentiate, age, undergo cell death, and are subsequently replaced by new cells if needed. However, some cells can become autonomous by not responding to mainstream signaling and can become cancerous. Breast cancers can originate from different tissues as seen in Figure 1.3.A. Carcinomas are the most common types of breast cancer and derive from epithelial sources, while sarcomas, less frequent, originate from connective tissues. Histologically, ductal cancers originate from milk ducts and lobular cancer derive from lobules [46].

Although some cancers initiate in the stroma, the majority of breast cancers originate from epithelial cells (Figure 1.3.B). Throughout years of studies, six major hallmarks have been identified which define cancer cells. They usually have uncontrolled proliferation, display a high capability of escaping growth suppressor signaling, develop high resistance to cell death, develop unlimited replicative properties, promote angiogenesis, and develop invasion and metastasis [47]. In addition, the ability to evade the immune system and to develop new metabolic pathways has recently been suggested.

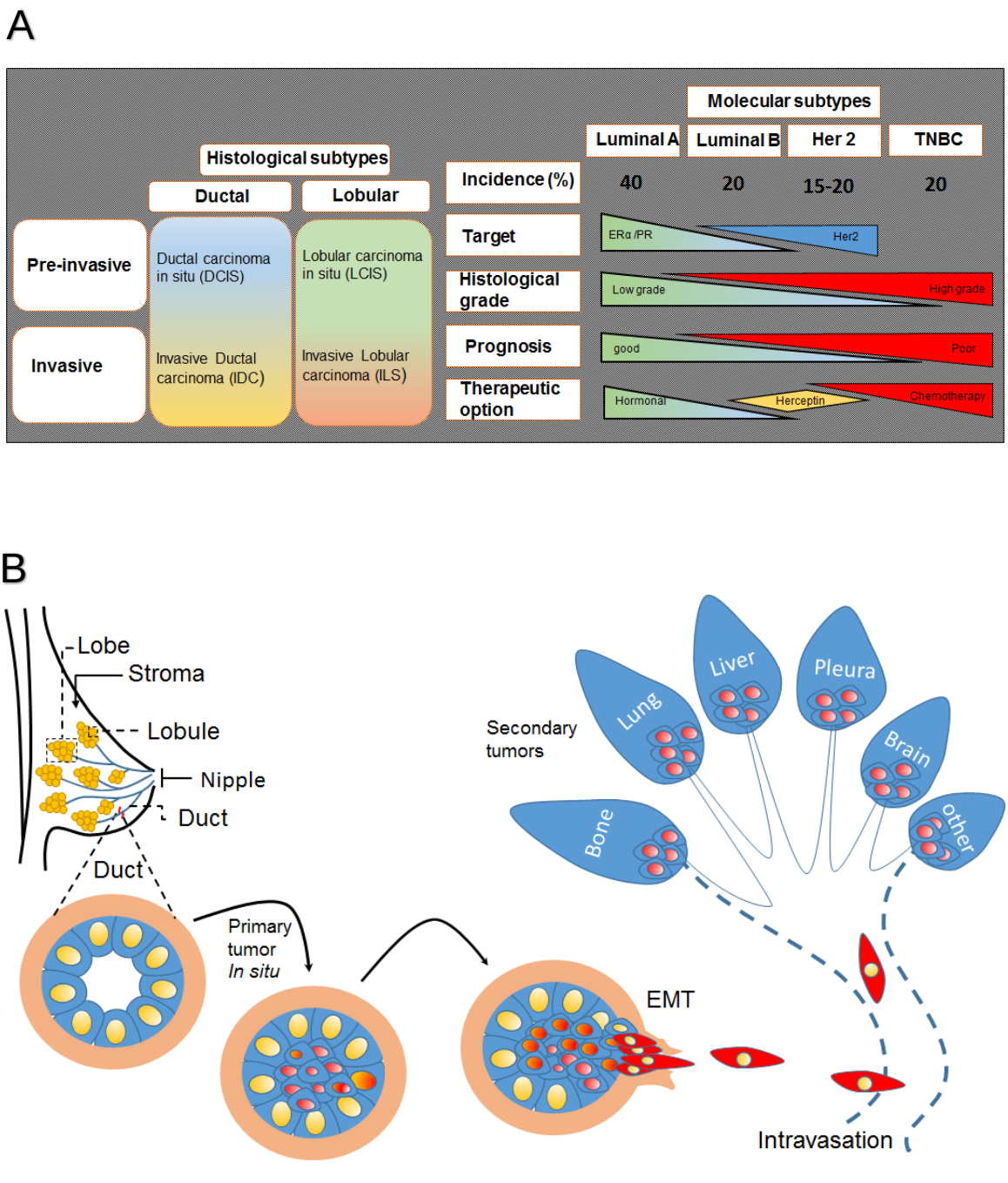


Figure 1.3: Breast cancer heterogeneity and metastasis. (A) Breast cancer classification based on histological and molecular features. (B) Process of metastasis and invasion of breast cancer from epithelial origin.

1.1.4 Breast cancer prevention

How do we prevent cancer? The answer to this question is quite difficult considering the complexity of the disease and the multiplicity of sources that can trigger it. In essence, healthy life style including healthy diet and exercise, stress management, avoidance of toxicants, breast removal for genetically predisposed women, skin protection from radiation, avoidance of late first-time pregnancy, and menopausal hormone replacement therapy (HRT) could significantly reduce the risk of breast cancer [53]. Several breast cancer vaccines targeting Her2+ and Triple negative breast cancers are still in trials and might reduce breast cancer incidence [21, 22].

1.1.5 Breast cancer diagnosis

Very few symptoms can inform about breast-cancer development. The most common is appearance of firm breast mass with irregular borders usually identifiable by touching. This is often associated with a nipple discharge containing blood which might indicate breast cancer. Breast pain or mastalgia (cyclic or not), might in rare cases be associated with breast cancer [14, 15].

Due to the frequent lack of symptoms at early stages of cancer, early diagnosis through regular breast screening is the best way to increase chances of survival in

cancer. Mammography or ultrasound is often used to image tumors in the breast. Although mammography can give false positive results, especially in premenopausal patients, it is indicated for the detection of calcifications and small solid lesions in non-dense breast [49, 50, 52]. Alternatively, ultrasound is more appropriate for detecting fluid-filled lesions and solid tumors in dense breast [55]. Breast magnetic resonance imaging (MRI), is also used for diagnosis if clinically relevant or as a complementary screening tool.

For further diagnosis, a tumor biopsy is often implemented. For instance, histologic analysis (immunohistochemistry) and molecular profiling are used to provide specific information on the malignancy of the tissue [16]. These analyses allow determination of the tumor grade based on how different the cancer is from normal cells. Tumor size and lymph node invasion are important in determining the tumor stage [85].

1.1.6 Breast cancer treatment

Breast cancer treatment options depend on pathological and clinical features [86]. The most used are the menopausal status, disease stage, tumor grade, and the breast cancer subtype [23, 24]. Breast cancer is organized in five different groups. The Luminal subtypes are positive for estrogen receptor (ER) α and progesterone

receptor (PR) and are considered Luminal A if negative for the Human epithelial growth factor receptor (Her) 2 and Luminal B if over-expressing Her2. The Her2 subtype is negative for ERα/PR and has an overexpression of Her2. The triple negative breast cancer subtype is negative for all classical receptors and is consequently not yet treated by targeted therapy. Some cancers are, however, difficult to classify since they do not fit into any of the four molecular subtypes previously described. Hence, they are considered unclassified [25]. Based on cancer features, several treatment options are available, and often consist of combining several of the following: tumor removal by surgery, radiotherapy, chemotherapy and targeted therapy [26, 27].

Surgery is indicated for localized breast cancers. Lumpectomy or Breast-conserving surgery (BCS) allows removal of tumors while keeping the majority of the breast intact. Massive and more invasive tumors often require a mastectomy (entire removal of the breast). In case of lymph node invasion, the axillary lymph nodes can also be removed [28]. Patients with larger tumors often undergo radiation or chemotherapy to reduce their size before surgery. Depending on the cancer metastatic stage, total body or breast specific irradiation follows most surgical procedures to prevent recurrence of the disease [29].

More specific treatment options have been implemented during the years to better target breast cancer. Hormonal therapy is used to treat patients with ERα/PR-positive tumors which often respond to endocrine therapy. ERα is expressed in the majority of breast cancers and its activity is highly dependent on the presence of estrogen. Therefore, endocrine therapy has been to date the most common treatment for breast cancer. Several endocrine therapeutic options exist. Traditionally, the level of hormone was reduced by ovariectomy and/or adrenalectomy which consists of ablating the ovaries and/or adrenal glands, which suppresses the production of androgen in females. Since these procedure do not completely abolish estrogen production, anti-estrogens such as Tamoxifen are often used to bind to and inhibit ERα. Further, aromatase inhibitors which inhibit estrogen synthase to reduce estrogen production also appear as an efficient therapeutic options [33]. Recently, anti-androgens have been suggested for ER-/Her+/AR+ breast cancer treatment based on the important role played by androgen receptor in promoting breast cancer growth [48].

Altogether, adjuvant therapy which may include targeted therapy, chemotherapy, and radiotherapy are used in combination to surgery. For instance, chemotherapy is used in cases of relapse of tumors after failure of hormonal therapy. It is also used in cases in which tumors are ERα/PR negative. Chemotherapeutic drugs

often target cell proliferation and induce cell death. Unfortunately, they are highly toxic and have major side effects [56].

1.2 Nuclear receptors in breast cancer

Nuclear receptors are a family of ligand-regulated transcription factors often activated by small molecules such as steroid hormones, thyroid hormone, vitamin D as well as metabolites of fatty acids, cholesterol, and bile acids. Nuclear receptors are involved in most aspects of cellular development, metabolism, differentiation, and reproduction [57]. Although many nuclear receptors have had their ligands identified, some receptors are still considered orphan and have no known endogenous ligands [34, 35]. Nuclear receptors are only found in metazoans, but there are different numbers of receptors in different species. In human and mouse, 48 and 49 different nuclear receptors have been identified, respectively.

In general, nuclear receptors are between 50 to 100 Kda molecular weight and are organized in five main domains. The N-terminal domain, also called the A/B domain, contains a supposedly weak activation function domain (AF1) whose activity can be independent of ligands. The N-terminal domain is not well conserved between nuclear receptors. In contrast, the DNA binding domain (DBD) which is adjacent to the AF1, is highly conserved between nuclear receptors. The

DBD has two zinc finger structures which allows interaction of nuclear receptors with specific DNA sequences [36]. The hinge region is a flexible region connecting the DBD to the Ligand binding domain (LBD) and was found to determine functional synergy between the AF1 and the activation function domain 2 (AF2). The LBD contains the AF2 which often depends on ligand binding for activation. The LBD is moderately conserved and is important for co-factor recruitment and dimerization of the receptor. The LBD is part of the C-terminus domain which is highly variable between receptors and may be absent.

1.3 Estrogen receptors

1.3.1 Nuclear estrogen receptors

The estrogen receptors are canonically known as intracellular receptors that recognize 17β-estradiol and other estrogenic ligands. Upon interaction with estrogens, ERs dimerize and become more tightly associated with nuclear chromatin where they directly bind to estrogen response elements (EREs) or to other transcription factors to regulate gene expression. There are two main nuclear estrogen receptors genes in mammals (Figure 1.4). The Estrogen Receptor α (ERα) or Nuclear Receptor subfamily 3, group A, member 1 (NR3A1) derives from the ESR1 (Estrogen Receptor 1) gene located on Chromosome 6q25.1, and was the first estrogen receptor to be discovered by Elwood V. Jensen in 1956 [30]. It is a 495

amino acid protein and stands out as the primary target in luminal breast cancer treatment. Genomic studies have revealed 3 splice variants of ERα designated ER$\alpha\Delta$3, ERα36, and ERα46 [32, 38, 39].

Estrogen Receptor β (ERβ) or NR3A2 (Nuclear Receptor subfamily 3, group A, member 2) is a paralog of ERα and derives from the ESR2 (Estrogen Receptor 2) genes located on chromosome 14q23.2. ERβ was discovered much later in 1996 by Dr Jan-Åke Gustafsson's research group at the *Karolinska Institutet* in Sweden [31]. The wild-type human ERβ1 protein has 530 amino acids. At least four splice variants of wild-type ERβ have been reported: ERβ2 or Erβcx, ERβ3, ERβ4, and ERβ5 [40]. They have different C-terminal sequences which significantly alter their ligand binding potential.

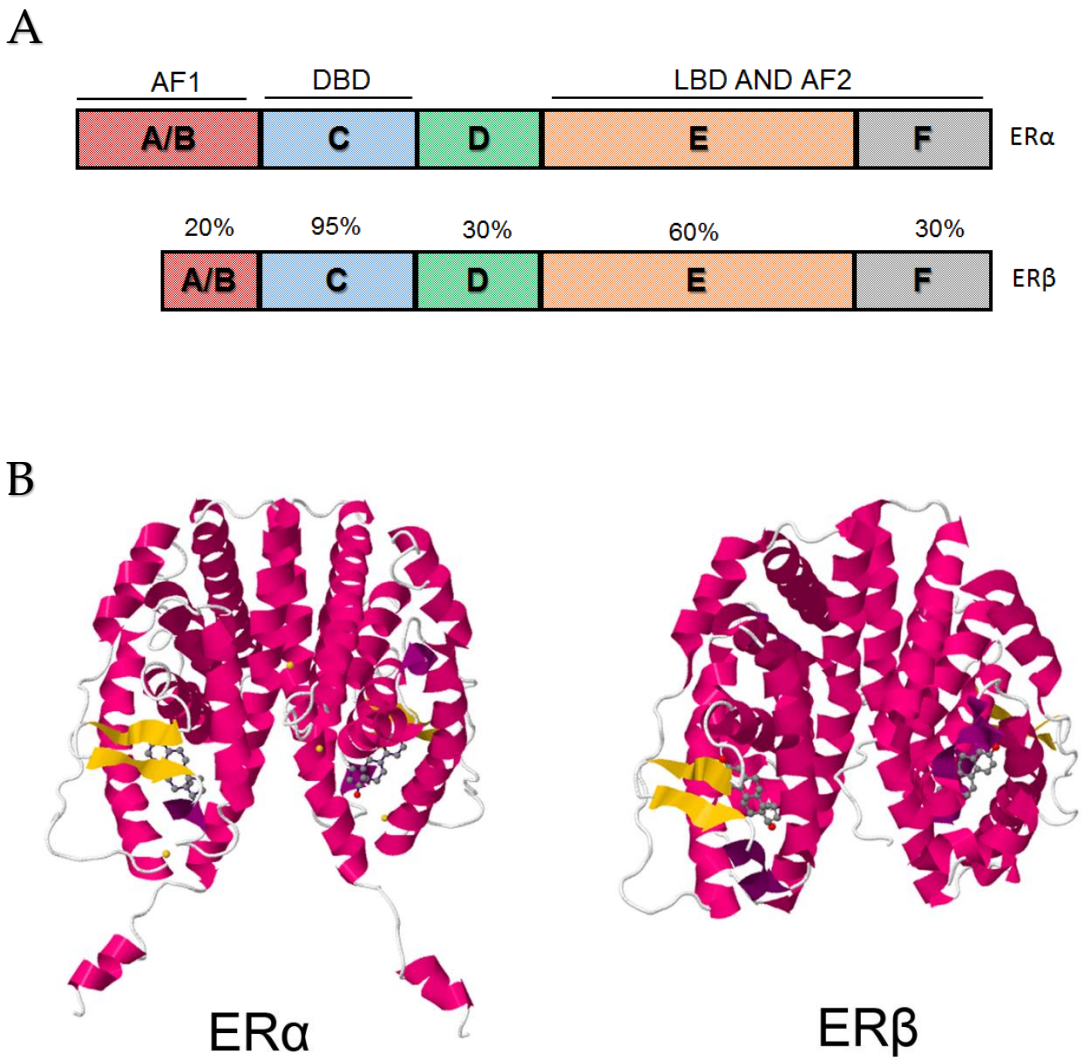


Figure 1.4: structure of nuclear estrogen receptors. (A) Schematics of ERα (top) and ERβ (bottom) protein Organization. (B) Crystal structures of Estrogen receptor ligand binding domains (LBD). (Left) Frontal view of estrogen receptor α LBD dimer complexed with estrogen. (Right) Frontal view of estrogen receptor β LBD dimer complexed with estrogen. The 3D structure was obtained from the Protein Data Bank (PDB) [99-100].

ERs display different patterns of expression. ERα is predominantly expressed in uterus, ovaries, breast, bone, white adipose tissues, and liver, while ERβ is found in ovary, central nervous system (CNS), cardiovascular system, lung, gonads, prostate, and immune system and breast [32]. While ERα expression is low in normal mammary gland and high in cancer tissues, ERβ expression is high in normal tissues but decreases when cancer progresses [76].

ERs have distinct functions in breast cancer. ERα is mostly reported to promote cell growth and proliferation, inhibit apoptosis, and activate Progesterone Receptor (PR) expression. ERβ on the other hand, correlates with anti-proliferation, apoptosis and PR repression [43]. Intriguingly, ERα and ERβ have been reported to heterodimerize [37]. Therefore, the ratio between the two receptors may be a key factor in ER signaling when they are co-expressed. More investigation needs to be done to address the role of hetero-dimerization and the effect of heterodimers on gene regulation.

Recently, genomic and transcriptomic analyses have shown that ERα and ERβ regulate a high number of common genes [49]. They both bind to Estrogen Response Elements (EREs) in the presence of estrogen. Although DNA binding seems to be ligand dependent, ERβ1 has been reported to also bind to non-ERE

sites through factors such as the Activating Protein (AP)-1, the Stimulating Protein (SP)-1, and the Nuclear Factor (NF)-Kβ in a ligand independent manner [37]. However, ligand effects on these interactions have not been well scrutinized and might explain some of the inconsistencies observed in ER signaling.

1.3.2 Membrane bound estrogen receptors

Cellular responses to estrogen signaling are mainly mediated by changes in transcriptional activity of classical estrogen receptors ERα and ERβ. The kinetics of ER-dependent gene expression is often slower in respond to ligand treatment. Intriguingly, non-transcriptional rapid responses have also been observed, which indicated that factors different from the common nuclear ERs might mediate these effects [61]. Membrane bounds ERs such as G protein-coupled estrogen receptor 1 (GPER) also known as G protein-coupled receptor 30 (GPR30), Estrogen receptor ER-X and Gq-mER have been reported to drive faster respond to estrogen [62, 63].

GPR30 mediates most of the non-genomic estrogen-induced rapid responses and localize both to the cytoplasmic membrane and, in contrast to other G-coupled protein receptors, to the endoplasmic reticulum membrane [61, 71, 98]. It is detected in the hypothalamus, pituitary gland, adrenal medulla, kidney medulla, and in developing follicles of the ovary as well as in the breast [68]. Both *in vivo*

and *in vitro* studies have shown that GPR30 drives cell proliferation in both normal and malignant cells by activating PI3K/AKT signaling [67, 69, 70]. Among endogenous estrogens, GPR30 has been shown to bind only estradiol, which promotes breast-cancer growth [64, 66]. It also binds synthetic ligands including Tamoxifen and the ER antagonists ICI-182,780/Fulvestrant, and was found to drive Tamoxifen resistance in breast and endometrial cancer [60, 62, 71].

Other membrane bound proteins including Gq-mER and ER-X are under investigation. Gq-mER plays an important role in homeostasis and ER-X shares sequential similarities with classical estrogen receptors. The latter is sensitive to anti-estrogens, and positively regulates the mitogen-activated protein (MAP) kinase pathway [61].

1.3.3 Estrogen receptor ligands

Several ligands have been found to bind estrogen receptors with a variety of binding affinities (Figure 1.5). The most common endogenous ligands in woman are 17β-estradiol (E2), estriol (E3), and estrone (E1). While 17β-estradiol is the most abundant estrogen, increased levels of estrone and estriol are detected during pregnancy.

Many ligands bind both receptors, but only some ligands display a good selectivity for ERα or ERβ. Propylpyrazoletriol (PPT), 16α-LE2 (Cpd1471), and SKF-82,958 are used as specific agonists for ERα while Methylpiperidinopyrazole (MPP) is commonly used as specific antagonist.

Several agonists of ERβ were founds to be selective over ERα. The endogenous agonists 3β-Androstanediol (3β-adiol), Dehydroepiandrosterone (DHEA), 8β-VE2 and AC-186, the phytoestrogens Apigenin, Daidzein, Genistein, Kaempferol, Liquiritigenin (Menerba), Penduletin, S-Equol (S)-4',7-isoflavandiol), and the synthetic agonists Diarylpropionitrile (DPN), ERB-196 (WAY-202196), FERb 033, Prinaberel (ERB-041, WAY-202041) were all reported to preferentially bind ERβ [58, 59]. Finding ERβ-specific antagonists have been more difficult. PHTPP shows more selectivity toward ERβ as an antagonist. However, others ligands such as (R,R)-Tetrahydrochrysene ((R,R)-THC) can antagonize ERβ activity, while being an agonist for ERα [65].

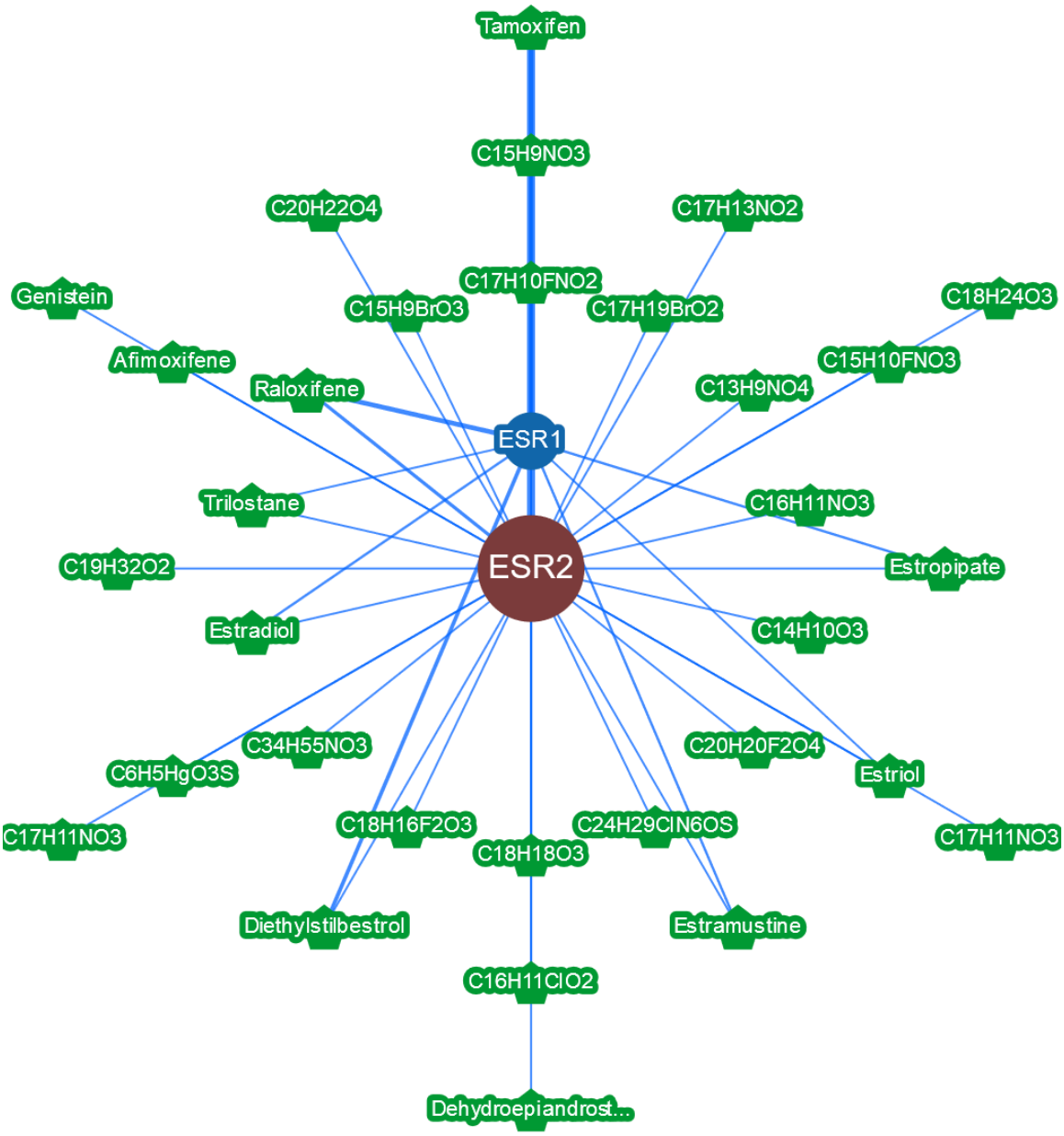


Figure 1.5: Global network of ERβ interacting chemicals. Representation of 32 chemicals found to interact with ERβ1 based on at least one instance of detectable physical association obtained from the Biogrid software (http://thebiogrid.org). The closest layers to the center are more highly connected.

1.3.4 Estrogen Receptors and Hormonal therapy

Several drugs have been approved to target ERα-dependent cell growth in breast cancer. Their main function is to bind and antagonize ERα function; intriguingly, some drugs display agonist and partial agonist properties depending on tissue location, which led to their designation as selective estrogen receptor modulators (SERMs). Clomifene was the first SERM to be approved in 1967 [75]. Tamoxifen was first synthetized 1966 but got approved for breast cancer treatment only in the 80's. Today, tamoxifen remains the most used SERM for ERα positive breast cancer treatment, and has been found to bind both ERs. Recently, a second binding pocket of Tamoxifen was discovered in ERβ1 Activation domain 1 (AF1) as illustrated in Figure 1.6. In contrast to its antagonistic role in the breast, tamoxifen induces ERα activity in the uterus. Raloxifene, on the other hand, shows antagonistic effect in both breast and uterus, with a reduced risk of endometrial cancer [74]. Other drug such as Fulvestran (ICI-182,780) were classified as selective estrogen receptor down-regulators (SERDs). Fulvestran intrinsically induces a conformational change upon binding to ER which promotes ubiquitination and degradation of the receptor [79].

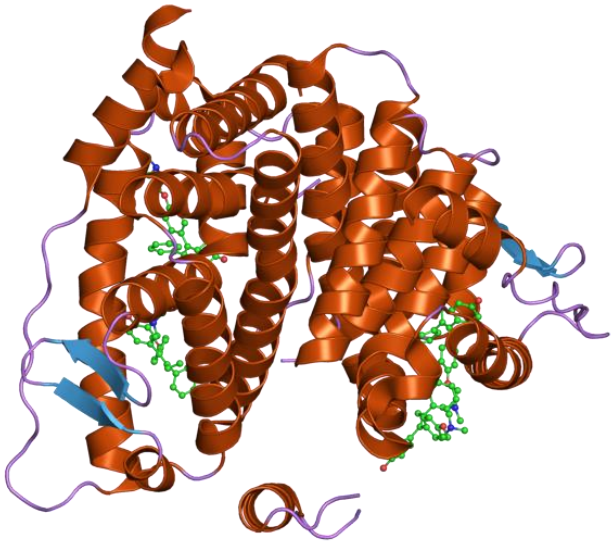


Figure 1.6: Crystal structure of ERβ in complex with 4-hydroxytamoxifen. A second binding pocket of 4-hydroxytamoxifen was found in the co-activator binding groove of ERβ. This publically available structure have been obtained from *http://www.ebi.ac.uk/*

1.4 Estrogen receptor co-factors in breast cancer

As transcription factors, ER interacts with numerous co-factors. Steroid Receptor Coactivator (SRC-1), Amplified in Breast 1 (AIB1), Breast Carcinoma Amplified Sequence 3 (BCAS3), Proline-, Glutamic acid-, Leucine-rich Protein (PELP-1). CREB-Binding Protein (CBP) and p300 can also bind both ERs in a ligand-independent manner [80].

Several independent studies on ERα interactions have reveal a large interactome for the receptor as found in the Biogrid database [103]. However, very few studies have investigated ERβ interacting proteins (Figure 1.7). Just to give few examples, ERβ can bind to Estrogen Receptor α (ERα), Activator Protein 1 (AP-1), Specificity Protein 1 (Sp-1), Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-KB) and can recruit several cofactors including p300, Nuclear Receptor Coactivator 3 (NCOA3) or AIB1, and Nuclear Receptor Coactivator 6 (NCOA6).

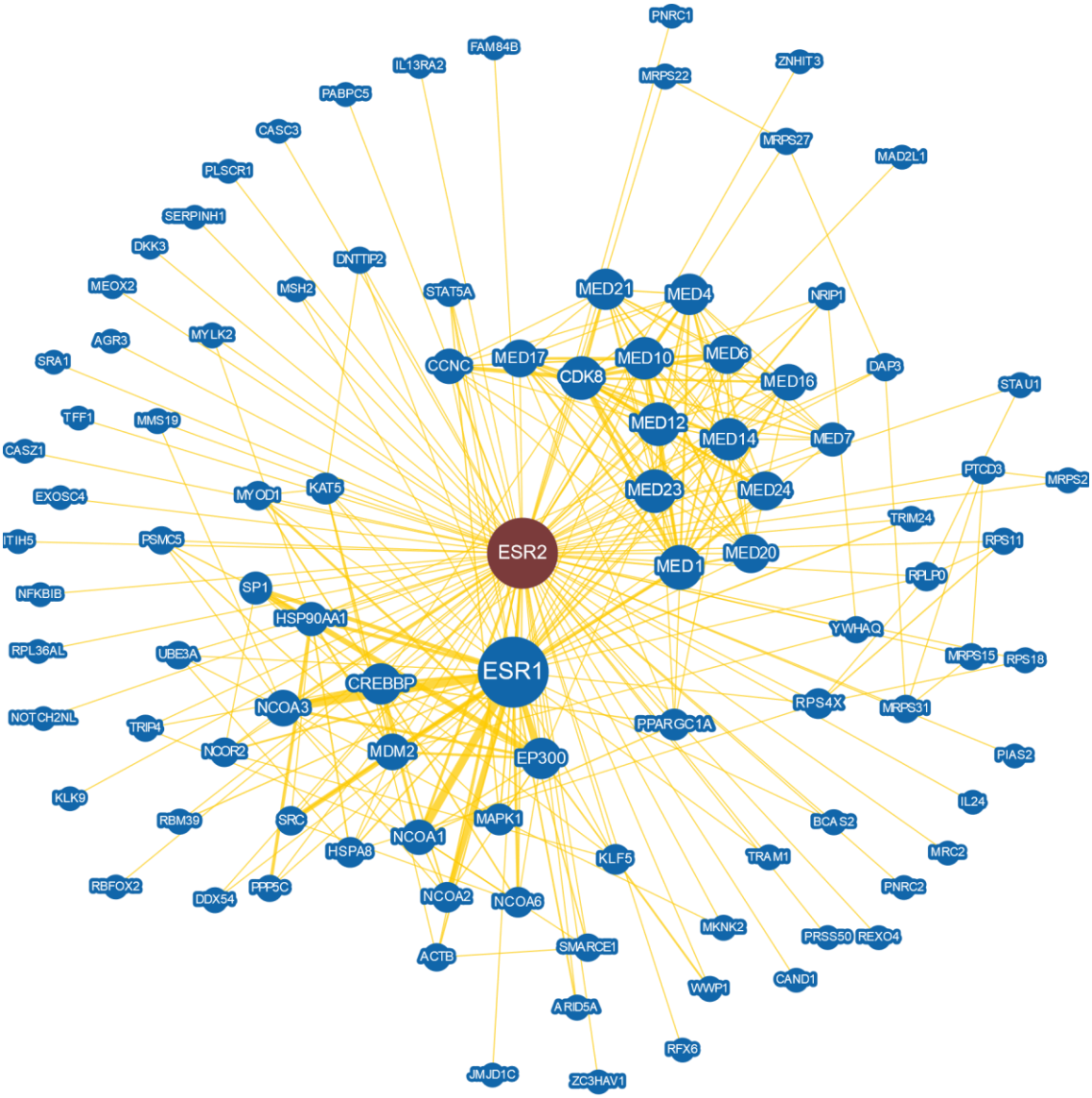


Figure 1.7: Global network of ERβ1 interacting proteins. Representation of 99 proteins associated to ERβ1 based on at least one minimum evidences of physical association *in vitro* or *in vivo*. The graph was obtained from the Biogrid software (http://thebiogrid.org). Greater node size represents increased connectivity and thicker edge sizes represent increased evidence supporting the association.

1.5 Targeting ERβ in breast cancer

1.5.1 ERβ in triple negative breast cancer

Several studies demonstrated a positive correlation between ERβ1 expression and better disease free and overall survival especially in Tamoxifen-treated patients [76]. Further, it was suggested that the ratio or ERβ to ERα might determine tumor response to hormonal therapy. To evaluate the importance of ERβ1 in TNBCs, several drugs have been taken into clinical trial [73]. Recently, a stage 4 trial was begun to validate the efficacy of adjuvant endocrine therapy in ERβ positive TNBC patients with removable tumors. In the treated group, pre- and peri-menopausal patients received 60 mg per day of the selective estrogen receptor modulator Toremifene, while postmenopausal patients were given 1 mg of Anastrozole, an aromatase inhibitor. The control group was observed for a month after chemotherapy and/or radiotherapy. All patients are being followed for a minimum time of five years (Clinical Trial ID: NCT02089854) [72]. Because the targeted group has ERα-/ERβ1+ tumors, the result of such studies will clearly determine the role of ERβ1 in TNBCs.

1.5.2 ERβ in luminal breast cancer

The role of ERβ in luminal breast cancer has been undermined by ERα and PR expression. However, one third to half of luminal breast cancers develop resistance to conventional hormonal therapy. Although still controversial, few studies have found ERβ1 to correlate with better survival in ERα-positive breast cancer patients [73], indicating that ERβ1 might still be an important factor in inhibiting breast cancer progression even in presence of ERα. Very recently, Jieqiong Liu and his collaborators have found from a cohort of 6769 patients that high ERβ1 expression correlates with better five-year disease-free survival (DFS) in both presence and absence of ERα, but with better five-year overall survival (OS) in ERα-positive patients only [77].

1.6 p53 family proteins in breast cancer

The p53 family of proteins are transcription factors composed by 3 main members which are p63, p53, and p73 (Figure 1.8). They derive from different genes located on chromosome 3, 17 and 1 respectively. Each member has several splice variants.

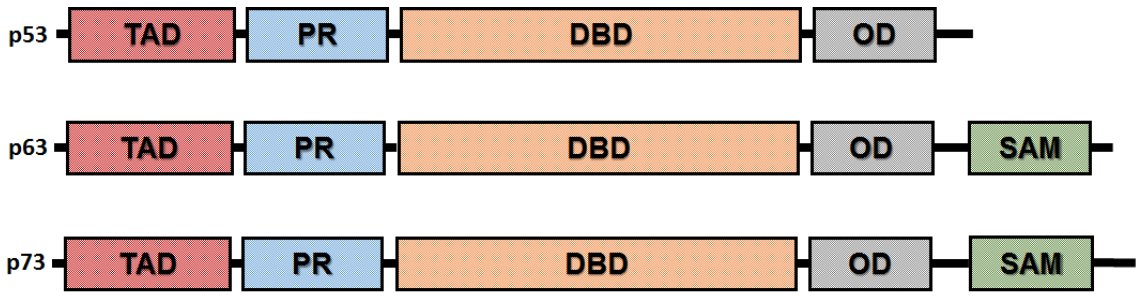


Figure 1.8: Structural representation of p53 family proteins. (Top to bottom) the major domains of p53, p63, and p73 are represented. From the C-terminus to the N-terminus, the domains are organized as followed: Transactivation domain (TAD), Proline-rich domain (PR), DNA binding domain, Oligomerization domain (OD) and sterile alpha-motif (SAM).

1.6.1 p53 in breast cancer

The tumor-suppressor protein p53 is a master regulator of cell-cycle that regulates the expression of proteins involved in DNA check-point, DNA repair, and apoptosis [78]. P53 originates from the TP53 gene located on chromosome 17 (17p13.1). p53 is expressed at low levels in normal cells and under normal conditions. However, it is stabilized in response to genotoxic stress through post-translational modifications to maintain genetic integrity or promote cell death if the DNA damage is too pronounced to be repaired. Depletion of p53 in mouse leads to cancer with 100% penetrance, strengthening the role of p53 as a tumor suppressor. Germline mutations of TP53 are frequent in cancer. Approximately 50% of cancers have single nucleotide polymorphisms or loss of the TP53 gene which often lead to Li-Fraumeni familial cancer syndrome. These mutations correlate with more aggressive cancers where the rate of mutation can reach 80 to 90% as seen in the case of TNBCs [81]. In contrast, the ERα-positive luminal breast cancers are mostly wild-type for p53, suggesting a possible inhibition of p53 tumor suppressive function in tumors expressing ERα.

P53 has several domains which are mostly structured as follow: the N-terminus transcription-activation domain (TAD), composed of a major activation domain (1-42) and a minor activation domain (43-63). TAD activates transcription factors

and is required for apoptotic induction. Adjacent to it is the proline-rich domain (64-92) which is important for p53 apoptotic activity in response to ionizing radiation [101]. The DNA-binding core domain (DBD: 102-292 aa) rich in arginine allows binding of p53 to the p53 response elements (p53Res). This domain is also targeted by co-repressors such as LIM domain only 3 (LMO3) to repress p53 transcriptional activity [82]. Subsequently to the DBD are the nuclear-localization signaling domain (316-325), the homo-oligomerization domain (OD: 307-355) which allows tetramerization of p53 monomers, and the C-terminal domain (356-393) that affects p53 stability and binding to specific p53 response elements [102].

At least 12 isoforms of p53 have been identified: p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α, Δ160p53β, and Δ160p53γ. They were reported to not only enhance p53 transcriptional activity in some cases (p53β), but to also have a dominant negative effect (Δ133p53) on full length p53 [83, 84]. However, it is important to acknowledge that scientists have not paid much attention to evaluating the importance of p53 isoforms.

The primary negative regulator of p53 stability is the E3 ubiquitin-protein ligase Mouse double minute 2 homolog (Mdm2) that binds to the TAD domain of p53. In human, Mdm2 is encoded by the MDM2 gene which is regulated by p53 based

on a negative feed-back loop mechanism. Mdm2-related protein (MdmX or Mdm4) also negatively regulates p53 activity by stabilizing mdm2 [87].

1.6.1.1 p53 tumor suppressive function

As the guardian of the genome, p53 is stabilized in response to genotoxic stress, oxidative stress, radiation, or starvation by several kinases including ATM, ATR, Chk1, and Chk2 [96]. These post-translational modifications prevent binding of Mdm2 to the TA domain of p53 allowing stabilization of the protein which thereby can bind to p53 response elements of target genes. The expression of genes such as Cyclin-dependent Kinase Inhibitor p21 (CIP1/WAF1), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase (13-4-4σ), Growth Arrest and DNA-Damage-inducible alpha (GADD45A) induces cell-cycle arrest; expression of DNA damage-binding protein p48, *Xeroderma pigmentosum* complementation group C (XPC), and GADD45A promote DNA repair, while p53-Upregulated Modulator of Apoptosis (PUMA), Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), Actin-Interacting Protein (AIP1), Bcl-2-Associated X (BAX), Death Receptor 5 (DR5), and Apoptosis antigen (1 APO-1 or FasR), are known to drive cell death [88]. In addition, cytoplasmic p53 interacts with pro-apoptotic BCL2 family members (BCL2, BCL-xL, BAX, and BAK) to induce apoptosis, demonstrating non-genomic regulatory function of p53. Further, p53 regulates

senescence, autophagy, and play an important role in angiogenesis where it regulates mammary serine protease inhibitor (Maspin), Adhesion G Protein-Coupled Receptor B1 (ADGRB1 or GD-AIF), Thrombospondin 1 (Tsp1) and B Aggressive Lymphoma Protein (Bal-1) [89, 82].

1.6.1.2 p53 mutations and gain of function (GOF)

The p53 gene is often mutated in cancer (Figure 1.9). The majority (75%) of these mutations are missense mutations that allow the full expression of the p53 protein in 90% of cases. These mutations mostly localize at the DNA binding domain as represented in Figure 1.10, suggesting an alteration of the normal binding properties of p53 to its binding site [90]. The most commonly mutated sites are R175, G245, R248, R249, R273, and R282. Depending on the involvement of the mutant in p53/DNA interaction, mutants will be considered as contact (R273H, R248Q, and R248W) if they directly interact with the major groove of the DNA, or structural (R175H, G245S, R249S, and R282H) when they only affect the conformation of the p53 protein [91].

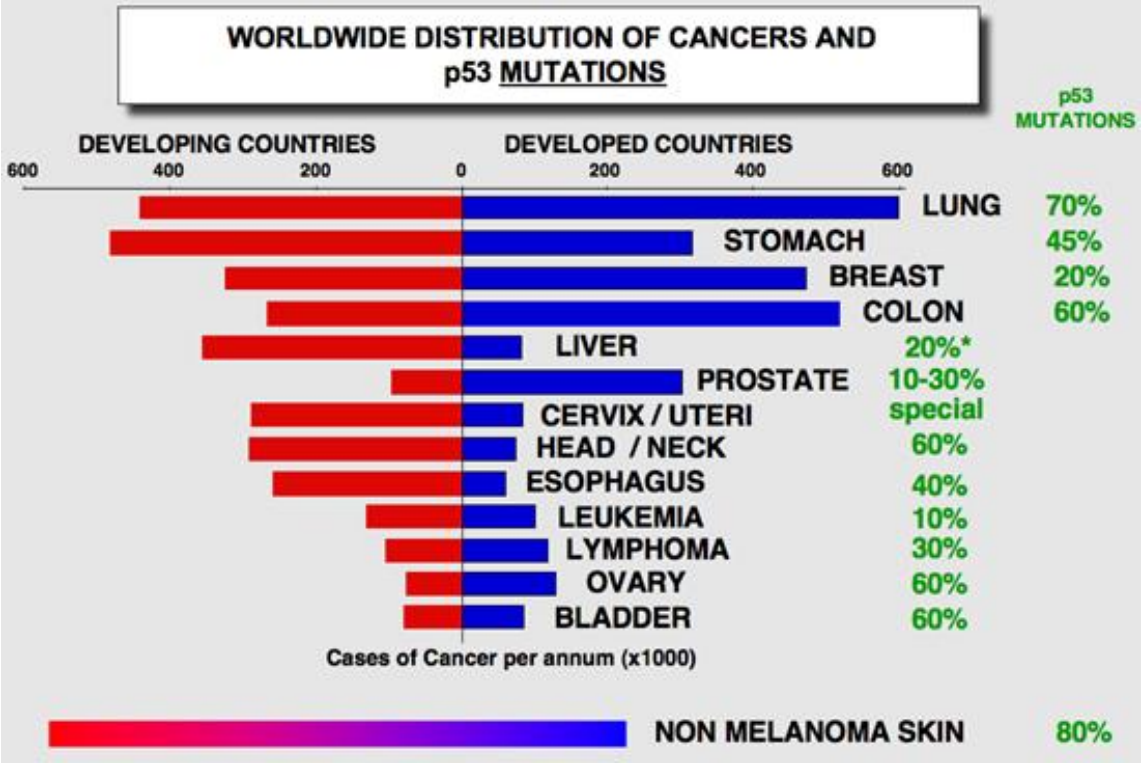


Figure 1.9: Worldwide distribution of cancer. The histograms compare cancer incidence in developing countries versus developed countries. In green is the rate of p53 mutations found in each category of cancer. This is a publically available data obtained from http://p53.free.fr

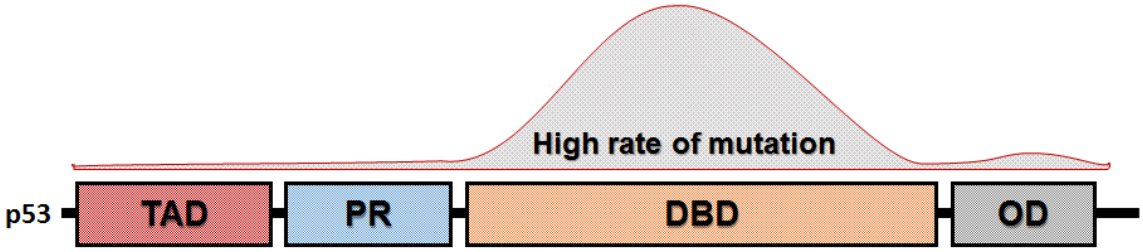


Figure 1.10: p53 mutation sites. Mutations of the p53 protein are predominantly located in the DNA-binding domain (DBD). The Transactivation domain (TAD), the Proline-rich domain (PR) and the Oligomerization domain (OD) have less mutations.

Previously thought to abolish p53 activity, the mutations are now recognized to promote a gain-of-function (GOF) of the protein. Indeed, *in vitro* and *in vivo* studies have shown a more aggressive phenotype when cancers expressing a mutant p53 were compared to tumors lacking p53 expression [92]. These mutations not only promote cancer initiation, but also drive tumor growth, drug resistance, epithelial to mesenchymal transition (EMT), invasion, and metastasis.

Mutant p53 GOF is mediated by its interaction with other transcription factors to alter their function. Mutant p53, but not wild-type, interacts with p53 family members to promote an aggressive cancer phenotypes in both cell lines and animal studies [93].

1.6.2 p63 in breast cancer

The tumor protein p63 originates from the TP63 gene which is believed to be the oldest gene from which p53 and p73 have derived. p63 is required for normal development and tissue formation. Mouse p63 knockout studies, have reported birth defects and morphological abnormalities in limbs, skin, mammary glands, and teeth. Despite being used as an epithelial marker in cancer, the role of p63 in breast cancer is still controversial.

The p63 protein has two main isoforms as seem in Figure 1.11. They derived from different promoters of the TP63 gene. Promoter 1 (P1) induces transcription of the longest isoform TAp63, which has a transactivation (TA) domain. P1 allows transcription of a shorter isoform (ΔNp63) which is lacking the transactivation domain. Furthermore, each isoform produces 3 different splice variants (α, β, and σ) at the C-terminal domain.

TAp63 and ΔNp63 isoforms have opposite functions in cancer. TAp63 have been shown to have salient anti-metastatic functions. Low expression of TAp63 was associated with more advanced stages of cancer. In contrast, ΔNp63 expression seems to promote invasion and metastasis by displaying a negative-dominant effect over p53, TAp63, and TAp73 [94].

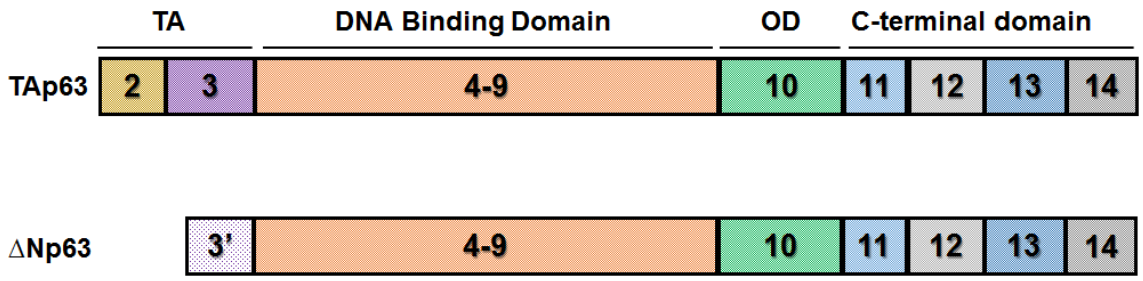


Figure 1.11: Structural representation of p63 main isoforms. The longest isoforms derived from of TP63 gene are represented. (Top) TAp63 and (bottom) ΔNp63 isoform. Numbers represent exons.

1.6.3 p73 in breast cancer

Similar to p63, p73 has two main isoforms derived from two distinct promoters of the TP73 gene. TAp73 but not ΔNp73 has the N-terminal transactivation domain. At least nine splice variants were found for TAp73 (α, β, γ, δ, ε, θ, ϕ, η, and η1) and six for ΔNp73 (ΔNp73α, ΔNp73β, ΔN'p73α, ΔN'p73β, Ex2Delp73, and Ex2/3Delp73).

TAp73 intrinsically displays tumor suppressor functions by promoting mitotic arrest and cell death. This property is, however, not shared with ΔNp73 which shows oncogenic properties by disrupting wild-type p53 and TAp73 activities. Besides, TAp73 depletion induces mitotic abnormalities resulting in polyploidy and aneuploidy. Despite the scarcity of p73 mutations in cancer, the inhibition of TAp73 by mutant p53 has provided options to target p73 for cancer therapy [95]. Although TAp73 has a specific gene signature, the majority of its targets genes are also regulated by wild-type p53 [97].

Due to functional discrepancies between TA and ΔN isoforms, the function of p53 family in cancer will be highly dependent on the ratio of their isoforms. Hence, TA/ΔN could be an important factor for predicting therapeutic responses and/or drug-resistance in breast cancer.

1.7 Significance and overall goals of the study

In the following studies, we tried to elicit the most important roles of ERβ, also called ERβ1, in breast cancer. ERβ1 is expressed in both epithelial and stromal cells of the breast, but tends to be lost in advanced cancer. Its expression in cancer correlates with better prognosis in both triple negative and luminal breast cancers. In that sense, the expression of ERβ1 makes cancer cells less aggressive in comparison to ERβ1-depleted cancer cells. Despite this evidence, ERβ1 protective role and how it maintains homeostasis is still enigmatic.

Here, we found evidence of ERβ1 being a tumor suppressor in the breast. We propose that ERβ1 be considered as a marker in breast cancer diagnosis, since the presence of ERβ1 could be associated with significantly reduced morbidity and mortality and better responses to tamoxifen. We also propose ERβ1 as a potential target for drug design. Although many ERβ1 ligands are not ERβ-subtype specific, a better understanding the function of the receptor might help design highly selective and more potent ligands with desirable activities.

Finally, ERβ1 interaction with p53-family proteins provides new opportunities to target these transcription factors and enhance their tumor suppressive activities.

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Chapter II

ERβ decreases the invasiveness of triple-negative breast cancer cells by regulating mutant p53 oncogenic function.

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2.1 Introduction

The basal-like subtype of breast cancer is the most challenging for clinical management and treatment due to the precariousness of effective targeted therapies. The majority of basal-like tumors are considered triple-negative breast cancers (TNBCs) for being negative for estrogen receptor α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor (HER)-2. TNBCs are most common among pre-menopausal women, African American, and BRCA1/2-mutant careers. They are very aggressive cancer with high potency for distant metastases [1, 2]. Interestingly, at least 80% of the tumors incorporate mutations in TP53 gene [1]. The majority of these mutations result in the expression of p53 proteins with single amino acid substitutions that cluster for the most part in the DNA-binding domain (DBD) [3]. Such changes often induce structural changes in

in the conformation of the protein which alter the DNA-binding ability of p53; as a result, the p53 protein loses its tumor suppressive activity or acquires new oncogenic functions. In fact, multiple studies have clearly demonstrated *in vitro* and *in vitro* that the gain-of-function derived from p53 mutations is independent from the loss of p53 tumor suppressor activities. Expression of mutant p53 in p53-null cell lines promotes proliferation and invasion [4]. Mice with tumor-associated p53 mutations, generate more invasive and metastatic tumors compared to p53-null mice [5, 6]. Beside, much of the mutant p53 pro-metastatic activity is mediated by the other p53 family proteins [7].

Differences in the p53 family members derive from transcriptional alternations between two different promoters resulting in N-terminal variants (full-length (TA) and truncated (ΔN)) and alternative splicing events which produce C-terminal isoforms (α, β, γ). Forming homo or hetero-oligomers within the family represents one of the mechanisms that regulate their activity [7–9]. Mutations are rare in p53 family members except for p53. Hence, only p53 with mutations inducing conformational changes involving the DNA-binding domain, was reported to interact with p73 and p63. TAp63 regulates gene expression to decrease the activity of cell surface receptors including EGFR and inhibits cell invasion [10–13]. As such, binding of mutant p53 to TAp63 will prevent p63 transcriptional activity,

thereby promoting cell invasion [10, 12, 14, 15]. Although mutant p53 retains some DNA-binding activity, it tethers to specific DNA sequences through other transcription factors including p63. This may account for the shared mutant p53 and p63 target genes that were identified in cancer cells [16]. Other mutant p53-interacting proteins that alter its gain-of-function include MDM2, PIN1, ANKRD11, and SMAD2 [7, 17, 18]. Another regulator of p53 is estrogen. Estrogen signaling is mostly mediated through two estrogen receptor (ER) subtypes: ERα and ERβ. ERα is the principal biomarker for directing endocrine therapies and the primary therapeutic target in breast cancer. Clinical studies found a correlation between wild-type ERβ (ERβ1) and better survival in patients with TNBC [10, 19–21].

Interestingly, ERs have been shown to alter wild-type and mutant p53 transactivation. They transcriptionally cooperate with p53 through two mechanisms. One functions when ERs and p53 bind to their cognate response elements without a physical interaction [22] and the other requires binding of ERα to wild-type p53 which results in repression of p53 function [23–25]. In contrast to ERα, the interaction between ERβ and p53 and its effects on transcription have not been studied and is the subject of the present chapter. We, and others, have previously shown that ERβ1 impedes epithelial to mesenchymal transition (EMT)

and decreases the invasiveness of mutant p53 TNBC cells by repressing EGFR signaling [26, 27]. However, the mechanism underlying the association of ERβ1 with the decreased EGFR activity and cell invasion has remained elusive. In the present study, we demonstrate the inhibition of mutant p53 oncogenic function as one of the mechanisms employed by ERβ1 to decrease invasion in TNBC cells.

2.2 Material and methods

2.2.1 Cells, reagents, and constructs

All cell lines were obtained from ATCC and cultured in RPMI-1460 or Dulbecco's Modified Eagle Medium media supplemented with 10% fetal bovine serum (FBS) at 37° C in 5% CO2. Phenol red-free medium supplemented with 0.5–1% dextran-coated charcoal (DCC)-treated FBS was used in ligand treatments. For stable expression of ERβ1, cells were infected with pLenti6/V5-FLAG-ERβ1 construct as described previously [27]. The pINDUCER20-FLAGERβ1 plasmid was transduced into cells and gradual ERβ1 expression was achieved following incubation of G418-selected cells with different concentrations of doxycycline (dox) for 24 hours (h) [52]. Cells were single- or co-transfected with the following plasmids: pIRESneo3, pIRESpuro3 (Clontech), pIRESneo-FLAGERβ1, pIRESpuro-FLAG-p53V143A, pIRESpuro-FLAGp53V175H, and pcDNA3-MYC-p53V143A. The

cDNA of p53V175H and p53V143A were PCR-amplified from pCMV-Neo-Bam-p53V175H and –p53V143A plasmids (Addgene, plasmids #16436 and 16435). FLAG-tagged ERβ1 domains [AF1 (amino acids: 1–150), DBD (145–220), and AF2 (211–530)] were cloned into a pIRESneo3 vector and MYC-tagged p53V143A domains [N-terminus (1–101), DBD (102–293) and C-terminus (296–393)] into a pcDNA3 plasmid. The pGEX4T-1 plasmid was used for the bacterial expression of GST-tagged proteins. Cells were transfected twice with p63-specifc siRNAs from Invitrogen, target sequences: 1# 5′-ATTCCATGGTCGTGTGAGACAGAAG-3′ and 2# 5′-AACTTAAGCGCCGAGTCGAGTACCA-3′. A siRNA targeting luciferase was used as control (Cat. No. 12935–146; Invitrogen). For p53 knockdown, cells were transfected twice with the pLKO-p53-shRNA-427 or scramble shRNA control plasmids (Addgene, plasmids #25636 and 1864).

All transfections were performed using Lipofectamine 2000 (Invitrogen) as previously described [27].

2.2.2 RNA extraction, real-time PCR

Total mRNA was isolated using the Aurum™ Total RNA Mini Kit (Biorad). RNA was reversed transcribed to cDNA using the iScript™ cDNA Synthesis Kit (Biorad). Real-time PCR was performed using the iTaq™ Universal SYBr Green

Supermix (Bio-Rad). All quantitative data were normalized to 36b4. Primer sequences for the real-time PCR experiments are listed in Supplementary Table 1.

2.2.3 Luciferase assays

For assessing ERE-dependent transcriptional activity, cells were maintained in phenol red free DCC containing media for 48 h, transiently co-transfected with 3-ERE-TATA-LUC reporter plasmid and a plasmid expressing β-galactosidase and incubated in the presence of ligands for 24 h. Luciferase activity was measured as previously described [41].

2.2.4 Co-immunoprecipitation and immunoblotting

Cells were plated at a density of 5 × 105−106 per 10-cm dish and lysed in immunoprecipitation (IP) buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.8 mM EGTA, 1 mM NP-40, 1 mM Glycerol, 2 mM PMSF, 1 mM Na3V04, 50 mM NaF, 1% protease inhibitor cocktail (Roche) and 1% phosphatase inhibitor mixture (Sigma). Supernatants were clarified by centrifugation and incubated with specific antibodies overnight and A/G agarose beads (Santa Cruz) or protein G magnetic beads (Biorad) for 3 h. Immunoprecipitates were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Biosciences). Membranes were probed with primary antibodies overnight at 4° C

and proteins were visualized using ECL detection kit (Amersham Biosciences) as previously described [39]. ERβ1 and its truncations were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) and the precipitates were immunoblotted with a rabbit antibody against p53 (Cell signaling). For the reverse experiments, antibodies against p53 (DO-1 or Pab 240; Santa Cruz) were used for immunoprecipitation and ERβ1 antibodies (rabbit polyclonal, Millipore or 14C8, GeneTex) for immunoblotting. MYC-tagged p53V143A and its truncations were immunoprecipitated with anti-MYC antibody (Cell signaling). Antibodies against p63 and ΔNp63 were from Abcam (BC4A4) and Biolegend, respectively. Antibodies against SHARP-1, Follistatin were from Santa Cruz and that of CCNG2 from Abcam.

2.2.5 Chromatin immunoprecipitation

Protein-DNA complexes were crosslinked with 1% formaldehyde for 10 min at room temperature (RT). Cells were harvested and cell suspensions were centrifuged. For nuclei purification, cell pellets were washed (PBS, NCP1, and NCP2 buffers) and incubated in lysis buffer (10 mM EDTA, 20 mM Tris-HCl pH 8.1, 0.5 mM Empigen, 1 mM SDS, and 1% protease inhibitor mixture) for 10 min at RT. Chromatin in the nuclear extract was sheared by sonication, clarified by centrifugation and the supernatant was used for immunoprecipitation as

previously described [53]. Chromatin-bound mutant p53 was incubated with an anti-p53 antibody or IgG and precipitated with protein G magnetic beads. Anti-FLAG M2 magnetic beads (Sigma) were used for the precipitation of the chromatin-bound ERβ1. Protein-DNA complexes were eluted and de-crosslinked at 70° C overnight and DNA enrichment was measured by real-time PCR using promoter-specific primers (Supplementary Table 1).

2.2.6 Protein expression and GST pull-down assay

GST-tagged proteins were produced as described previously [54]. Briefly, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture of transformed bacterial cells with pGEX4T-1-p53143Arecombinant plasmid to induce expression of GST-tagged protein. Protein interaction was assessed using a GST pulldown kit (Thermo Scientific) according to the manufacturer's protocol. GST-tagged proteins were used as bait to capture the full length or ERβ1 truncations that were expressed in vitro using the TnT® Quick Coupled Transcription/Translation System (Promega). Glutathione agarose beads were used to precipitate the GST-tagged protein complexes overnight. The interacting proteins were eluted and subjected for analysis to SDS-PAGE and immunoblotting.

2.4.7 Migration and invasion assays

In the wound-healing assay, the cell monolayer was scratched with a pipette tip to form the wound. Images of the wound were taken when the cells were scraped and 12 h later, and the area of cell migration in the wound was measured. Invasion assay was performed as described previously [27]. Cells were plated in matrigel-coated 6.5 mm Transwell champers (BD Biosciences). Six hours later, the cells that had invaded through the filter and attached to its bottom surface were stained with crystal-violet and counted in five independent fields in each Transwell.

2.4.8 Statistical analysis

Student's t-test and ANOVA were used for statistical analysis. Statistical significance was obtained when p-value ≤ 0.05.

2.3 Results

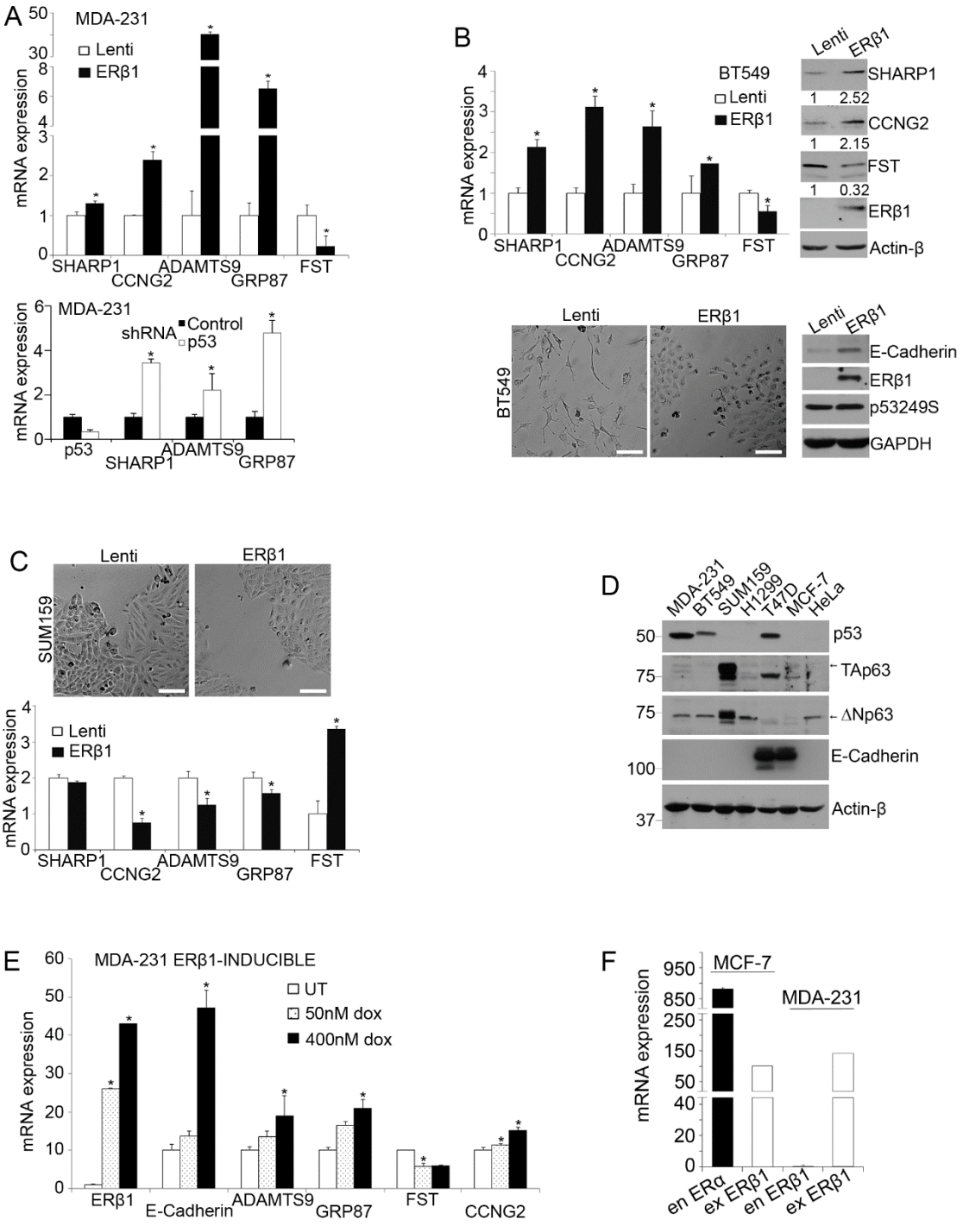
2.3.1 Anti-migratory activity of ERβ1 correlates with inhibition of mutant p53 function

In the present study we searched for ERβ1-interacting proteins and target genes that may account for the decreased invasiveness of ERβ1-expressing TNBC cells [26, 27]. We focused on mutant p53 signaling since p53 is frequently mutated in TNBC and mutant p53 proteins promote tumor metastasis [10, 12, 17, 28]. We used

as an indicator of mutant p53 gain-of-function the expression of genes that are regulated by mutant p53. We focused on those genes that inhibit metastasis in breast cancer including SHARP-1 and the ERα-regulated CCNG2 [3, 10, 29–31] and the pro-metastatic factor Follistatin [32]. As shown in Figure 2.1A (top), expression of ERβ1 in mutant p53 (p53280K)-expressing MDA-MB-231 cells upregulated SHARP-1, CCNG2, and the tumor suppressor ADAMTS9 [33] and downregulated Follistatin. The relevance of mutant p53 to the expression of these genes was further demonstrated by the upregulation of SHARP1, ADAMTS9, and GRP87 following knockdown of mutant p53 in MDA-MB-231 cells (Figure 2.1A, bottom). A similar gene expression signature was observed following upregulation of ERβ1 in another TNBC cell line. BT549 cells have mesenchymal-like morphology and express a different hot spot p53 mutant (p53249S). The changes in the expression of SHARP-1, CCNG2, and Follistatin mRNAs were also confirmed at the protein level (Figure 2.1B, top). In addition to altering the expression of metastasis-associated genes, ERβ1 induced epithelial transformation in these cells as it did in MDA-MB-231 cells (Figure 2.1B, bottom) [27]. In contrast to mutant p53-expressing TNBC cells, ERβ1 did not alter the epithelial-like morphology of the p53 null SUM159 TNBC cells (Figure 2.1C, top). In these cells, ERβ1 was found to regulate the expression of Follistatin, ADAMTS9, and CCNG2

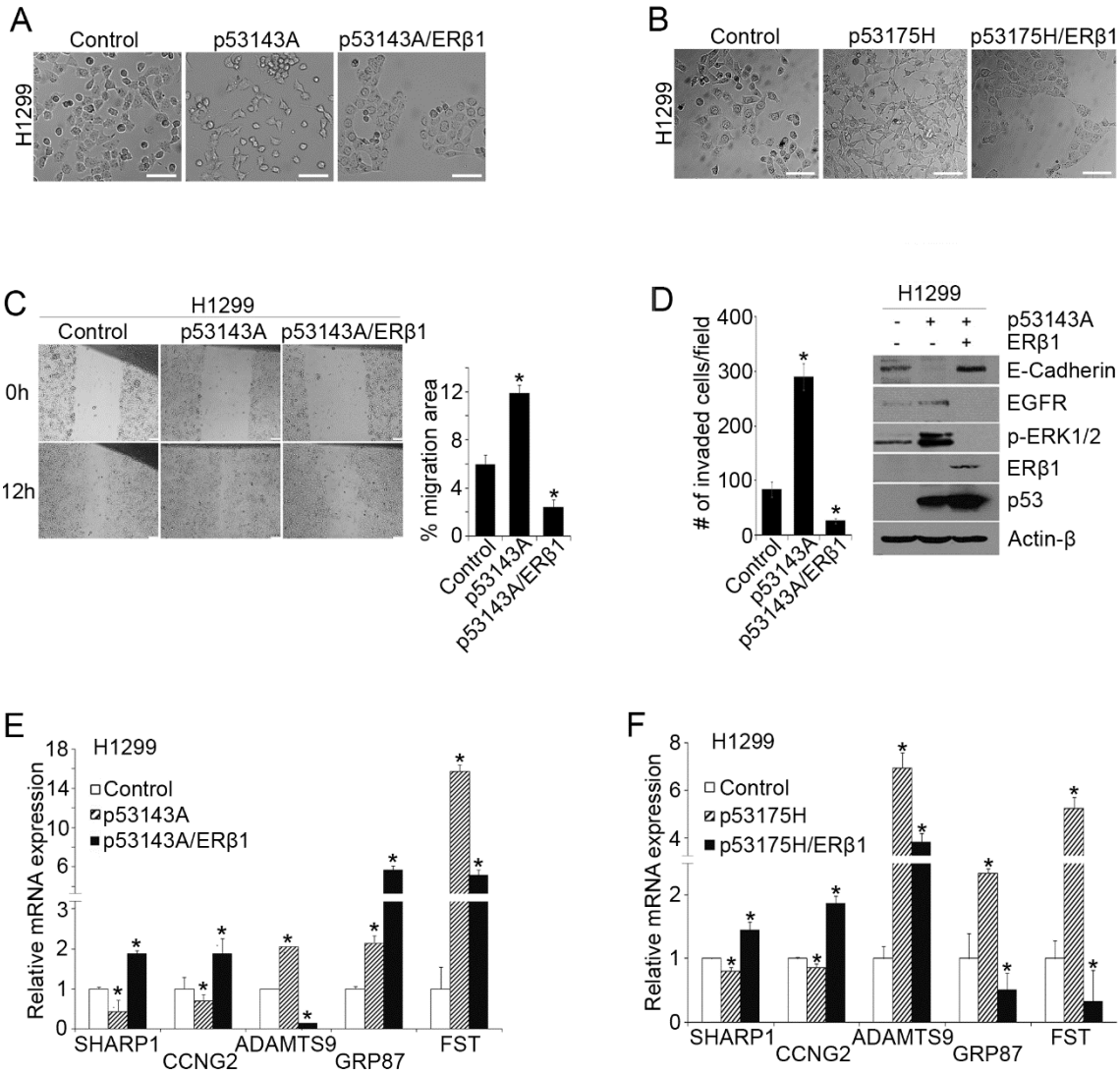
in opposite direction compared with the mutant p53-expressing cells (Figure 2.1C, bottom). This may be associated with a different mode of gene regulation in ERβ1-expressing cells that lack p53 and may depend on p63 isoforms that target the same group of genes and show unique expression in these cells (Figure 2.1D) [10]. We also analyzed the same genes in MDA-MB-231 cells with differential expression of ERβ1. Gradual upregulation of ERβ1 resulted in a progressive increase of ADAMTS9, GRP87, and CCNG2 and decrease of Follistatin levels (Figure 2.1E and Supplementary Figure 2.1). We previously showed that the levels of the transfected ERβ1 in TNBC cells are comparable with those of the endogenous receptor in MCF-7 cells [27]. In the present study, we compared the levels of the transfected ERβ1 in TNBC cells with the expression of endogenous ERα in MCF-7 cells, an indicator of biologically relevant ER expression in breast cancer cells. As seen in Figure 2.1F, the transfected ERβ1 in TNBC cells is expressed at lower levels compared with the endogenous ERα in MCF-7 cells. Despite an expected variation in the expression due to transfection, these results indicate the relevance of the ERβ1 expression system to human breast cancer cells.

Figure 2.1: ERβ1 regulates mutant p53 target genes. (A–C) TNBC cells were stably infected with lentivirus containing an empty vector (Lenti) or pLenti-FLAG-ERβ1 plasmid (ERβ1). (A) Top: The mRNA levels of mutant p53 target genes were quantified in control (Lenti) and ERβ1-expressing MDA-MB-231 (MDA-231) cells by real-time PCR and normalized to control cells (Follistatin, FST). Values are mean ± standard deviation (S.D.) of three independent experiments; *P ≤ 0.05. Bottom: mRNA levels of mutant p53 target genes in MDA-MB-231 cells after transfection with p53shRNA. (B) Top: mRNA and protein levels of mutant p53 target genes in control and ERβ1expressing BT549 cells. Band intensities were analyzed by densitometry and normalized to Actin-β. The numbers under each immunoblot show the fold change compared to the control cells and represent the median of three experiments. Bottom: Morphology (left) and protein levels of ERβ1, mutant p53249S and E-cadherin (right) in control and ERβ1-expressing BT549 cells (scale bars, 100 μm). (C) Morphology (top) and mRNA levels of mutant p53 target genes (bottom) of control and ERβ1-expressing p53 null SUM159 cells. Values represent the mean ± S.D. of three independent experiments; *P ≤ 0.05. (D) Protein levels of p53, TAp63, ΔNp63 and E-cadherin in wild-type cell lines. I MDA-MB-231 cells were stably infected with lentivirus containing the pINDUCER-FLAG-ERβ1 plasmid and left untreated (UT) or treated with 50 nM or 400 nM doxycycline for 24 h for gradual induction of ERβ1 expression. Graph indicates the mean of three experiments; *P ≤ 0.05. (F) mRNA levels of exogenous (ex) ERβ1 in MCF-7 and MDA-MB-231 cells are compared with those of endogenous (en) ERα in MCF-7 cells. Graph shows the mean of three independent experiments.


A
MDA-231
Lenti
ERβ1
mRNA expression
SHARP1
CCNG2
ADAMTS9
GRP87
FST
MDA-231
shRNA
Control
p53
p53
SHARP1
ADAMTS9
GRP87
B
BT549
Lenti
ERβ1
mRNA expression
SHARP1
CCNG2
ADAMTS9
GRP87
FST
Lenti
ERβ1
SHARP1
1
2.52
CCNG2
1
2.15
FST
1
0.32
ERβ1
Actin-β
BT549
Lenti
ERβ1
Lenti
ERβ1
E-Cadherin
ERβ1
p53249S
GAPDH
C
Lenti
ERβ1
SUM159
Lenti
ERβ1
mRNA expression
SHARP1
CCNG2
ADAMTS9
GRP87
FST
D
MDA-231
BT549
SUM159
H1299
T47D
MCF-7
HeLa
p53
TAp63
ΔNp63
E-Cadherin
Actin-β
E
MDA-231 ERβ1-INDUCIBLE
UT
50nM dox
400nM dox
mRNA expression
ERβ1
E-Cadherin
ADAMTS9
GRP87
FST
CCNG2
F
MCF-7
MDA-231
mRNA expression
en ERα
ex ERβ1
en ERβ1
ex ERβ1

To corroborate our findings, we examined the effects of ERβ1 upregulation on mutant p53 function in H1299 lung cancer cells. These cells are null for p53 and undergo mesenchymal reprogramming after mutant p53 expression [10, 12, 34]. We established pooled colony cell lines that express the frequently altered in human cancers p53 gain-of-function mutants p53143A and p53175H alone or together with ERβ1 [35, 36]. Upregulation of ERβ1 reversed the mutant p53-induced mesenchymal-like phenotype (Figure 2.2A and 2.2B), the increase in cell migration and invasion (Figure 2.2C and 2.2D, left) [10, 12], the downregulation of the epithelial marker E-cadherin and upregulation of EGFR signaling (Figure 2.2D, right). In addition, expression of ERβ1 reversed the downregulation of SHARP-1 and CCNG2 and upregulation of Follistatin. A difference in the expression pattern of ADAMTS9 and GRP87 may be associated with a different function of p53 mutants in the presence of varying expression of TA and ΔNp63 (Figures 2.2E and 2.2F).

Figure 2.2: ERβ1 decreases cell invasion by regulating mutant p53 target genes. (A–B) H1299 cells after stable transfection with empty vectors (control), or recombinant mutant p53143A or p53143A and ERβ1 plasmids (A) as well as mutant p53175H or p53175H and ERβ1 plasmids (B) (scale bars, 100 μm). (C) Left: Migration in control, p53143A- and p53143A/ERβ1-expressing H1299 pooled colony cells was assessed with wound-healing assay. Right: The area of cell migration in the wound was measured at the time of scratching the cell monolayer and 12 h later using Image J software. Values represent the mean ± S.D. of fold changes in migration area (12 h/0 h) from three experiments; *P ≤ 0.05. (D) Left: Invasion was assessed in control, p53143A- and p53143A/ERβ1-expressing H1299 cells with matrigel-coated Transwell chambers. The cells that invaded were quantified in five independent fields. The graph indicates the mean (cell number per field) of three experiments; *P ≤ 0.05 indicated. Right: E-cadherin, EGFR and 66andomiz-ERK1/2 levels in control, p53143A- or p53143A/ERβ1-expressing H1299 cells. (E–F) mRNA levels of mutant p53 target genes in control, p53143A-, p53143A/ERβ1-, p53175H-, and p53175H/ERβ1-expressing H1299 cells (*Follistatin*, FST).

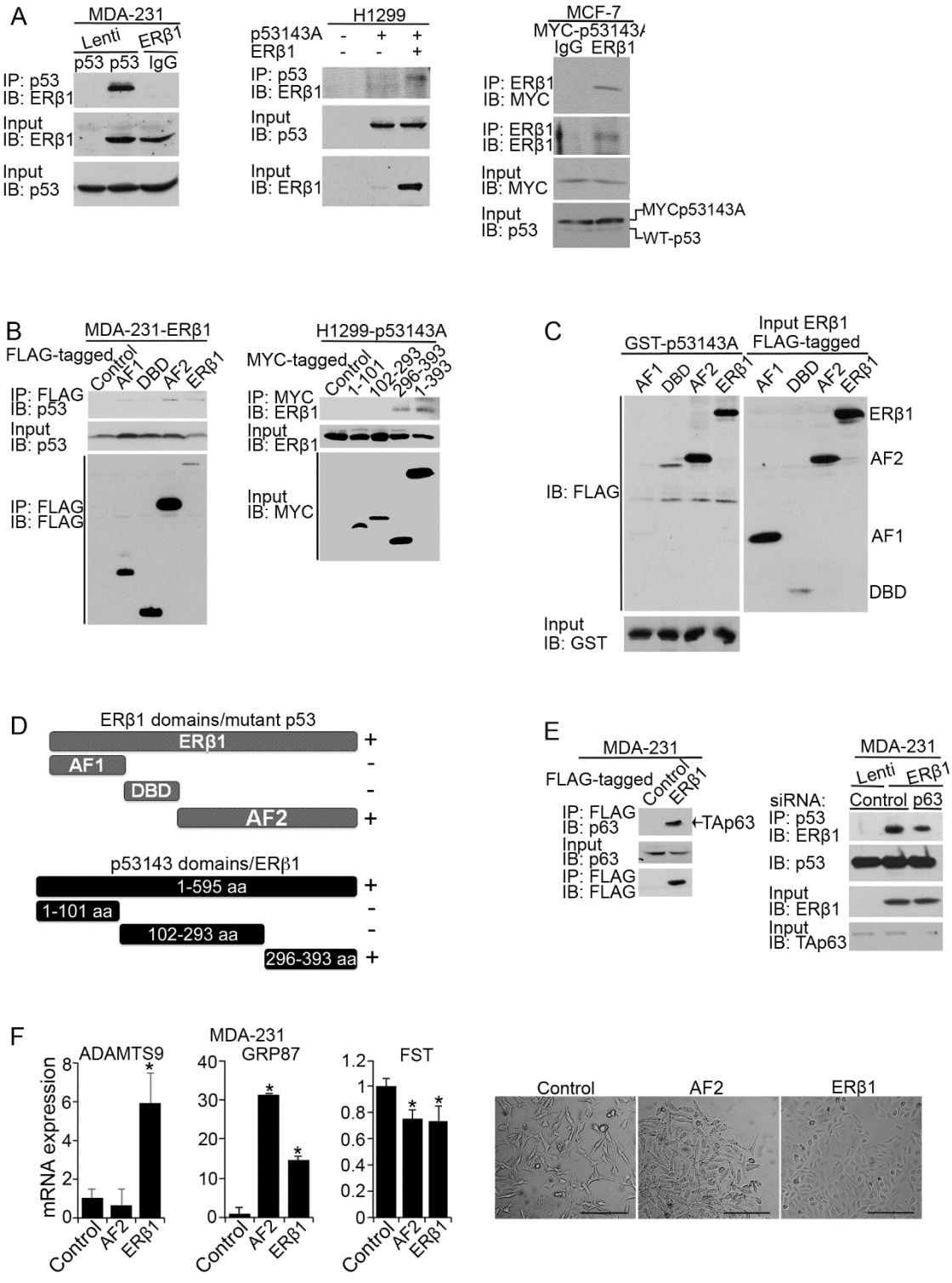

A
Control
p53143A
p53143A/ERβ1
H1299
B
Control
p53175H
p53175H/ERβ1
H1299
C
H1299
Control
p53143A
p53143A/ERβ1
0h
12h
% migration area
Control
p53143A
p53143A/ERβ1
D
H1299
# of invaded cells/field
p53143A
ERβ1
E-Cadherin
EGFR
p-ERK1/2
ERβ1
p53
Actin-β
Control
p53143A
p53143A/ERβ1
E
H1299
Control
p53143A
p53143A/ERβ1
Relative mRNA expression
SHARP1
CCNG2
ADAMTS9
GRP87
FST
F
H1299
Control
p53175H
p53175H/ERβ1
Relative mRNA expression
SHARP1
CCNG2
ADAMTS9
GRP87
FST

2.3.2 ERβ1 interacts with mutant p53

A transcriptional cooperation between ERβ and p53 was previously associated with the binding of the proteins to their cognate response elements [22]. To examine whether an ERβ1-mutant p53 binding occurs, we performed co-immunoprecipitation (CoIP) experiments in lysates from p53280K-expressing MDA-MB-231 and p53143A-transfected H1299 cells. As seen in Figure 3A, an interaction between ERβ1 and p53 mutants was observed in both MDA-MB-231 and H1299 cells. In addition to transfected ERβ1, mutant p53 bound to endogenously expressed receptor in MCF-7 cells (Figure 3A, right). To define the interacting regions of the two proteins, we examined a series of N-terminal, C-terminal and DBD deletion mutants of ERβ1 and p53143A. Analysis of MDA-MB-231 cells expressing ERβ1 domains and H1299 cells expressing p53143A truncations revealed that the C-terminus (296–393 aa) of mutant p53 and AF2 of ERβ1 are indispensable for their interaction (Figure 2.3B and Supplementary Figure 2.2). GST pull-down experiments with GST fusion mutant p53 expressed in bacteria and in vitro translated ERβ deletion mutants confirmed the AF2 of ERβ1 as the mutant p53 interacting region (Figure 2.3C and 2.3D). In addition to ERβ1, the C-terminus of mutant p53 was previously shown to bind to many other proteins and is required for the inhibition of p63 function and promotion of

invasion [3]. Given that mutant p53 is tethered to specific chromatin regions through p63 [16], we examined whether ERβ1 binds to p63. As seen in Figure 3E, p63 interacted with ERβ1 and silencing of p63 in MDA-MB-231 cells decreased the association of ERβ1 with mutant p53. These findings suggest a role for p63 in the regulation of the ERβ1-mutant p53 interaction. Furthermore, we tested whether the AF2 of ERβ1 alone is able to suppress mutant p53 function. As seen in Figure 3F (left), the expression patterns of two of the mutant p53 target genes (GRP87, Follistatin) in AF2-expressing MDA-MB-231 cells were similar with those in cells expressing full length ERβ1. This was associated with the more epithelial-like morphology of AF2-expressing cells, suggesting that the AF2 is essential for the p63 affects the regulation of mutant p53 function by ERβ1.

Figure 2.3: ERβ1 interacts with mutant p53 and p63. (A) Lysates from control and ERβ1-expressing MDA-MB-231 cells (left) or control, p53143A-, and p53143A/ERβ1-expressing H1299 cells (middle) were immunoprecipitated with anti-p53 antibody, followed by immunoblotting with ERβ1 antibody. Right: Lysates from MCF-7 cells transfected with MYC-p53143A were immunoprecipitated with anti-ERβ1 antibody or IgG, followed by immunoblotting with anti-MYC or anti-ERβ1 antibodies. The bottom panels are the input controls of cell lysates. (B) Left: Lysates from MDA-MB-231 cells stably expressing FLAG-tagged full-length ERβ1 or its activation function 1 (AF1), DNA-binding (DBD) and activation function 2 (AF2) domains were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-p53 and anti-FLAG antibodies. Right: Lysates from H1299 cells stably co-transfected with empty vectors (control) or full-length ERβ1 together with MYC-tagged full-length mutant p53143A or its N-terminal (1–101 aa), DBD (102–293 aa) and C-terminal (296–393 aa) domains were immunoprecipitated with anti-MYC antibody followed by immunoblotting with anti-ERβ1 antibody. (C) GST-tagged p53V143A expressed in bacteria was used as bait protein to capture in pull-down assay in vitro translated FLAG-tagged full-length ERβ1 or its AF1, DBD, and AF2 domains. (D) Schematic representation of ERβ1 truncations interacting with mutant p53 (top) and mutant p53 truncations interacting with full-length ERβ1 (bottom). I Left: Lysates from control and FLAG-tagged ERβ1-expressing MDA-MB-231 cells were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-p63 and anti-FLAG antibodies. Right: Lysates from control and ERβ1-expressing MDA-MB-231 cells after transfection with control or p63 siRNA (#1) were immunoprecipitated with anti-p53 antibody, followed by immunoblotting with ERβ1 antibody. (F) mRNA levels of ADAMTS9, GRP87, and Follistatin (FST) (Left) and morphology (right) of MDA-MB-231 cells expressing full-length ERβ1 or its AF2 domain (scale bars, 200 μm).


A
MDA-231
Lenti
ERβ1
p53 p53 IgG
IP: p53
IB: ERβ1
Input
IB: ERβ1
Input
IB: p53
H1299
p53143A - + +
ERβ1 - - +
IP: p53
IB: ERβ1
Input
IB: p53
Input
IB: ERβ1
MCF-7
MYC-p53143A
IgG ERβ1
IP: ERβ1
IB: MYC
IP: ERβ1
IB: ERβ1
Input
IB: MYC
Input
IB: p53
MYCp53143A
WT-p53
B
MDA-231-ERβ1
FLAG-tagged
Control AF1 DBD AF2 ERβ1
IP: FLAG
IB: p53
Input
IB: p53
IP: FLAG
IB: FLAG
H1299-p53143A
MYC-tagged
Control 1-101 102-293 296-393 1-393
IP: MYC
IB: ERβ1
Input
IB: ERβ1
Input
IB: MYC
C
GST-p53143A
Input ERβ1
FLAG-tagged
AF1 DBD AF2 ERβ1 AF1 DBD AF2 ERβ1
IB: FLAG
ERβ1
AF2
AF1
DBD
Input
IB: GST
D
ERβ1 domains/mutant p53
ERβ1 +
AF1 -
DBD -
AF2 +
p53143 domains/ERβ1
1-595 aa +
1-101 aa -
102-293 aa -
296-393 aa +
E
MDA-231
FLAG-tagged
Control ERβ1
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Lenti ERβ1
siRNA: Control p63
IP: p53
IB: ERβ1
IB: p53
Input
IB: ERβ1
Input
IB: TAp63
F
ADAMTS9
mRNA expression
Control AF2 ERβ1
MDA-231
GRP87
Control AF2 ERβ1
FST
Control AF2 ERβ1
Control
AF2
ERβ1

2.3.3 p63 affects the regulation of mutant p53 function by ERβ1

p53 mutants have been shown to promote invasion by preventing normal TAp63 function [10, 12, 28]. Given that ERβ1 inhibited both invasion and mutant p53 function in TNBC cells, we investigated whether activation of TAp63 is essential for the anti-invasive activity of ERβ1. We initially found that ERβ1 alters the expression of the p63 target genes K14 and BCL-2 in MDA-MB-231 cells that express both TA and ΔNp63 (Figure 2.4A and 2.1D) [37]. Importantly, transfection with siRNA that downregulates both p63 isoforms reversed to a significant extent the epithelial-like morphology and upregulation of E-cadherin in ERβ1-expressing MDA-MB-231 cells (Figure 2.4B and 2.4C and Supplementary Figure 2.3) [27]. In addition, knockdown of p63 significantly reversed the ERβ1-induced expression of the mutant p53 target genes SHARP-1, ADAMTS9, and CCNG2. In contrast, the levels of Follistatin were further decreased which may reflect a different effect of TAp63 and ΔNp63 downregulation on the expression of some of the ERβ1/mutant p53-regulated genes (Figures 2.4C and 2.1A). These results suggest that p63 is involved in the mechanism employed by ERβ1 to inhibit the invasive phenotype of TNBC cells and are consistent with the regulation of mutant p53-ERβ1 interaction by p63 (Figure 2.3E). To examine whether ERβ1 regulates p63 target genes in a mutant p53-independent manner, we analyzed the expression of

SHARP-1, CCNG2, and Follistatin in p53 null H1299 cells that express endogenous p63 (Figure 2.1D). As seen in Figure 4D and 4E, upregulation of ERβ1 altered the expression of these genes and the morphology of cells in absence of p53, albeit to a lesser degree than in presence of mutant p53 suggesting that ERβ1 can directly act on p63.

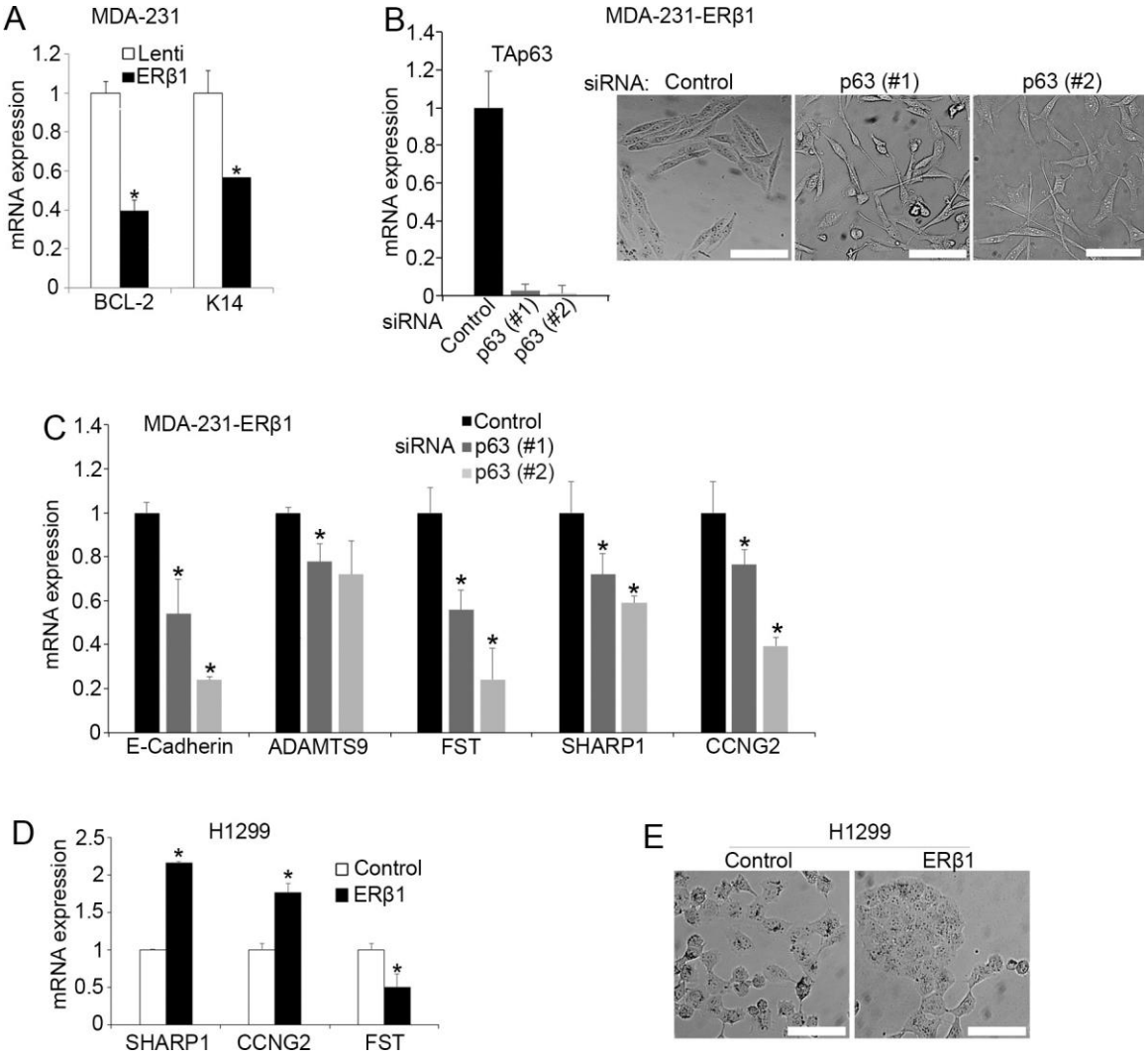


Figure 2.4: p63 affects the regulation of mutant p53 by ERβ1. (A) mRNA levels of the p63 target genes K14 and BCL-2 in control and ERβ1-expressing MDA-MB-231 cells. The graph shows the mean of three experiments; *P ≤ 0.05. (B) Levels of TAp63 and morphology of ERβ1-expressing MDA-MB-231 cells following transfection with control or two siRNAs targeting p63 (scale bars, 100 μm). (C) mRNA levels of E-cadherin and mutant p53 target genes in ERβ1-expressing MDA-MB-231 cells after transfection with p63 siRNA (Follistatin, FST). Values represent the mean ± S.D. of three experiments; *P ≤ 0.05. (D–E) mRNA levels of mutant p53 target genes and morphology of control and ERβ1-expressing H1299 cells (scale bars, 100 μm).

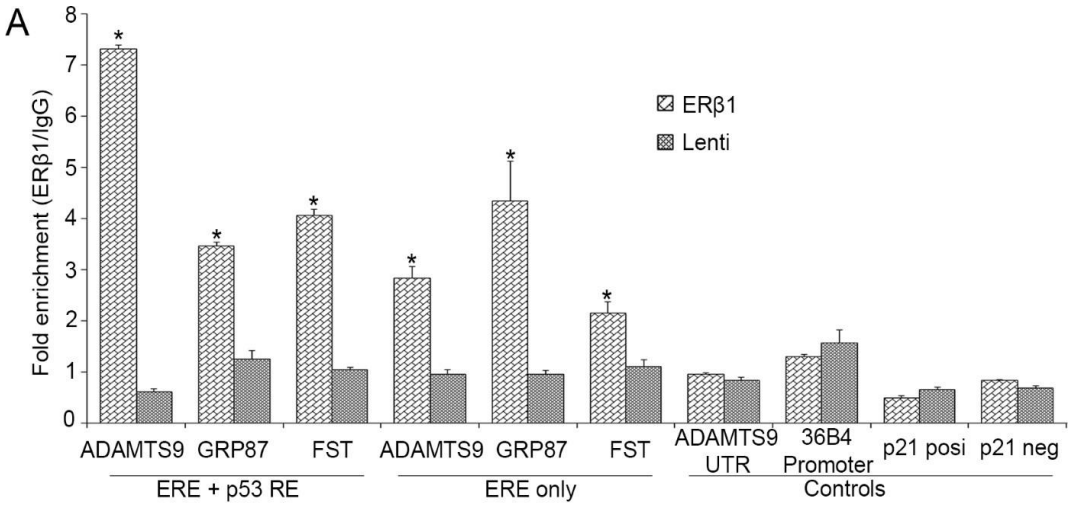
2.3.4 ERβ1 interferes with the regulatory elements of mutant p53/p63 target genes

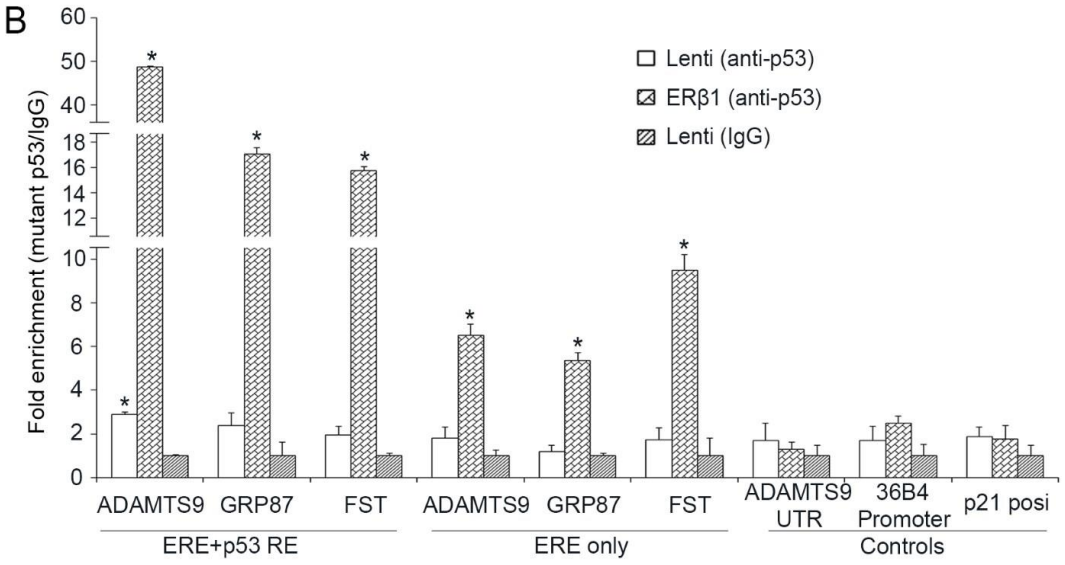
Given the binding of ERβ1 to both mutant p53 and p63, we investigated whether ERβ1 interacts with regulatory elements of mutant p53/p63 target genes. We performed chromatin-immunoprecipitation (ChIP) experiments in MDA-MB-231 cells to examine whether ERβ1 binds to sites that contain either both ERE and p53Res or exclusively ERE within the promoter region and close to the first exon of ADAMTS9, GRP87, and Follistatin genes (Supplementary Figure 2.4). We also analyzed ERE/p53Res-negative sites from 36B4 promoter and downstream of the ADAMTS9 gene as well as a 5′ p53RE from CDKN1A (p21) promoter that binds to wild-type p53 [38]. As seen in Figure 5A, a strong association of ERβ1 with ERE/p53Res-containing sites of ADAMTS9, GRP87, and Follistatin was detected in ERβ1-expressing cells. The association of ERβ1 with the ERE-containing sites of the same genes was also induced in ERβ1-expressing cells, albeit at significantly lower degree in case of ADAMTS9 and Follistatin. These results suggest that both direct DNA binding and interaction with mutant p53 may be necessary for ERβ1 to associate with promoters of mutant p53 target genes (Figure 2.5A).

To examine whether upregulation of ERβ1 alters the binding of mutant p53 to promoters of its target genes, we performed ChIP for p53 in control and ERβ1-

expressing MDA-MB-231 cells. As seen in Figure 5B, in contrast to p21 promoter, the association of mutant p53 with ADAMTS9, GRP87, and Follistatin sites was dramatically induced in ERβ1-expressing cells. This suggests that ERβ1 may specifically increase the binding of mutant p53 to genes that are associated with metastasis. An enrichment of the mutant p53-bound sequences that contain exclusively ERE motifs was also observed in ERβ1-expressing cells, suggesting that mutant p53 may tether to specific DNA sequences through ERβ1 (Figure 2.5B). To examine whether p63 alters the binding of ERβ1 to regulatory elements of mutant p53 target genes, we performed ChIP in ERβ1-expressing MDA-MB-231 cells following knockdown of p63. As shown in Figure 2.5C, the binding of ERβ1 to ERE/p53Res of ADAMTS9, GRP87, and Follistatin promoters was induced by p63 downregulation. In contrast, p63 knockdown decreased the ERβ1 association with the ERE motif of GRP87 promoter suggesting that p63 may regulate direct ERβ1-DNA binding (Figure 2.5C). Taken together, these results suggest an interaction of ERβ1 with promoters of mutant p53 target genes, which is regulated by mutant p53 and p63.

Figure 2.5: ERβ1 binds to promoters of mutant p53 target genes. (A) Chromatin immunoprecipitation (ChIP) analysis in control and ERβ1-expressing MDA-MB-231 cells for the presence of ERβ1 at sites of mutant p53 target genes that contain both ERE and p53Res or exclusively ERE (Follistatin, FST). ERE/p53Res-negative sites from 36B4 promoter and downstream of the ADAMTS9 gene and a 5′ p53RE from p21 promoter that binds wild-type p53 were used as controls. Anti-FLAG antibody was used to immunoprecipitate ERβ1 and normal mouse IgG was used as experimental control. Fold enrichment of p53 target sequences was normalized to IgG ChIP. (B) ChIP analysis for binding of mutant p53 at sites of mutant p53 target genes in control and ERβ1-expressing MDA-MB-231 cells. (C) ChIP analysis for the presence of ERβ1 at sites of mutant p53 target genes in control and ERβ1-expressing MDA-MB-231 cells following transfection with control or p63 siRNA (#1). Fold enrichment of target sequences in ERβ1 precipitates was normalized to that of IgG precipitates. All graphs represent the mean ± SEM of three experiments; *$P \leq 0.05$.


A
Fold enrichment (ERβ1/IgG)
ERβ1
Lenti
ADAMTS9
GRP87
FST
ADAMTS9
GRP87
FST
ADAMTS9 UTR
36B4 Promoter
p21 posi
p21 neg
ERE + p53 RE
ERE only
Controls


B
Fold enrichment (mutant p53/IgG)
Lenti (anti-p53)
ERβ1 (anti-p53)
Lenti (IgG)
ADAMTS9
GRP87
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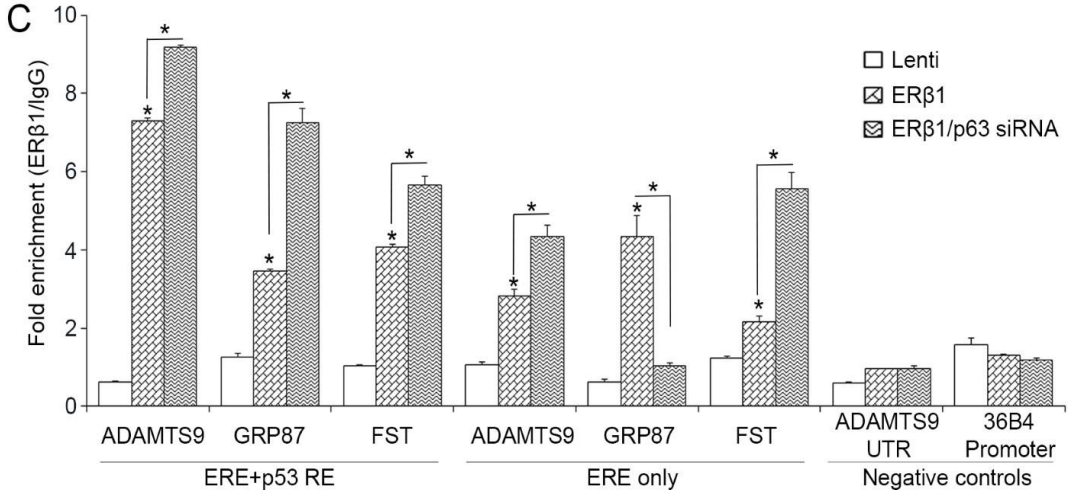
ADAMTS9
GRP87
FST
ADAMTS9 UTR
36B4 Promoter
p21 posi
ERE+p53 RE
ERE only
Controls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Fold enrichment (ERβ1/IgG)
Lenti
ERβ1
ERβ1/p63 siRNA
ADAMTS9
GRP87
FST
ADAMTS9
GRP87
FST
ADAMTS9 UTR
36B4 Promoter
ERE+p53 RE
ERE only
Negative controls

2.3.5 Effects of ligands on ERβ1-mutant p53 interaction

Upregulation of ERβ1 in cancer cells elicits tumor repressive actions in both ligand-dependent and –independent manner. Similarly, ligand-dependent and –independent target genes were identified in ERβ1-expresing cancer cells [27, 34, 39–41]. In this study, we investigated whether ligands that bind ERβ1 alter the function of mutant p53. The ERβ1 agonists 17β-estradiol (E2), 5α-androstane-3β,17β-diol (3β-Adiol) and Diarylpropionitrile (DPN) but not the ERα antagonist tamoxifen significantly increased the ERE-dependent transcription in ERβ1-expressing MDA-MB-231 cells (Figure 2.6A). In contrast, tamoxifen significantly increased the expression of all the mutant p53 target genes in ERβ1-expressing cells but not in control cells (Figure 2.6B and 2.6C). This may suggest that tamoxifen increases ERβ1 transcriptional responses when the receptor cooperates with other transcription factors. In addition to tamoxifen, the ERβ1-selective agonist DPN altered the expression of SHARP-1, CCNG2, and Follistatin but not ADAMTS9 and GRP87 in the same mode as the upregulation of ERβ1 did (Figure 2.6B and 2.6C and Figure 2.1A and 2.1B). These results support the presence of a functional receptor in TNBC cells and the ability of ER ligands to regulate mutant p53 function.

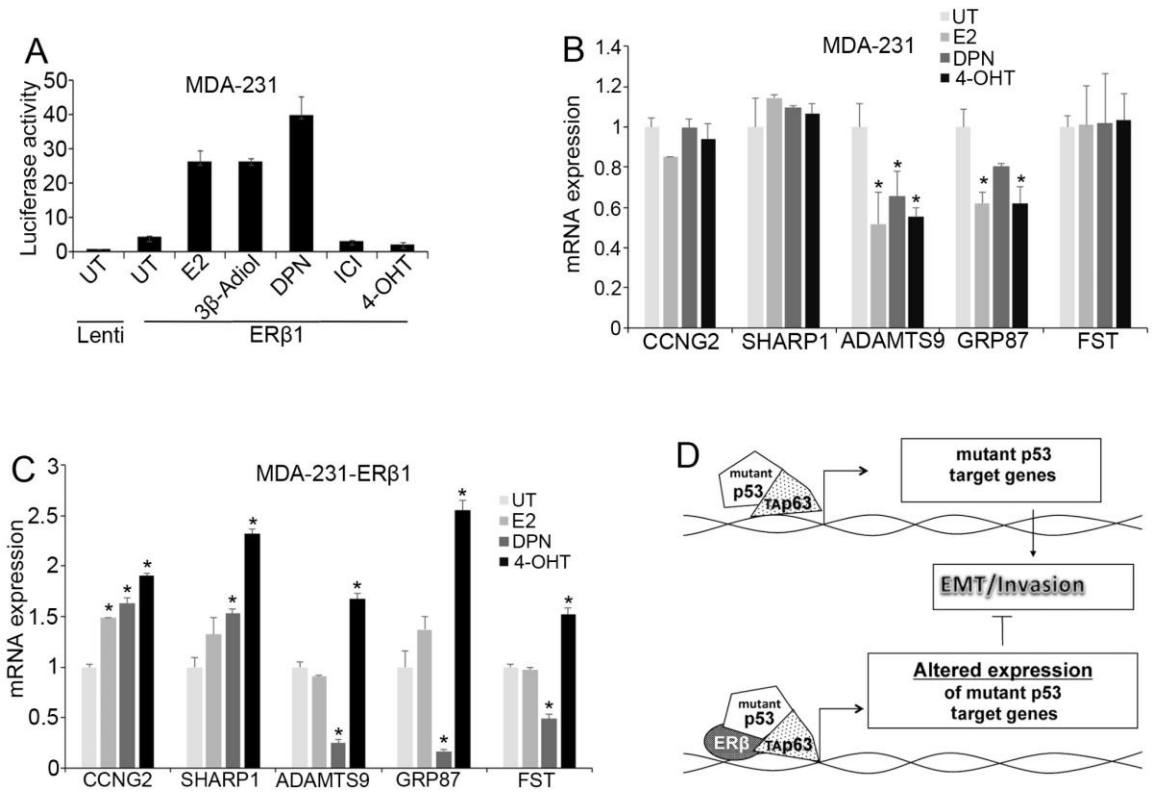


Figure 2.6: Effects of ER ligands on mutant p53 function. (A) ERE-driven luciferase activity in control and ERβ1-expressing MDAMB-231 cells after treatment with 10 nM 17β-estradiol (E2), Diarylpropionitrile (DPN), ICI 182780 (ICI) or 1 μM 5*α*-androstane- 3β,17β- diol (3β-Adiol) or 4-hydroxytamoxifen (4-OHT) for 24 h. The graph indicates the mean ± S.D. of three experiments. (B–C) mRNA levels of mutant p53 target genes in control (B) and ERβ1-expressing (C) MDA-MB-231 cells after treatment with 10 nM E2, 10 nM DPN or 1 μM 4-OHT in DCC-containing media for 24 h (Follistatin, FST). Values represent the mean ± S.D. of three experiments; *P ≤ 0.05. (D) Proposed mechanism illustrating the regulation of mutant p53 function by ERβ1 in breast cancer cells. Mutant p53 alters the activity of transcription factors including p63. This results in a gene expression program that promotes cell invasion. By interacting with mutant p53, ERβ1 alters mutant p53-dependent gene expression impeding EMT and inhibiting invasion.

2.4 Discussion

In the present study we examined whether inhibition of mutant p53 oncogenic function accounts for the decreased invasiveness of ERβ1-expressing TNBC cells.

We found that ERβ1 upregulates mutant p53 target genes that are associated with normal phenotype and decreases the expression of pro-metastatic factors. This effect was observed in TNBC and other human cancer cell lines that harbor different tumor-associated p53 mutations indicating the ability of the receptor to alter mutant p53-dependent transcription across different forms of mutant p53. Small divergence in the expression of some of the p53 target genes across ERβ1-expressing cell lines may be attributed to the different function of mutant p53 in cells with varying expression of TA and ΔNp63 and other cofactors.

We also examined whether an ERβ1-mutant p53 interaction is required to modify the mutant p53 function. A strong binding of ERβ1 to different p53 mutants was observed in triple-negative and other types of cancer cells. Further analysis revealed that the AF2 domain of ERβ1 interacts with the C-terminus of mutant p53. This region of mutant p53 binds to many other proteins [42, 43] and inhibits the function of p63 [18]. Our experiments showed that ERβ1 interacts with p63. This suggests that ERβ1 may repress mutant p53 function through its recruitment

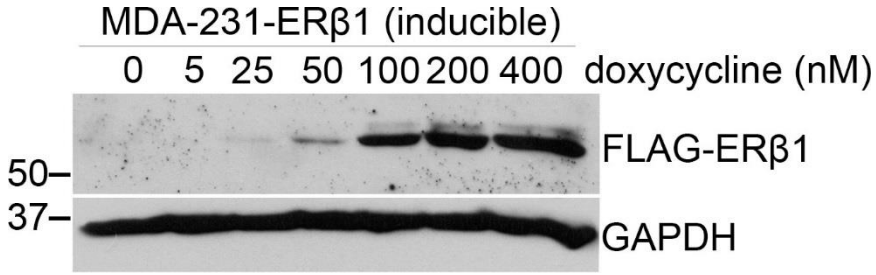
to mutant p53-p63 complexes at promoters of mutant p53/p63 target genes (Figure 2.6D). ChIP assays confirmed the association of ERβ1 with such promoters through both direct binding and tethering mechanisms. The direct binding is also supported by the ability of ERβ1 to regulate the same genes in absence of p53. Importantly, ERβ1 was also found to increase the association of mutant p53 with p53Res. Wild-type p53 binds the same promoters [16] and mutant p53 shows impaired binding to standard p53Res [3]. Thus, our findings may imply that the ERβ1-mutant p53 interaction readjusts the folding of mutant protein into a wild-type conformation that leads to reactivation of wild-type function. Notably, binding of mutant p53 to ERE motifs of the same promoters in the presence of ERβ1 suggests that mutant p53 may regulate ERβ1 transcriptional activity. This model of mutant p53 function was previously described with other transcription factors [3].

In this study, we propose that the anti-invasive activity of ERβ1 in breast and perhaps in other types of cancer cells may correlate with its ability to interact with oncogenic mutant p53. The relevance of this interaction to breast cancer may depend on the expression of the two proteins in tumor cells. Although the expression of ERβ has been suggested to decline in invasive carcinomas [44– 46], a significant of number of breast tumors including TNBCs express the receptor [19,

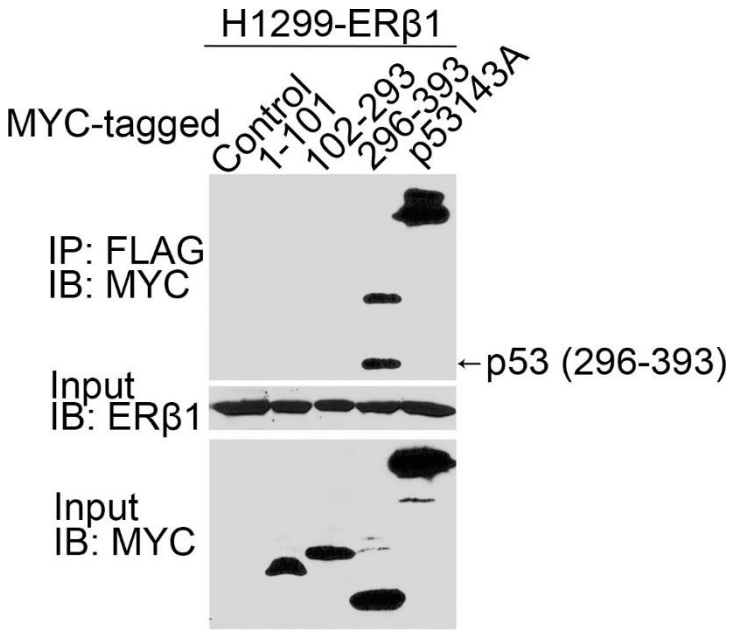
21, 47, 48]. In this study, lower levels of ERβ1 were found to interact with mutant p53 in TNBC cells compared with those of ERα that interact with wild-type p53 in ERα-positive breast cancer cells [24, 25]. Importantly, the ERβ1-mutant p53 binding was also detected in breast cancer cells that naturally express the receptor. The functionality of this interaction is supported by the ligand-dependent inhibition of mutant p53 function in ERβ1-expressing cells. This is consistent with the previously identified ligand-dependent ERβ1 target genes in TNBC cells [40]. Some of the ER ligands that act as ERα antagonists have been reported to induce ERβ1-mediated tumor repressive actions [40, 49–51]. The inhibitory effect of tamoxifen on mutant p53 function in the presence of ERβ1 suggests that patients whose p53-defective breast tumors are positive for the receptor may benefit from treatment with ER ligands. This effect may also explain the previously reported association of ERβ1 with better survival in patients treated with tamoxifen [19].

Further understanding of the mechanism that regulates the occupancy of both proteins at promoters of common target genes should establish ERβ1 as an important regulator of mutant p53 oncogenic function in breast cancer.

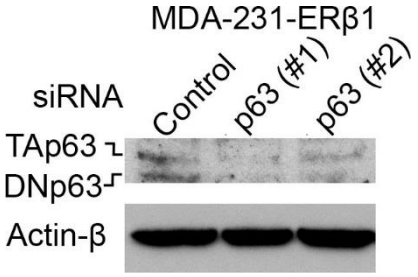
2.5 Supplementary figures



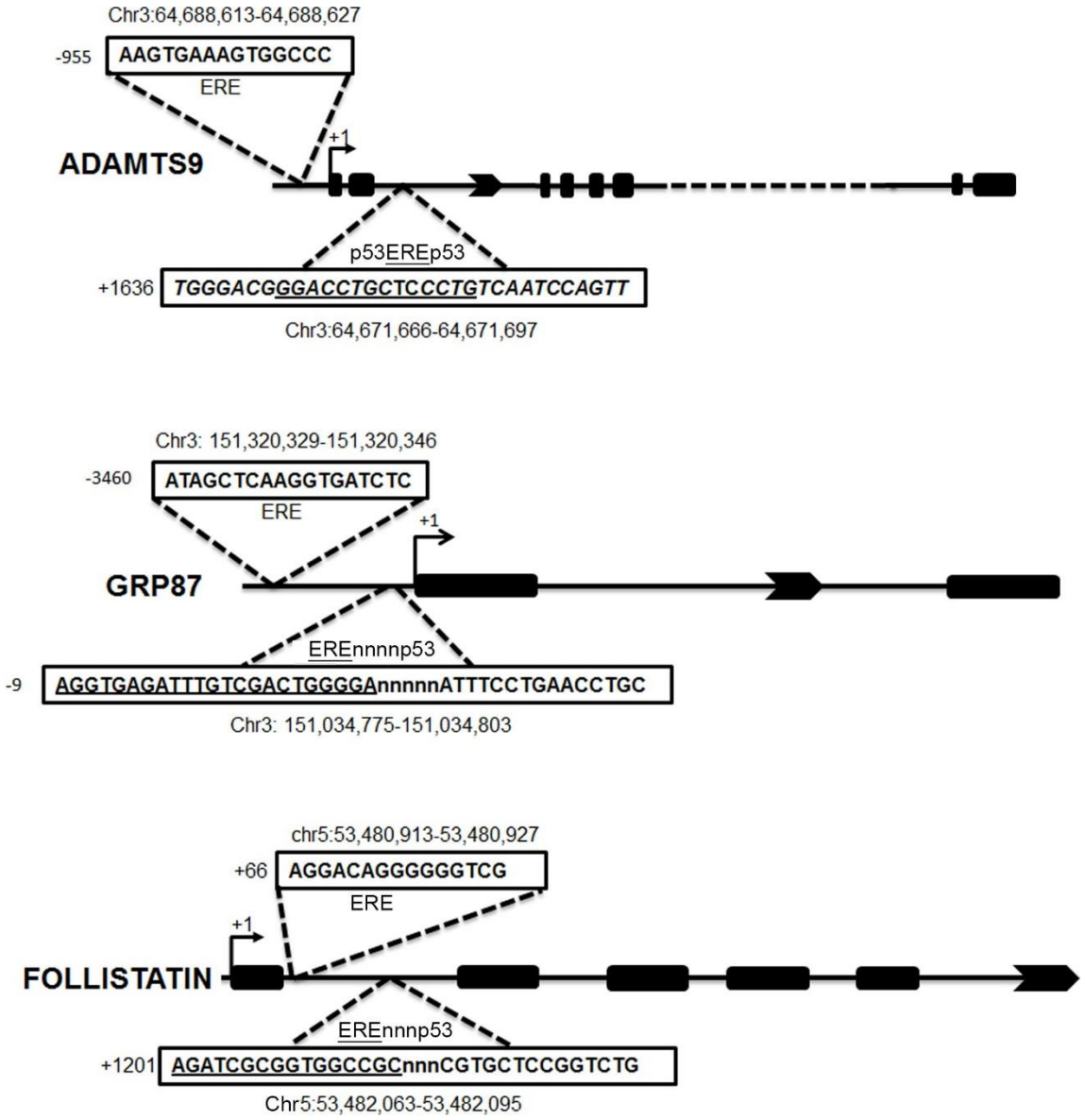
Supplementary Figure S2.1: **Gradual expression of ERβ1**. MDA-MB-231 cells were infected with lentivirus containing the pINDUCER20-FLAG-ERβ1 recombinant plasmid. Gradual ERβ1 expression was achieved following incubation of G418-selected cells with different concentrations of doxycycline (dox) for 24 h. ERβ1 expression was analyzed by immunoblotting.



Supplementary Figure S2.2: ERβ1 interacts with mutant p53. Lysates from H1299 cells stably co-transfected with empty vectors (control) or FLAG-tagged ERβ1 together with MYC-tagged full-length mutant p53143A or its N-terminal (1–101 aa), DBD (102–293 aa) and C-terminal (296–393 aa) domains were immunoprecipitated with anti-FLAG antibody followed by immunoblotting with anti-MYC antibody. The bottom panels are the input controls of cell lysates.



Supplementary Figure S2.3: siRNA-mediated downregulation of p63. Protein levels of TAp63 and ΔNp63 in ERβ1-expressing MDA-MB-231 cells after transfection with control or two siRNAs targeting p63.



Supplementary Figure S2.4: Binding sites of ERβ1 on mutant p53 target genes. ADAMTS9, GRP87 and FOLLISTATIN genomic sequences were extracted from University of California Santa Cruz (UCSC) genome browser. Gpminer and DBTSS were used to identify potential transcription start sites. The start sites of first exons were labeled as +1. Jaspar database was used to find potential EREs and p53/ p63Res. Their chromosomal locations and relative distances to the first exons are indicated. Schemes of GRP87 and ADAMTS9 have been adjusted to illustrate sequences of the negative strands. Values are given with an error of ± 1.

Supplementary Table S2.1: Oligonucleotides used in ChIP (5′⟶ 3′)

ERE + P53 binding motifs	**ADAMTS9-prom FW**	CAATTCAAAGCGTCCGGGG
	ADAMTS9-prom RV	TTATTCACGCGCCAGGAACC
	GRP87-prom FW	GT GAGATTT GT CG ACTGGGGA
	GRP87-prom RV	TCTAAGCTCCAGCCCTCTGT
	FST-prom FW	GCAGCCCTCGGAGATTTCG
	FST-prom RV	CCGTTTTGCAATCCGCCAGA
ERE only	**ADAMTS9-ERE FW**	CT CTT CCGT CCCCATCTCTTG
	ADAMTS9-ERE RV	GT GCC AAAT CT CTT CCCGAAT G
	GRP87-ERE FW	CCTT GTTGCATGTTCCAGCC
	GRP87-ERE RV	TGCACATATAAAGGGCGTCA
	FST-ERE FW	TT CAT GGAGGACCGCAGTG
	FST-ERE RV	AGACCACAGAAAAGTCGACCC
Controls	**ADAMTS9 dwst-UTR FW**	GAAACTGAGT GGATGCCTGC
	ADAMTS9 dwst-UTR RV	CAATATCAAGGGGCCTGGGA
	36B4-prom FW	GTTGAGCAACATTAGGCAAGC
	36B4-prom RV	ACAGGCACCTTTTTAAAATCTCATT
	P21-FW	CTGGACT GGGCACT CTT GT C
	P21-RV	CTCCTACCATCCCCTTCCTC

Supplementary Table S2.1: Oligonucleotides used in real-time PCR (5′⟶ 3′)

TAp63-FW 1	AAGATGGTGCGACAAACAAG
TAp63-RV 1	AGAGAGCATCGAAGGTGGAG
TAp63-FW 2	CCTGACCCTTACATCCAGCG
TAP63-RV 2	CGGTTCATCCCTCCAACACA
kl4-FW	GCAGTCATCCAGAGATGTGAC
kl4-RV	GCCTCAGTTCTTGGTGCGA
BCL-2-FW	GATAACGGAGGCTGGGATGC
BCL-2-RV	TCACTTGTGGCCCAGATAGG
SHARP1-FW	CGTCTTTGGAGTTGACATGG
SHARP1-RV	GGGCAGCTTTGAGAACTAGC
CCNG2-FW	TGGACAGGTTCTTGGCTCTT
CCNG2-RV	GATGGAATATTGCAGTCTTCTTCA
ADAMTS9-FW	GAACGCGACGGAGCATTAAC
ADAMTS9-RV	TAGAAACTGCTGGCCGAAGG
GRP87-FW	TCCTGACACGCATCTTTGCT
GRP87-RV	CCGTGCAGCTCGTTATTTGG
FST-FW	CAATGCCACTTATGCCAGCG
FST-RV	TCGGTGTCTTCCGAAATGGAG

2.6 Significance of Chapter II

In this chapter, we investigated a new mechanism used by ERβ1 to oppose cancer progression and metastasis in triple negative breast cancers (TNBCs). Because no FDA approved targeting therapy is available for TNBC patients, they have to rely on chemotherapy and/or radiotherapy and endure all the painful side effects of these treatments. We suggest that ERβ1 should be used as a diagnostic marker in breast cancer. With regard to the significant increase in survival of TNBC patients undergoing tamoxifen treatment, and considering our finding that Tamoxifen stabilizes ERβ1, we recommend treating TNBC cases with Tamoxifen especially when ERβ1 is expressed. Our study open new perspectives for targeting ERβ1 as well as its interaction with mutant p53. Overall, ERβ1 is a potential candidate that might be used as an alternative to the lack of targeted therapy in TNBC treatment.

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Chapter III

ERβ1 a co-regulator of wild-type p53 activity in luminal breast cancer

3.1 Introduction

Breast cancer is the predominant type of cancer in women worldwide. Early detection along with better clinical options have greatly increased survival rate in developed countries. Breast cancer has been divided into subtypes based on molecular features to provide more appropriate care options to patients. The large majority of diagnosed breast cancers (2/3) is classified as luminal implying that they are positive for the estrogen receptor α (ERα) and/or the progesterone receptor (PR) [1]. While the luminal A subtype is negative for the Human epithelial growth factor receptor (Her) 2, the luminal B subtype overexpresses that receptor. Luminal breast cancers have a higher survival rate compared to other breast cancer subtypes because they can be targeted by hormonal therapy and/or the anti-HER2 antibody Herceptin in the case of luminal B. Alas, a considerable number of the ERα-positive cancers develop resistance or fail to respond to tamoxifen [2]. For the most part, luminal cancers are wild-type for p53 (p53.free.fr). Hence, there might

be other mechanisms inhibiting the tumor suppressive function of p53. Several pathways have been reported to affect p53 activity [4, 5]. Mdm2, the key posttranscriptional regulator of p53 is upregulated in cancer, resulting in more pronounced p53 degradation [5]. In response to genotoxic stresses, p53 is phosphorylated by kinases such as ATM and ATR. P53 phosphorylation prevents its binding to E3 ubiquitin ligase Mdm2 and its subsequent degradation [6]. Therefore, mutations of p53 kinases prevent p53 stabilization and activation [4]. Although much less in known about p53 transcriptional regulators, methylation of TP53 have been observed [7]. p53 tumor suppressive function is mediated by transcription of target genes which include p21 and PTEN. Alteration of these genes, significantly dampen p53 function [8]. Altogether, p53 function depends on the normal activity of its upstream regulators and downstream effectors. Still, it is unclear how p53 tumor suppressive function is mitigated to allow tumor growth in most ERα-positive tumors that retain wild-type p53 alleles.

ERα abrogates p53 tumor suppressive activity as a result of interacting with the wild-type p53 protein. Upon radiation, this interaction was disrupted, thereby restoring p53 function [9, 38]. Further, c-MYC was also reported to mediate ERα-dependent estrogenic effects by inhibiting p21 which is critical for p53 induced cell-cycle arrest and apoptosis [10]. Crystal E. Berger and colleagues identified p53

as a transcriptional target of ERα, which contrast with previous finding suggesting ERα as inhibitory to wild-type p53 activity [11]. Hence, ERα-dependent inhibition of wild-type p53 in breast cancer might mainly be posttranscriptional. Although the role of ERα is still controversial, TCGA data analysis showed a significant decrease in p53 expression in luminal cancer while no significant difference was observed in ERα expression, indicating that ERα regulation is not the prominent determinant of p53 expression *in vivo*. One important transcription factor that is known influence ERα activity in breast cancer is ERβ1.

ERβ1 shares a large repertoire of regulated genes with ERα [12, 13]. ERβ1 can heterodimerize with ERα and oppose the effect of ERα on the transcription of certain estrogen responsive genes. [14, 15]. This inhibitory property of ERβ1 was associated with repression of growth of ERα-positive MCF-7 and T47D cells *in vitro* and *in vivo*, in xenograft models [40, 41]. According to TCGA database, ERβ expression decreases in ERα-positive breast tumors. A considerable number of clinical studies have found a positive correlation between ERβ1 expression and better disease free survival (DFS) and overall survival (OS) in both presence and absence of ERα [16-21]. However, the mechanisms that account for these associations are not fully understood. We have previously shown that ERβ1 interacts with mutant p53 and modulate its gain of function in ERα-negative breast

cancer cells [22]. The role of ERβ1 in regulating wild-type p53 function in ERα-positive tumors remains unexplored. Here we show that ERβ1 can enhance wild-type p53 tumor suppressor function. ERβ1 can affect p53 activity by both ERα-dependent and independent mechanisms. In addition, we found that ER ligands differentially regulate wild-type p53 activity. We thus propose ERβ as a potential therapeutic target to modulate p53 activity in luminal breast cancer.

3.2 Methods

3.2.1 Cell culture and constructs

MCF-7 were obtained from ATCC, MCF10A from Dr Preethi Gunaratne. Cells were kept in complete DMEM or DMEM/F12 media and supplemented with 10% FBS, D-Glucose; insulin and EGF were added to DMEM/F12 only. For ligand treatment, serum free DMEM media containing 1% DCC was used. Stable cell lines were produced as previously described [22].

3.2.2 Ligand and drug treatments

To assess ER activity, breast cancer cells were maintained in serum free 1% DCC media for 48 hours and treated with 17β-estradiol (E2), Diarylpropionitrile (DPN), ICI-182,780 (ICI) and 4-hydroxytamoxifen (4-OHT) alone or in combination with 17β-estradiol (E2) for 24 hours. To induce genotoxicity, Cisplatin was freshly

dissolved in DMSO and used at concentrations of 10 μM or 20 μM. Cells were stressed for 24 h in presence or absence of ER ligand.

3.2.3 RNA extraction and RT-PCR.

Total RNA was extracted using the Aurum Total RNA Mini Kit (Biorad). Copy DNA was generated from purified mRNA using the iScript cDNA Synthesis Kit (Biorad). Real-time PCR will be conducted using the VeriQuest Fast SYBR Green qPCR Master Mix (2X) (Affymetrix).

3.2.4 Co-immunoprecipitation and Immunoblotting

All co-immunoprecipitations, pull down assays and immunoblottings were done as previously described [22]. Briefly, GST tagged proteins were produces from bacteria (Rosetta) and solubilized by sonication combined with a freeze and thaw cycle. Glutathione sepharose beads were used for protein purification follow by *in vitro*-translated protein pull-down. Proteins were immunoblotted after electrophoresis using specific antibodies: p53 was detected with DO1 antibody obtained from Santa cruz, ERβ detection was possible using 14C8 (Genetex), polyclonal 51-7700 (Invitrogen) or anti-FLAG (Cell signaling) antibodies. For the GST pull-down experiment whole bacterial lysate was stained with Coomassie Brilliant Blue R-250 (Thermo) to detect transduced-protein expressions.

3.2.5 Statistical analysis

Student's t test and ANOVA were used for statistical analyses. Only p-value <0.05 were considered significant.

3.3 Results

3.3.1 ERβ1 regulates wild-type p53 transcriptional activity

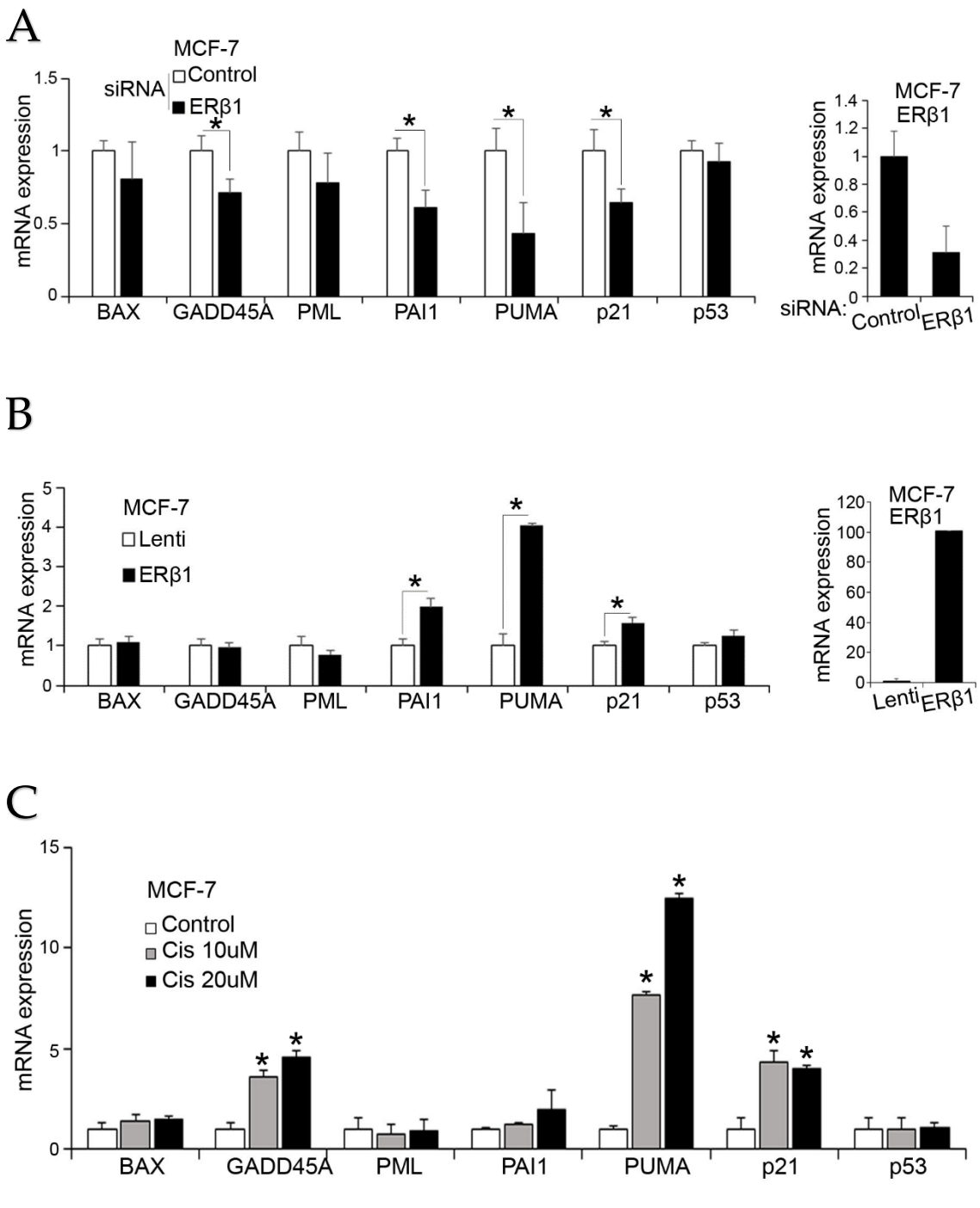
ERβ1 expression correlates with better survival in breast cancer [17, 20]. We, and others, have investigated the mechanism of ERβ1 action that may account for these associations, proposing the tumor suppressor function of the receptor [22-24]. In this present study, we transfected wild-type p53 breast cancer cells with scrambled (Control) or specific siRNA against ERβ1 and analyzed the cells by real time PCR for the expression of p53 target genes (Figure 3.1A). We found that down-regulation of ERβ1 decreased the expression of well-known p53 target genes. We observed decreased mRNA levels of Plasminogen activator inhibitor-1 (PAI-1), p53 upregulated modulator of apoptosis (PUMA), cyclin-dependent kinase inhibitor 1 ($p21^{Waf1}$), and growth arrest and DNA-damage-inducible alpha (GADD45A) in MCF-7 cells following downregulation of ERβ1 as seen in Figure 3.1A. These genes induce cell cycle arrest, DNA repair or cell death [25, 26, 28].

PAI-1 is required to drive replicative senescence in mouse and human fibroblasts [32]. PUMA, a Bcl-2 homology 3 (BH3)-only Bcl-2 family member, is a potent driver of apoptosis and is upregulated by transcription factors in response to stress. It primarily acts in mitochondria where it releases the pro-apoptotic proteins BAX and/or BAK from inhibition by anti-apoptotic factors [33]. p21 is the most studied p53 target gene. Its expression induces G1/S phase arrest [28]. GADD45A induces mitotic arrest in response to stress [39]. In contrast to PAI-1, PUMA, p21, and GADD45A, downregulation of ERβ1 did not significantly alter the expression of the p53 target genes Bcl-2-like protein 4 (BAX), Promyelocytic Leukemia Protein (PML), and p53 expression itself (Figure 3.1A). Consistent with the results in MCF-7 cells after ERβ1 downregulation, upregulation of ERβ1 induced the expression of PAI-1, PUMA, and p21 (Figure 3.1B). In contrast, GADD45A expression was not altered in ERβ1-expressing cells, which can be explained by the different transcriptional activity of the transfected ERβ1. Taken together these results strengthen our hypothesis that ERβ1 plays important role in wild-type p53-dependent transcription.

Further, to corroborate that the above transcriptional responses are p53-dependent, we exposed MCF-7 cells to genotoxic stress using different concentrations of cisplatin (0, 10, and 20 μM). Cisplatin is primarily known to

stabilize the protein levels of p53 and induce p53-dependent transcription [42]. As it was expected, strong increases in the expression of PUMA, GADD45A, and p21 were observed after cisplatin treatment. PAI-1 was slightly induced only after treatment with a higher concentration of cisplatin (20 μM) (Figure 3.1C). This result is consistent with previous studies and indicates the strong association between cisplatin-induced DNA damage and p53 activation [26]. On the other hand, BAX and PML did not respond to cisplatin treatment. BAX is known to be activated by cytoplasmic wild-type p53 to induce apoptosis through a mechanism that involves mitochondrial membrane permeabilization [29]. In addition to mediating p53 effects, PML embodies autonomous pro-apoptotic functions [30]. Based on these observations, it is possible that p53 affects the expression and the function of these genes at post-transcription level.

Figure 3.1: ERβ1 enhances transcription of wild-type p53 target genes. (A) mRNA expression of WT p53 target genes in wild-type (control) and ERβ1-siRNA knockdown MCF-7 cells (Left). ERβ1 expression in control and ERβ1-siRNA knockdown MCF-7 cells. Values represent the mean ± S.D. of three experiments; *P ≤ 0.05. (B) mRNA expression of WT p53 and its target genes in stably transfected control cells (Lenti) and ERβ1-expressing MCF7 (Left) and mRNA expression of ERβ1 in stably transfected control cells (Lenti) and ERβ1-expressing cells (Right). Values represent the mean ± S.D. of three experiments; *P ≤ 0.05. (C) Relative mRNA expression of p53 target genes after 24h treatment of wild-type MCF7 cells with 0uM (Control), 10uM and 20uM cisplatin. Values represent the mean ± S.D. of three experiments; *P ≤ 0.05


A
MCF-7
siRNA
Control
ERβ1
mRNA expression
1.5
1
0.5
0
BAX
GADD45A
PML
PAI1
PUMA
p21
p53
MCF-7
ERβ1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
siRNA:
Control
ERβ1
B
MCF-7
Lenti
ERβ1
5
4
3
2
1
0
BAX
GADD45A
PML
PAI1
PUMA
p21
p53
MCF-7
ERβ1
120
100
80
60
40
20
0
Lenti
ERβ1
C
MCF-7
Control
Cis 10uM
Cis 20uM
15
10
5
0
BAX
GADD45A
PML
PAI1
PUMA
p21
p53

3.3.2 ERβ1 enhances p53 activity in response to genotoxic stress.

The p53 protein is stabilized under stress that leads to DNA damage [31]. Therefore, it was important to evaluate the effect of ERβ1 on p53 activity under genotoxic stress conditions. We knocked down ERβ1 in MCF-7 cells that were exposed to either vehicle or 20 μM of cisplatin for 24 hours (Figure 3.2). In the absence of genotoxic stress, as it was seen above, ERβ1 downregulation caused a reduction in PAI-1, PUMA, p21 and GADD45A expression (Figure 3.1A). More importantly, ERβ1 downregulation prevented the cisplatin-mediated upregulation of the same genes, suggesting that ERβ1 regulates wild-type p53 activity both in the absence and presence of genotoxic stress. In contrast, as in the absence of stress, no significant change in mRNA expression was observed in the other p53 target genes that are BAX and PML. These results demonstrate a critical role of endogenous ERβ1 in regulating wild-type p53 transcriptional activity.

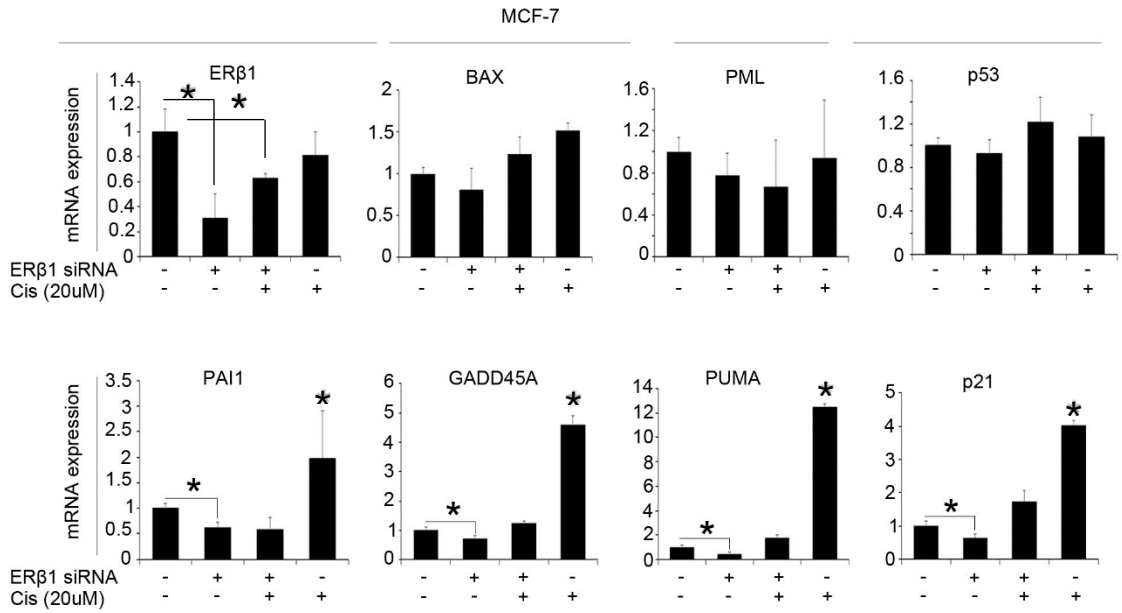


Figure 3.2: ERβ is necessary for p53 activity. (Top) relative mRNA expression of ERβ1 and p53 target genes non-responsive to cisplatin in MCF7 cells following ERβ1 siRNA knock-down and subsequent treatment with cisplatin (0 μM or 20 μM) for 24 h. (Bottom) relative mRNA expression of ERβ1 and p53 target genes responsive to cisplatin in MCF7 cells following ERβ1 siRNA knock-down and subsequent treatment with vehicle or 20 μM cisplatin for 24 h. Values represent the mean ± S.D. of three experiments; *P ≤ 0.05

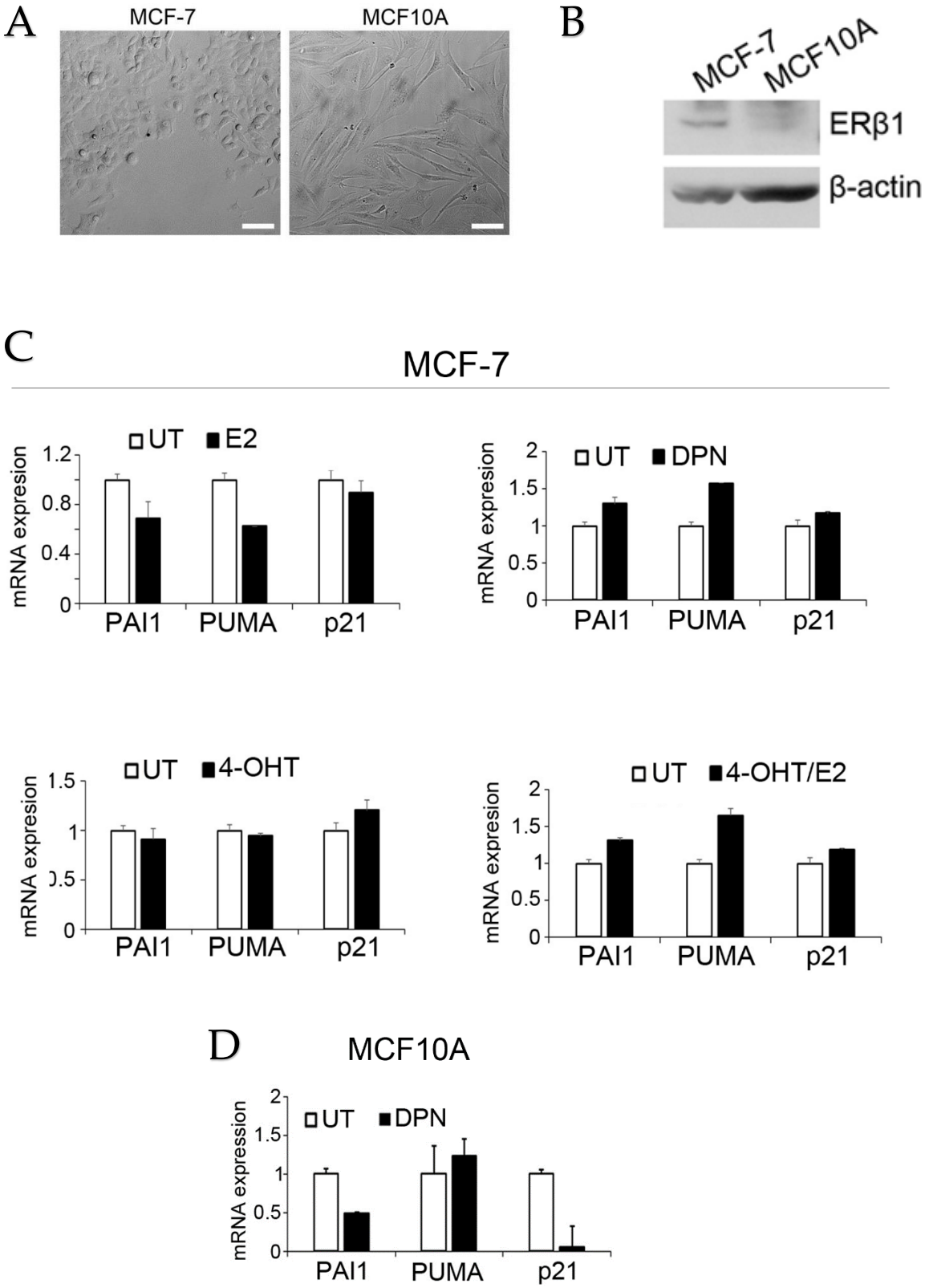
3.3.3 ER ligands modulate wild-type p53 function

To substantiate the importance of ERβ1 in regulating p53 tumor suppressive activity, we evaluated the effect of ER ligands. As seen in Figure 3.3A, we examined the effects of ER ligands on p53 activity in luminal cell line MCF-7 and the immortalized mammary epithelial cell line MCF10A. MCF-7 are smaller in size than MCF10A and grow in clusters which may be associated with the high expression of the epithelial marker E-cadherin [22, 37]. We evaluated the expression of ERβ1 in MCF10A that are already known to be negative for ERα. In contrast to MCF-7 cells that express relatively low levels of endogenous ERβ1, the receptor could not be detected in MCF10A at protein level (Figure 3.3B). MCF-7 cells were treated for 24 hours with either vehicle, the ER ligand 17β-estradiol (E2), the ERβ1 selective agonist Diarylpropionitrile (DPN) or the selective estrogen receptor modulator 4-hydroxytamoxifen (Figure 3.3C) and the mRNA levels of PAI-1, PUMA, and p21 were analyzed by real-time PCR. E2 treatment inhibited expression of PAI-1 and PUMA, implying a negative effect of the ERα agonist on p53 activity (Figure 3.3C, top). Interestingly, treatment with DPN increased the expression of p53 target genes in a similar manner as ERβ1 upregulation did. The stronger gene expression changes in cells following transfection of ERβ1 compared with the ligand treatment may be explained by the moderate level of endogenous

ERβ1 in MCF-7 cells. 4-OHT treatment alone did not considerably affect p53 activity. However, when 4-OHT was used in combination with E2, 4-OHT reversed the E2-dependent decrease in p53 target genes, especially for PAI-1 and PUMA (Figure 3.3C, bottom).

To ascertain that the observed transcriptional responses following treatment with ERβ agonist were mediated by ERβ, we treated the ERβ1-negative MCF10A with DPN (Figure 3.3D). DPN treatment did not have any effect on PUMA, and in contrast to MCF-7 cells, inhibited PAI-1 and p21 expression. These results further indicate the regulation of p53-dependent gene expression by ERβ.

Figure 3.3: ER ligands modulate WT p53 function. (A) Morphology of wild-type MCF-7 and MCF10A cells (scale bars, 100 μm). (B) Lysates for MCF-7 and MCF10A were run and immunoblotted with ERβ1and p53 antibodies. β-actin was used as an internal control. (C) Relative mRNA expression of p53 target genes (PAI-1, PUMA, p21) following 24 h of MCF-7 treatment with solvent (UT), 10 nM 17-β estradiol (E2), Diarylpropionitrile (DPN), and 1 μM 4-hydroxytamoxifen (4-OHT) alone or in 17-β estradiol (E2). (D) Relative mRNA expression of p53 target genes following 24 h of MCF10A treatment with solvent (UT), and Diarylpropionitrile (DPN). Values represent the mean ± S.D. of three experiments.


A
MCF-7
MCF10A
B
MCF-7
MCF10A
ERβ1
β-actin
C
MCF-7
UT
E2
mRNA expresion
1.2
0.8
0.4
0
PAI1
PUMA
p21
UT
DPN
2
1.5
1
0.5
0
PAI1
PUMA
p21
UT
4-OHT
1.5
1
0.5
0
PAI1
PUMA
p21
UT
4-OHT/E2
2
1.5
1
0.5
0
PAI1
PUMA
p21
D
MCF10A
mRNA expression
UT
DPN
2
1.5
1
0.5
0
PAI1
PUMA
p21

3.3.4 ERβ specific agonist DPN and 4-OHT enhance p53 tumor suppressive function in luminal cells

To better illustrate the different effects of ligands on wild-type p53 tumor suppressive activity, the expression of PAI-1, PUMA, and p21 in response to different ligands were plotted in the same line chart (Figure 3.4). Each treatment affected the expression of p53 target genes in the same manner. While E2 tends to inhibit the expression of p53 target genes, the ERβ ligand DPN was significantly upregulating them. Moreover, 4-OHT did not show any effect alone, but induced a considerable increase in the presence of E2 (Figure 3.4A). Conversely, MCF10A cells which lack classical ER expression, did not show the pattern in gene expression that was observed in ERβ-positive MCF-7 cells in the presence of ERβ ligand (Figure 3.4B).

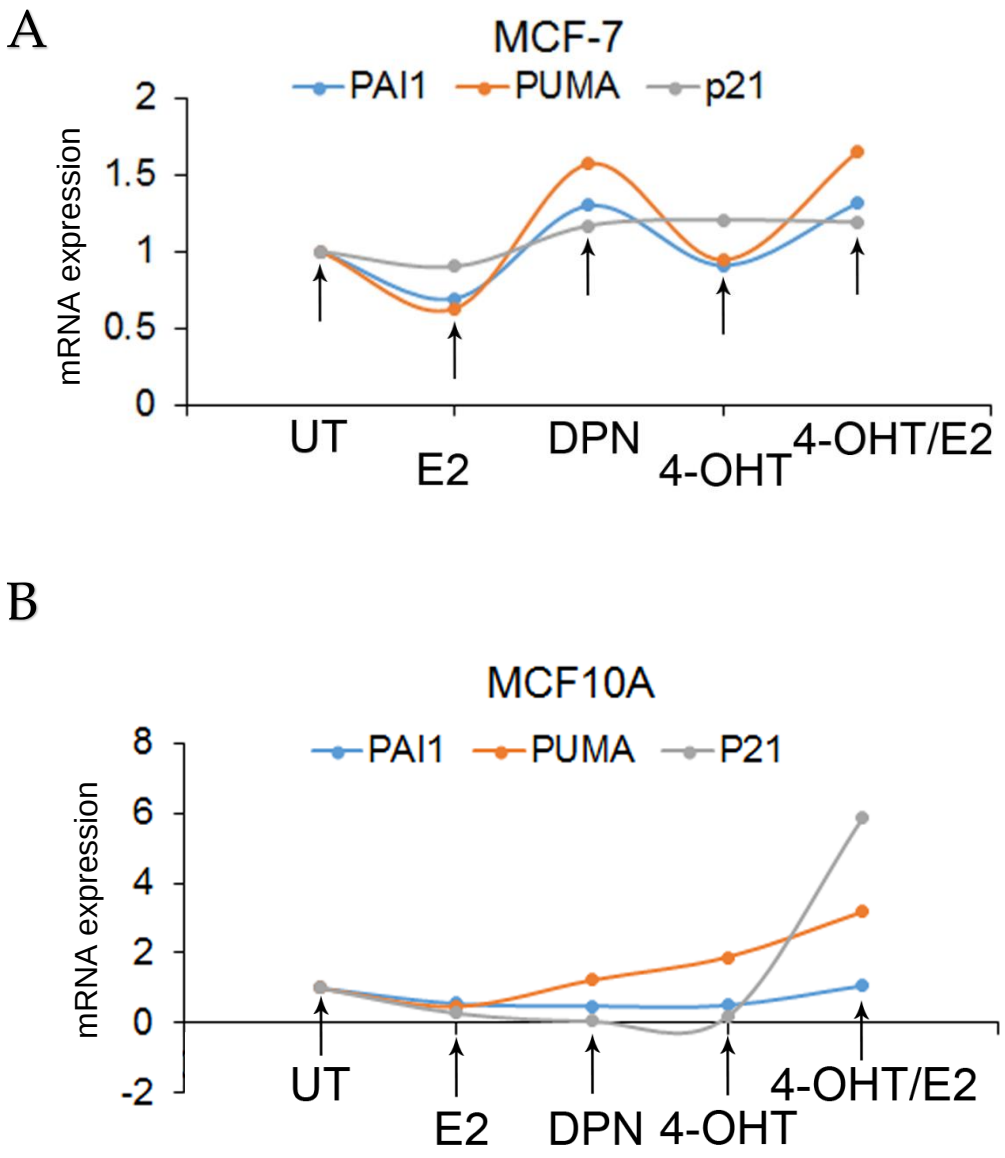


Figure 3.4: ERβ1 activation correlates with increased wild-p53 response. mRNA expressions of PAI-1, PUMA, and p21 were assessed from MCF-7 (A) and MCF10A (B) after treating the cells with vehicle (UT), 10 nM 17β-estradiol (E2), Diarylpropionitrile (DPN), 1 μM 4-hydroxytamoxifen (4-OHT) alone or with 17β-estradiol (4-OHT/E2) and normalizing them to the untreated samples. mRNA levels following each treatment (dots), were plotted in the same line graph (Blue: PAI-1, Orange: PUMA, and Grey: p21).

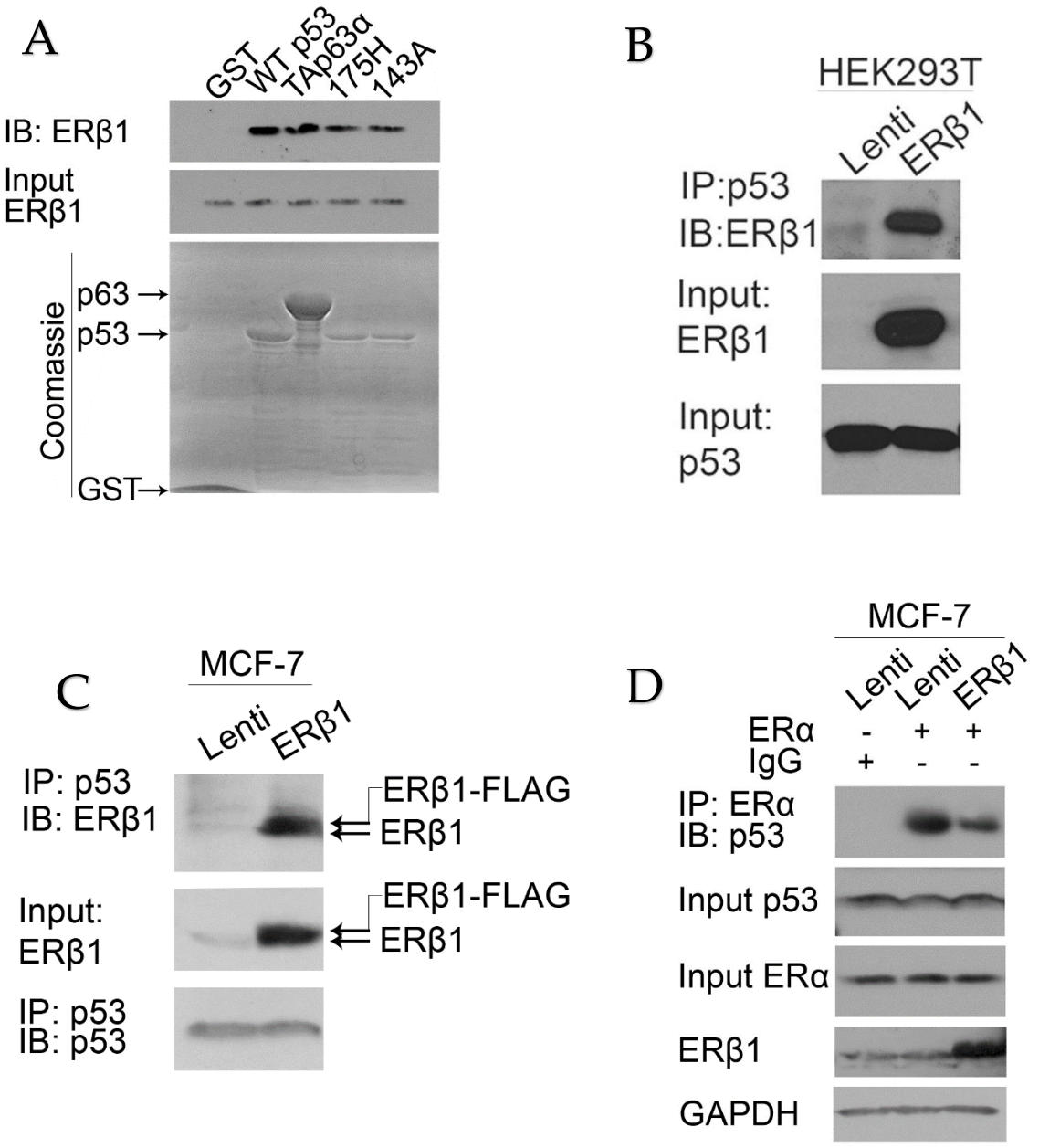
3.3.5 ERβ1 interacts with wild-type p53

To better understand the molecular mechanism employed by ERβ1 to regulate wild-type p53 function, we examined for possible interactions between the two proteins. We previously showed association between ERβ1 and the C-terminal domain of p53 that has missense mutations in its DBD [22]. This implies that ERβ1 may interact with wild-type p53. Indeed, GST pull-down assay showed a direct interaction between ERβ1 and wild-type p53 (Figure 3.4A). In addition to wild-type p53, the same analysis confirmed the binding of ERβ1 to TAp63, and p53 mutants R175H and V143A (Figure 3.4A). To validate the association of ERβ1 with wild-type p53 *in vivo*, we carried out a co-immunoprecipitation (CoIP) assay in ERβ1- transfected HEK-293 and MCF-7 cells that express endogenous WT p53. As seen in Figure 3.4B-C, in MCF-7 cells that express low levels of endogenous ERβ1, both the transfected ERβ1 which is FLAG-tagged and the endogenous ERβ1 were found to interact with wild-type p53. The interaction between the two proteins was also observed in HEK293 cells.

Although MCF-7 cells have low levels of endogenous ERβ1, they express high levels of ERα which was previously described to inhibit wild-type p53 activity [9]. To examine whether ERα is involved in the effect of ERβ1 on wild-type p53 function, ERα was immunoprecipitated in control (expressing the empty vector)

and in ERβ1-expressing MCF-7 cells. We found a strong reduction in interaction between ERα and p53 in ERβ1-expressing cells (Figure 3.4D). These results suggest that ERβ1 can induce p53 tumor suppressor function by interacting with p53 and regulating the interaction of ERα with p53.

Figure 3.5: ERβ1 interacts with wild-type p53. (A) ERβ1 interaction with p53 and p63. GST-tagged p53 (WT p53), TAp63, p53 mutant R175H, and V143A proteins were produced from rosetta bateria and purified using glutathione sepharose beads. The GST-protein and bead complex was used to pull down Flag-tagged ERβ1 generated from in vitro translation and all samples were denatured and used for electrophoresis (B) Lysate from empty vector (Lenti)- and ERβ1-expressing HEK293T cell was used to immunoprecipitate WT p53 and ERβ1 immunoblotting using p53 antibody and ERβ1 antibody, respectively. (C) Lysate from empty vector (lenti)- and Flag-tagged ERβ1-expressing cells MCF-7 were used to immunoprecipitate p53 followed by ERβ1 immunoblotting using p53 and ERβ1 antibodies. (D) Lysate from control cells (Lenti) and ERβ1 expressing MCF-7 were used for p53 immunoprecipitation and ERα immunoblot. GAPDH was used as a control.


A
GST
WT p53
TAp63α
175H
143A
IB: ERβ1
Input
ERβ1
Coomassie
p63 →
p53 →
GST→
B
HEK293T
Lenti
ERβ1
IP:p53
IB:ERβ1
Input:
ERβ1
Input:
p53
C
MCF-7
Lenti
ERβ1
IP: p53
IB: ERβ1
ERβ1-FLAG
ERβ1
Input:
ERβ1
ERβ1-FLAG
ERβ1
IP: p53
IB: p53
D
MCF-7
Lenti
Lenti
ERβ1
ERα - + +
IgG + - -
IP: ERα
IB: p53
Input p53
Input ERα
ERβ1
GAPDH

3.4 Discussion

In this study, we investigated the role of ERβ1 on wild-type p53 activity in luminal breast cancer cells. We found that ERβ1 regulates p53 target genes even in presence of ERα. It is known that a significant proportion of ERα-positive breast cancers become resistant to hormonal therapy [14]. ERα and wild-type p53 are both important transcription factors that exert opposite actions in cell growth. While wild-type p53 acts as a tumor suppressor by inducing apoptosis, cell-cycle arrest, and senescence and decreasing cell survival, ERα promotes cell proliferation and survival. Further, ERα was reported to sequester p53 through a protein-protein interaction. Importantly, the therapeutic potential of this association was demonstrated when the ERα-p53 interaction was disrupted under ionizing radiation allowing p53 to be more functional [9]. In contrast to ERα, upregulation of ERβ1 in cancer cells has been shown to elicit tumor repressive functions similarly to wild-type p53 [22]. The biological significance of ERβ1 in ERα-positive cells was demonstrated by several studies. ERβ shRNA knockdown was reported to promote cell growth in MCF-7 [41]. ERβ1 expression in T47D cells inhibited xenograft tumor growth and angiogenesis [40]. Our findings indicate that ERβ1 triggers an important p53 activation in ERα-positive breast cancer cells. Upregulation of ERβ1 alone was able to promote the transcriptional activity of p53.

These results may imply that ERβ1 is a potential indicator of the wild-type p53 function in ERα-positive breast cancers, and thus a potential marker that determines better outcome and response to endocrine therapy. More importantly, our findings show that ligands that activate ERβ1 can induce the expression of p53 target genes. This suggests the importance of ERβ1 as a potential therapeutic target for the treatment of luminal breast cancers that express wild-type p53.

One of the mechanisms we propose is that ERβ1 protects p53 tumor suppressor function from ERα-dependent inhibition. Two major facts can explain such a mechanism: ERα/ERβ dimerization and p53 interaction with ERβ1. It is well known that ERβ1 and ERα can heterodimerize [15]. Therefore, binding of ERβ1 to ERα will eventually reduce ERα nuclear availability and binding properties toward wild-type p53. The second option is based on ERβ1 affinity to p53. We previously demonstrated that ERβ1 directly interacts with mutant p53 [22]. Our recent findings indicate that ERβ1 also interacts with wild-type p53 (Figure 3.5C). This must create a competitive environment between both ERs in cells that have to share a common target. Hence, the ratio of ERβ versus ERα and their affinity to p53 are crucial factors that may determine p53 activity in estrogen responsive tissues. Elucidating the precise effects of ERs on p53 activity may have important

clinical implications in prognosis given that the ERβ/ERα ratios have previously been identified as determinants of clinical outcome.

3.5 Supplementary Table I: list of real-time PCR primers

	FW	RV
PUMA	AACTGAAAAAGAAACGGAATGGAA	CTCCCTGGGGCCACAAATC
BAX	GCCCTTTTGCTTCAGGGTTTC	TGAGACACTCGCTCAGCTTC
PAI1	ACAACCCCACAGGAACAGTC	GATGAAGGCGTCTTTCCCCA
PML	TGGACGAGAACCTTGCTGAC	GCTGGCTAATCTTCTGGGTTTC
GADD45A	AAGGATGGATAAGGTGGGGGA	CACGTTATCGGGGTCGACGTT

3.6 Significance of Chapter III

In this chapter, we demonstrated the importance of ERβ1 for the regulation of wild-type p53 activity. The increased expression of well-known p53 target genes seems to derive from a direct transcriptional cooperation between ERβ1 and wild-type p53. Most importantly, this association can function as a potent barrier against estrogen-induced ERα pro-proliferative function. With further investigations, ERβ expression in luminal cells might be used as a good marker to predict better survival and responses to endocrine therapy. ERβ1 positive response to ligands such as DPN might also open alternative ways to target resistance in ERβ-positive luminal cancers.

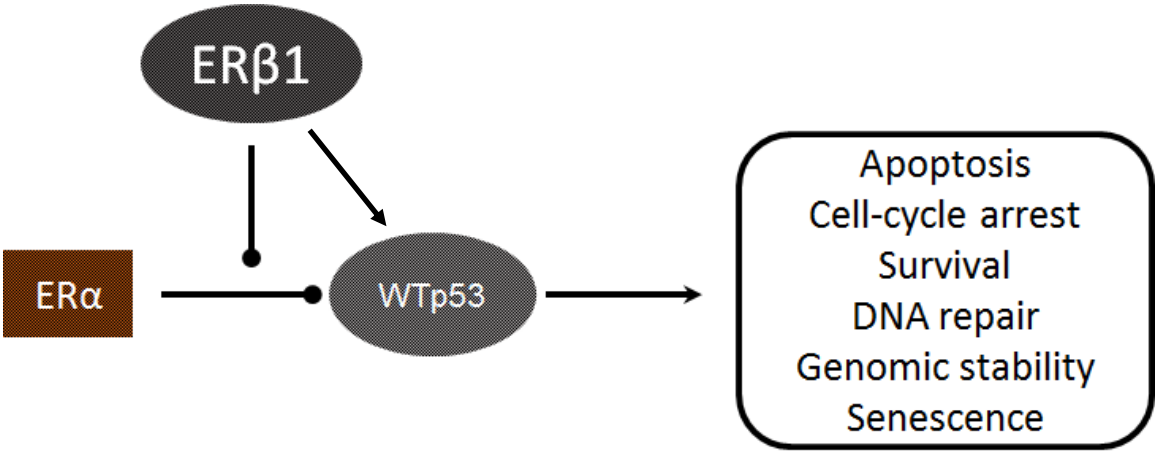


Figure 3.6: ERβ1 opposes ERα-dependent inhibition of p53 and enhances wild-type p53 function.

3.7 References

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Chapter IV

Global Conclusions

Through multiples studies, we have tried to understand some of the functions and elucidate some of the mechanisms of ERβ in breast cancer. We gathered significant pieces of evidence that indicate tumor suppressive roles of ERβ1 in cancer. We found that many of these tumor suppressive functions of ERβ1 associate p53. ERβ1 can enhance wild-type p53 tumor suppressive activity and promote cell-cycle arrest, DNA repair or cell death. Hence, ERβ1 may prevent cancer initiation especially in cancers expressing wild-type p53. Unfortunately, many mutations of p53 exist in cancer which make cancer cells more aggressive as seen in the case of TNBCs. Mutant p53 often loses its tumor suppressive functions and develops oncogenic properties. We observed that ERβ1 can oppose mutant p53 gain-of-function and restore some of the wild-type p53 functions. ERβ1 interacts with mutant p53 to modulate the expression of target genes involved in EMT, invasion and metastasis. Therefore, ERβ1 may oppose cancer progression, strengthening its role as a tumor suppressor. Overall, ERβ1 can protect cells from becoming cancerous and metastatic and appears as good candidate to improve cancer survival rate.

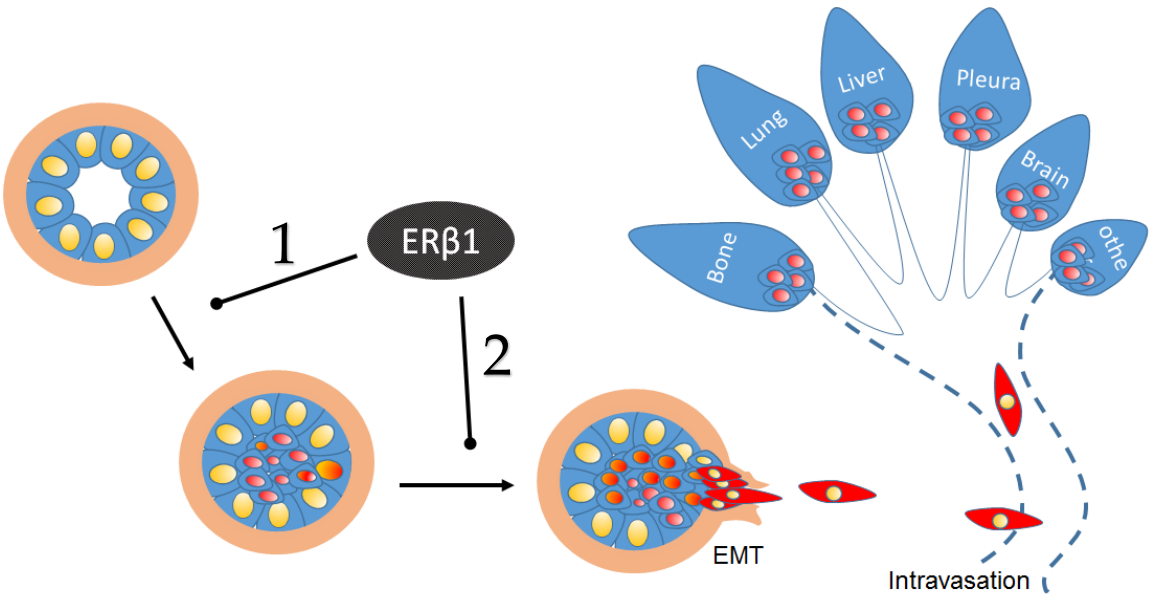

Figure 4.1: Tumor suppressive functions of ERβ1 in breast cancer. (1) ERβ1 inhibits genetic mutations and tumor formation. (2) ERβ1 inhibits epithelial to mesenchymal transition (EMT), invasion and metastasis.