IL-6/STAT3 MEDIATED DOWN-REGULATION OF ESTROGEN RECEPTOR ALPHA (ERα) EXPRESSION IN BREAST CANCER

by

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DEDICATION

I would like to dedicate this thesis to Jesus, my husband Sola Adeleye, my parents, Kanene & Philip Nnoli and my sister, Adaobi Nnoli. Your love, encouragement and support have seen me through the past few years.

ABSTRACT

Approximately seventy percent of the breast cancer patients present with estrogen receptor alpha (ERα) positive disease. Targeting ERα with hormonal therapy (HT) is effective in reducing the risk of disease recurrence and for treating disease. Expression levels of ERα vary in tumors and metastases, which correlates with response to HT. Thus, understanding the mechanisms underlying ERα expression could lead to improved therapeutic strategies. The tumor microenvironment has been shown to promote tumor growth and metastases by secreting cytokines and growth factors during disease progression. Interleukin-6 (IL-6). IL-6, a pleotropic pro-inflammatory cytokine has been described *in vivo* as a potent growth factor in ERα positive breast cancers. IL-6, which signals through the IL-6 and gp130 receptors leads to the tyrosine phosphorylation of signal transducer and activator of transcription (pStat3), a known mediator of tumorigenesis activated in a variety of cancers including breast cancers.

Using ERα positive breast cancer cell lines treated with IL-6 at various time points, we determined expression of ERα and ERα regulated genes via qRT-PCR and western blots. We also used chromatin immuno-precipitation (ChIP) to determine the presence of transcriptional modifiers on the ERα promoter and changes in histone marks.

We observed an inverse relationship between the IL-6/pStat3 signaling pathway and ERα expression in breast cancer patient samples and breast cancer cell lines. We show that in ERα positive breast cancer cell lines (MCF-7, T47D, BT-474), IL-6 induced activation of Stat3 led to the recruitment and binding of Stat3 to the ERα promoter. We demonstrated that Stat3 is acetylated and interacts to HDAC1 and known transcriptional repressor on the ERα promoter. We observed a decrease in ERα mRNA and protein, and a decrease in expression of ERα target genes (PGR, GREB1, RET, EGR3) as a

consequence of IL-6 signaling. Our results demonstrated that the recruitment of Stat3 and HDAC1 to the ERα promoter led to decreased histone H3K9 acetylation and H3K4 mono-methylation and increased histone H3K9 tri-methylation.

Our results suggest a model whereby Stat3, functions as a transcriptional repressor of ERα by recruiting the histone-modifying enzyme HDAC1 to the ERα promoter.

BIOGRAPHICAL SKETCH

Jennifer is the third child of Kanene & Philip Nnoli. She was born on the 31st of May 1985 in Lagos, Nigeria. At the age of sixteen, Jennifer and her siblings migrated to the United States of America to join their father who had migrated to the United States much earlier. Jennifer attended Torrance high school for a couple of years and after finishing high school, she enrolled at California State University, Northridge (CSUN) in the fall of 2003. After four years of studying at CSUN, Jennifer earned at bachelors degree in Chemistry. During her time at CSUN, Jennifer was awarded a MARC U*STAR scholarship which allowed her to work in a research lab as an undergraduate. Jennifer chose to work with Dr. Steven Oppenheimer, a cancer biologist, who studied the effects on carbohydrates on cell-cell interactions. Jennifer's time in the Oppenheimer lab fueled her interest in cancer research and led her apply to Gerstner Sloan-Kettering Graduate School of Biomedical Sciences for her PhD. In July of 2007, Jennifer moved to New York City and enrolled in Gerstner Sloan-Kettering, where she began the first year of her PhD.

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LIST OF ABBREVIATIONS

5-aza-dC: 5-aza-2'-deoxycytidine

Ac: Acetylation

CDC: Center for disease control

ChIP: Chromatin Immunoprecipitation

CNTF: Ciliary neurotrophic factor

CTRL: Control

DNMTs: DNA methyltransferases

DTT: Dithiothreitol

EGR3: Early growth response 3

EMSA: Electro-mobility shift assay

ERE: Estrogen response element

ERα: Estrogen receptor alpha

ESR1: Estrogen receptor 1 (gene name)

FoxM1: Forkhead box protein M1

FoxO3a: Forkhead box protein O3a

G-CSF: Granulocyte colony-stimulating factor

gp130: Glycoprotein 130

GREB1: Growth regulation by estrogen in breast cancer 1

H3K4Me1: Histone 3 mono-methyl (lysine 4)

H3K9Ac: Histone 3 acetyl (lysine 9)

H3K9Me3: Histone 3 tri-methyl (lysine 9)

HATs: Histone acetyltransferase

HDAC1: Histone Deacetylase 1

HP1: Heterochromatin protein 1

IC50: half maximum inhibitory concentration

IL-6: Interleukin-6

IVD: In vitro-methylated DNA

JAK: Janus Kinase

LIF: Leukemia inhibitory factor

MMP-9: Matrix metalloproteinase-9

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OSM: Oncostatin M

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

PGR: Progesterone receptor

PI: Protease inhibitor

PMSF: Phenylmethanesulfonylfluoride

qRT-PCR: Real-time polymerase chain reaction

RET: Rearranged during transfection

SD: Standard deviation

SERM: Selective estrogen receptor modulator

SOCS3: Suppressor of cytokine signaling 3

STAT3-C: Constitutively active Signal transducer and activator of transcription 3

STAT3: Signal transducer and activator of transcription 3

TNF: Tumor necrosis factor

TSA: Trichostatin A

TYK2: Tyrosine kinase 2

WGA: Whole genome amplification

CHAPTER ONE
INTRODUCTION

Breast cancer is the most common invasive cancer in females worldwide. It currently affects approximately 230,000 women and 2500 men in the United States each year. In 2009, approximately 41,000 women died from breast cancer, (CDC data). Currently, the survival rate of breast cancer depends on the stage at which the disease was discovered. Typically, cancers discovered at stage 0, I & II have a 90-100% survival rate, while those discovered at stage III have a 72% survival rate. Unfortunately, those discovered at much later stage IV have the lowest survival rate of only 25 percent. Classifying breast cancer into molecular subtypes has been crucial in planning treatment, developing new therapies and improving the survival rate. The different molecular subtypes of breast cancer include; Luminal A – (ER+ and/or PR+, HER2-), Luminal B – (ER+ and/or PR+, HER2+), Triple negative/basal like (ER-, PR-, HER2-) and HER2 type – (ER-, PR- and HER2+). The prevalence of the molecular subtypes are as follows; luminal A and B – (60-70%), triple negative (15-20%) and HER2 – (10-15%).

The objective of my research is to provide further understanding into how luminal A and B breast cancers (ER+) are regulated by the pleiotropic cytokine – IL-6, using luminal A and B breast cancer cell lines. My focus was on determining whether ERα positive breast cancer cell lines can be made ERα negative through STAT3 transcriptional repression of the ERα gene. My research revealed a novel role for the IL-6/JAK/STAT3 pathway in modulating ERα expression through epigenetic modifications of the ERα promoter.

The Estrogen Receptor alpha (ERα)

The estrogen receptor alpha (ERα) is the most important target in breast cancer treatment. About 70 percent of breast cancers express ERα, a key indicator of prognosis and response to endocrine therapy [1]. The role of ERα, and its ligands in breast carcinogenesis has been recognized for some time[2]. Estrogens play a crucial in sexual development, reproduction and many physiological processes [3]. ER plays a vital role in the development, progression, treatment and outcome of breast cancer [3]. In the absence of ligand, ERα is sequestered in target cell nuclei within a large inhibitory heat shock protein complex [4]. In the classic pathway, estrogen binding to the estrogen receptors a and b induces a dynamic conformational change that leads to ER dimerization and association with co-regulatory proteins with the subsequent transcriptional activation of estrogen-responsive genes [5]. The interaction of ERα with target gene promoters can occur directly, through specific estrogen response elements (ERE) or indirectly by using other DNA bound transcription factors such as AP1, SP1 or NF-κB [6-8]. ERα co-factors interact with different target proteins that link it to other signaling pathways. These proteins often affect ERα signaling directly or indirectly [9] . A number of studies using Chromatin Immunoprecipitation (ChIP) with microarrays or high throughput sequencing in MCF-7 breast cancer cells have mapped ERα binding events genome wide. It shows that Forkhead motifs were enriched in ER binding events [10, 11]. The Forkhead protein FoxA1 has been shown to be present in many ERα binding regions [12]. Hurtado et al. showed that FOXA1 is a critical determinant that influences differential chromatin interactions and that almost all ER-chromatin interaction and gene expression changes are dependent on the presence of FOXA1 [13]. Clinically, it's been shown to predict outcome in ERα positive breast cancer patients [14].

The full human ERα gene spans about 300kb of chromosome 6 [15]. Two years after cloning the human ERα cDNA, the genomic organization of ERα was described. ERα consists of 8 exons, spanning 140kb of chromosome locus 6q25.1 [15, 16]. Further analysis of nuclear receptors indicated that multiple promoters might be common feature of steroid hormone receptors and to date, several exons encoding 5'-untranslated regions of ERα mRNA have been identified. The human ERα gene has also been shown to be transcribed from at least seven promoters into multiple transcripts that differ in their 5'-UTR.

Estrogen receptor alpha (ERα) therapy

ERα is the principal biomarker for response of breast cancer to tamoxifen treatment [17]. Anti-estrogens such as selective estrogen receptor modulators (SERMs) act as competitive blockers of estrogen-ER binding, and have been successfully used in the treatment of ERα positive breast cancer [1]. In the adjuvant setting, tamoxifen reduces the rate of disease recurrence and has led to a significant reduction in breast cancer mortality in the past few decades [18].Tamoxifen is able to inhibit the expression of ERα target genes that regulate cell cycle and apoptosis. Tamoxifen leads to repression of cyclin D1 and MYC, reduces the activity of transcription factors SP1 and NF-κB and down-regulates NF-κB target gene BCL2 [19-22]. Overall, a third of the women treated with tamoxifen for 5 years will have recurrence of breast cancer within 15 years [21]. Reported mechanisms of tamoxifen resistance include alterations in levels of CUEDC2 and LMTK3, and over expression of ERBB2 [21]. Data suggests that level of ERα expression correlates with response to tamoxifen therapy [23]. Kim et al. [23] showed that patients with low levels of ERα do not respond to tamoxifen when compared to patients on placebo (Figure 1.1). Essentially, low-level expression of ERα is a key

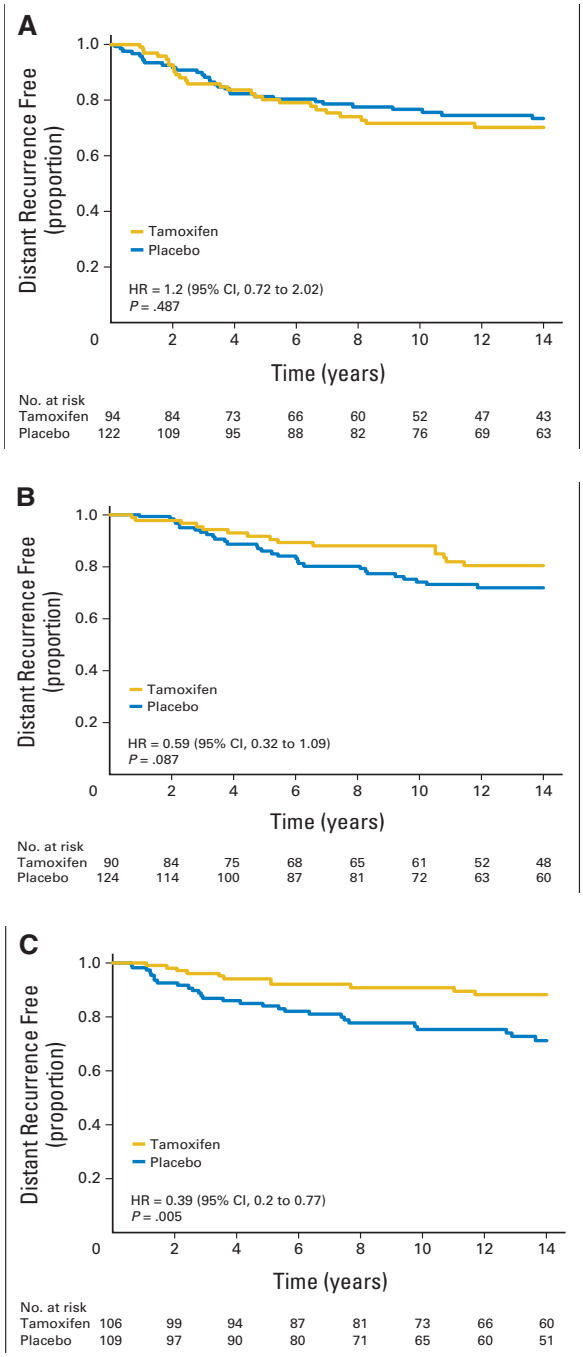


Figure 1.1. Quantitative estrogen receptor expression by reverse transcriptase polymerase chain reaction and distant recurrence at 10 years. Each Kaplan-Meier plot represents tamoxifen and placebo arms of patients diagnosed with tumors that express (A) low, (B) middle, and (C) high tertile levels of *ESR1* mRNA. HR, hazard ratio. Re-printed with permission Kim C, Tang G, Pogue-Geile KL et al. Estrogen receptor (ESR1) mRNA expression and benefit from tamoxifen in the treatment and prevention of estrogen receptor-positive breast cancer. Journal of clinical oncology [23].

determinant of tamoxifen resistance in ERα positive breast cancers [23]. Aromatase inhibitors, which target estrogen synthesis, have also been successfully used in the treatment of breast cancer. Aromatase inhibitors have also been noted to have greater efficacy than tamoxifen when used in late-stage disease [24, 25]. Besides tamoxifen, another SERM, used in the treatment of breast cancer is toremifene [26]. Toremifene has also been shown to prevent progression of high-grade prostatic intraepithelial neoplasia (PIN) to prostate cancer [26]. SERMS and aromatase inhibitors have limitations. This is because tamoxifen is able to inhibit the expression of ERα target genes that regulate cell cycle and apoptosis. Tamoxifen leads to repression of cyclin D1 and MYC, reduces the activity of transcription factors SP1 andNF-κB and down-regulates NF-κB target gene BCL2 [19-22].

Transcriptional regulators of Estrogen receptor alpha (ERα)

The transcription of ERα is regulated by several factors, which include GATA binding factor 3 (GATA-3), Forkhead box protein O3a, (FoxO3a), Forkhead box protein M1 (FoxM1) and ERα which can regulate it own transcription[11, 13, 27, 28].

GATA-3 is a direct positive regulator of ERα expression, it binds to two cis-regulatory elements located within the ERα gene [27]. GATA-3 is also required for the recruitment of RNA Pol II to the ERα promoter and is crucial for the response of ERα positive breast cancers to estradiol [27]. Interestingly, ERα has been shown to directly stimulate the transcription of GATA-3 [29].

Madureira et al. [28] identified FoxM1 as a physiological regulation of ERα expression in breast cancers. They revealed that FoxM1 expression led to up-regulation of ERα mRNA and protein and showed that FoxM1 can activate the transcriptional activity of ERα promoter through two closely located Forkhead response elements located at the

proximal region of the ERα promoter [28]. Interestingly, they observed that FoxO3a co-immunoprecipitated with FoxM1 in vivo, suggesting the possibility that both Forkhead box proteins cooperatively regulate ERα gene transcription [28].

FoxO3a, is a key regulator of ERα gene transcription [30]. Levels of FoxO3a have been correlated with ERα expression in breast cancer. FoxO3a expression was shown to induce ERα promoter activity and protein levels [30]. Electro-mobility shift assays (EMSA's) showed that FoxO3a directly binds to the ERα promoter, and this was confirmed *in vivo* by ChIP. Although FoxO3a has been shown as a transcriptional activator of ERα, some reports have also linked it to ERα repression [31].

Recently, a number of negative transcriptional regulators of the estrogen receptor have been reported. One of those regulators is Twist. 'Twist contributes to hormone resistance in breast cancer by down-regulating estrogen receptor-α' [32] and 'TWIST Represses Estrogen Receptor-alpha Expression by Recruiting the NuRD Protein Complex in Breast Cancer Cell' [33] both discuss Twist as down-regulators of ERα. Both show an inverse correlation between Twist and ERα in breast cancer cell lines. They also show that forced expression of Twist in ERα positive breast cancer cell lines reduced ERα expression and that knockdown of Twist in ERα negative breast cancer cells such as MDA-MB-435 increased ERα expression [33]. Twist was also shown to recruit DNA methyltransferase 3B to the ERα promoter, leading to higher promoter methylation in ERα positive cell lines compared to parental cells. Also Twist was shown to recruit HDAC1 to the ERα promoter and further reduce ERα transcript levels [32].

SNAIL [34] has also been reported as a transcriptional regulator of the estrogen receptor. Wade et al showed that an inverse relationship exists between Snail and ERα in breast cancer cell lines. Over-expression of Snail in MCF-7 cell line, led to decrease in

cell-cell adhesion and increased cell invasive. ERα mRNA and protein were also decreased/lost in response to Snail binding to regulatory DNA sequences at the ESR1 locus. Essentially, the transcription factor Snail mediated epithelial to mesenchymal transitions by repression of ERα. [34].

It is important to note here that although Twist and Snail, genes involved in EMT have been shown to be transcriptional regulators of ERα expression, ERα has also been shown to transcriptionally regulate Snail and Slug expression in breast cancer cell lines [35, 36].

Epigenetics & Estrogen receptor alpha (ERα)

Epigenetic modifications principally regulate ERα expression [37]. Epigenomics refers to the study of heritable changes in gene expression, which occur without a change in DNA sequence. ERα synthesis is repressed by the methylation of the ERα promoter [38]. Promoter hypermethylation is significantly associated with the loss of ERα in primary breast cancer and breast cancer cell lines [38].

DNA methylation

One of the ways in which ERα is epigenetically regulated, is by DNA methyltransferase 1 (DNMT1) mediated promoter methylation, which leads to a decrease in ER expression [39]. DNMT1 is a large enzyme composed of a C-terminal catalytic domain and a large N-terminal regulatory domain with several functions [40]. Methylation of the ERα promoter mediates transcriptional silencing of the *ER* gene in ER negative breast tumors [41].

DNA methylation is an epigenetic mark that involves the addition of a methyl group on the fifth position of cytosine within CpG dinucleotides. DNA methylation is mediated by three conserved DNA methyltransferases: DNMT1, DNMT3A, DNMT3B. DNMT1 has

been shown to maintain DNA methylation patterns, while DNMT3A and DNMB3B are responsible for de novo methylation [42]. DNA methylation occurs in repetitive regions across the genome at dense CG regions, called CpG islands. CpG islands mostly occur around transcriptional regulatory regions of house keeping and essential development regulatory genes [43]. In ERα, the CpG island is found in exon1, adjacent to promoters A and B. Overall, DNA methylation leads to stable gene silencing [44].

DNA methylation not only leads to methylation of DNA around the promoter region, but it can also lead to inhibition of transcription via two main mechanisms: methyl groups at CpG islands can hinder the binding of transcription factors to the promoter and methylation at the CpG dinucleotide can create a docking site for the binding of methyl-CpG-binding proteins and their associated repressors, resulting in constant gene suppression [43] [45]. These effects of DNA methylation interfere with transcription.

Approximately 20-30% of breast cancers are diagnosed as ERα negative and some cancers loose ERα expression as they progress. In many of these breast cancers DNA methylation plays a role in loss of ERα expression. Yang et al [46] demonstrated that reduced ERα expression is due to increased DNA methylation. DNA methylation is no longer considered a permanent epigenetic mark. In breast cancer, it's been shown that inhibiting DNMT1 using 5-aza-2'-deoxycytidine induced ERα expression. Treatment of cancer cell lines with this inhibitor has been shown to enhance re-expression of a gene [47].

Histone Methylation

Recently, there have been several studies demonstrating the importance of histone methylases and histone demethylases in regulating estrogen receptor alpha (ERα) activity and expression [48]. Histone methylating enzymes directly interact with DNA

methylating enzymes [49]. Methylation of histones occurs on either lysine or alanine residues, resulting in either condensation of relaxation of chromatin structure [50]. Methylation provides binding sites for regulatory proteins with specialized binding domains [49]. The main sites of methylation of histones occur on either heterochromatin or euchromatin. Heterochromatin is a tightly packed form of DNA that is considered transcriptionally silent, whereas euchromatin is less densely packed and transcriptionally active. Within heterochromatin, there are methylation lysine residues which demarcate subdomains [49]. Methylated histones also serve as a docking site for repressive proteins, including the polycomb protein (PC) and heterochromatin protein (HP1), which recognize histone H3, K27, or H3 K9 respectively [51]. HP1 and PC recognize methylated lysine residues through their chromo domain [50]. Other proteins recognize methylated lysine through two other motifs, known as the Tudor domain and the WD40 repeat domain. Histone lysine methylases share a common Suvar Enhancer of Zeste, Trithorax (SET) domain.

Histones package euchromatin DNA into nucleosomes containing 147 base pairs of DNA and core histone proteins (H2A, H2B, H3 and H4) [50]. Alterations of chromatin structure are modulated through post translational modification of lysine tails [52]. The amino terminal tails of all four core histones contain conserved lysine residues. One of the marks that occur on lysine residues is acetylation, which was initially thought to neutralize the basic charge of histone tails, in order to decrease affinity between histone and negatively charged DNA [52]. However further research has proven that acetylation provides recognition motifs for docking of proteins that recruit transcriptional activators or repressors, such as acetyllysine-binding bromodomain [53].

Histone Acetyltransferases (HATs)

Histone acetyl-transferase (HAT) enzymes acetylase lysine amino acids on histone proteins by transferring an acetyl group from acetyl CoA to form N-acetyllisine [53]. They are described as Type A and Type B. Type A HATs are located in the nucleus while type B HATs are located in the cytoplasm. Type B HATs perform housekeeping roles, acetylating newly synthesized free histone, while Type A HATs acetylate nucleosomal histones in the nucleus within the chromatin [52]. Histone acetylation is generally associated with transcriptional activation, eurchromatin, and an increase in gene expression [52].

In ERα positive breast cancer cell lines, the lysines on histones in the promoter region are on average acetylated [41]. Co-activator and co-repressors, which encode enzymes with HAT-modulating activity, mediate ERα functional activity in the nucleus. HAT activity is encoded in co-integrators CBP/p300 (CREB-binding factor). Binding of HATs to ERα allows for acetylation of local histones [53].

Histone deacetylases (HDACs)

Histone deacetylates (HDACs) have been shown bind to ERα and over-expression of HDACs leads to silencing of the ERα gene [54]. HDACs are divided into three different groups of proteins. Class I HDACs, which include HDACs 1, 2, 3 and 8 are related to the *Scacchromyces Cerevisea* transcriptional regulator RPD3. Class II HDACs include HDAC 4, 5, 6, 7, 9 and 10 are expressed in a cell specific manner. Class III HDACs are Sir2/Hst homologues, and their structure and enzymatic mechanisms are different from Class I and II HDACs [55]. HDAC proteins are responsible for transcriptional repression [55]. Together with co-repressor complexes such as N-COR and transcription factors they mediate gene repression. HDAC inhibitors for class I and II are currently been used in clinical trials for patients that lack ERα expression[55, 56]. HDAC inhibitors such as Trichostatin A (TSA) have led to re-expression of ERα. However, it is important to note

that the use of both TSA and 5-aza-dc (DNMT inhibitor) enhanced re-expression of both ERα mRNA and protein [46, 47, 54].

Other mechanisms of ERα silencing include several multi-molecular ERα repression complexes. pRB2/p130-E2F4/5-HDAC1-SUV39H1-p300 or pRB2/p130-E3F4/5-HDAC1-DNMT1-SUV39H1 proteins were found on the ERα promoter. These complexes included HDACs, DMNTs, histone methyltransferase, SUV39H1 and cell cycle regulatory protein pRb2/p130 [57].

Regulation of ERα by phosphorylation

Phosphorylation of ERα is one of the mechanisms by which ERα signaling can be regulated. Phosphorylation of ERα induced by growth factors play an important role in enhancing estrogen signal activation [58]. Identified phosphorylation sites of ERα include S104/106, S118, S167, S236, T311, Y537 [59]. These sites are targeted by kinases, which include MAPK, Akt and c-Src [58]. There is still a lot unknown about the roles of each phosphorylation site. Chen et al. [60]showed that phosphorylation of ERα on S118 promotes dimerization and Kikhite et al [61] showed that kinase-specific phosphorylation of ERα changes receptor interactions with ligands, DNA and estrogen associated co-regulators. Thus far, phosphorylation sites S118 and S167 are located within the action function (AF-1) region and this seems to be the most important component for activation of ERα signal because phosphorylation at these sites leads to enhanced activation of genomic action in both an estrogen-dependent and estrogen-independent manner [62].

Estrogen Receptor alpha (ERα) regulated genes

ERα is the driving transcription factor in ERα positive breast cancers and via its target genes; it dictates cell growth and endocrine responses [63]. It is therefore important to

identify and study ERα target genes to better understand and manage the disease. Several studies have identified ERα target genes. More recently two papers used chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) in primary breast cancers to better identify ERα target genes[64]. They performed ERα ChIP-seq on eight ERα positive, PR positive and HER2 negative breast tumors and seven ER+ PR- HER2- or ER+ PR+ HER2+, stating that PR- and HER2+ tumors are more likely to be aggressive. The results identified hundreds of binding events [64]. I'll specifically discuss ERα target genes that were examined in this study.

Progesterone Receptor (PGR)

Progesterone is an important hormone in breast cancer. Just like ERα, PGR signaling has a distinct role in mammary gland biology. PGR levels are regulated by estrogen-dependent and estrogen-independent pathways [65]. PGR is a nuclear receptor that regulates the expression of many downstream target genes [66]. In human breast cancer cells, the proximal promoters controlling PGR transcription contain estrogen response elements (ERE), which are recognized by ER plus binding sites for other transcription factors which ER interacts with [67, 68].

Growth Regulation by Estrogen Receptor in Breast Cancer (GREB1)

GREB1 is an ERα target gene and its expression correlates with ERα expression in breast cancer cell lines and breast cancer tissue [69]. GREB1 expression also inversely correlates with HER2 status. Similarly to ERα patients, patients that express GREB1 exhibit significant sensitivity and prolonged survival compared to GREB1 negative expression [70, 71]. A recent paper found that transducing MCF-7 cells with GREB1 increased the metabolic activity of the cells suggesting the GREB1 may function as a growth promoter in breast cancer. Also IL-6 inhibited E2-induced GREB1 transcription

activity and led to a reduction of GREB1 mRNA. Likewise over-expression of a constitutively active form of STAT3, STAT3-C led to a decrease in GREB1 mRNA [69]. This suggests that IL-6/STAT3 pathway plays a role in regulating ERα in ERα positive breast cancers and cell lines.

Early Growth Response 3 (EGR3)

Analysis of estradiol treated breast cancer cell lines identified EGR3 as a bonafied ERα target that plays a key role in ERα signaling [72]. EGR3 is a member of the EGR family and shares the common EGR response element with other members involved in DNA binding and transactivation [73]. Very little is known about EGR3 and breast cancer other than it's an intracellular mediator of the estrogen-signaling pathway in breast cancer. My research study provides further evidence that indeed EGR3 is a mediator of ERα signaling [72].

Rearranged during transfection (RET) proto-oncogene

Ret encodes a receptor tyrosine kinase for members of the glial cell line derived neurotropic factor family of extracellular signaling molecules. Although Ret is a target of ERα, its regulation has also been shown to the independent of the estrogen receptor [74]. A recent study revealed that down-regulation of Ret using a RET inhibitor blocks a feed-forward loop of decreasing Fak, an integrator of IL-6-Ret signaling [75].

Triple negative breast cancers

About 20% of breast cancers are triple negative. Triple negative breast tumors lack expression of estrogen receptor (ERα), progesterone (PR), and HER2 [76]. This cancer is challenging to deal with because they do not respond to endocrine therapy or other available targeted agents. Although the metastatic potential of triple-negative breast cancer is similar to that of other breast cancer subtypes, these tumors are associated

with a shorter median time to relapse and death [77]. Also, once a metastatic triple-negative breast cancer is present, there is a much shorter median time from relapse to death. Higher rates of triple negative breast cancer have been observed in women who are younger and woman of African or Hispanic ancestry have been shown to have higher rates of triple negative breast cancer [78, 79]. Chemotherapy remains the main treatment for triple negative breast cancers. Current treatment includes the use of anthracyclines, taxanes, ixabepilone, platinum agents and some biologic agents [80, 81]. The lack of effective therapies for triple negative breast cancers has led researchers to look for further sub-classifications of triple negative tumors. A recent study by Brown et al revealed that IL-6 inhibition in addition to IL-8 inhibition dramatically inhibited colony formation and cell survival *in vitro* and staunched tumor engraftments and growth in vivo in triple negative breast cancer cell lines; thereby linking IL-6 pathway as an important pathway in triple negative breast cancer progression [82].

I will employ the use of triple negative breast cancer cell lines MDA-MB-231, MDA-MB-468, HCC1806, HCC38, HCC 1937, in my research study. The IL-6/STAT3 pathway is active in triple negative breast cancer cell lines and inactive ERα positive cell lines, thus these cell lines provide a way to explore the importance of IL-6 in tumor progression. In conjunction with ERα positive cell lines (T47D, MCF-7 and BT-474), I am particularly interested in determining whether sustained IL-6 signaling can lead to loss of ERα expression. Understanding the mechanisms of *ERα* gene regulation is of fundamental importance to the management of breast cancers.

Interleukin- 6 (IL-6)

Interleukin-6 (IL-6) is both a pro-inflammatory and an anti-inflammatory cytokine [83]. It is involved in the regulation of immune response, hematopoiesis and acute phase reactions as well as cell growth and differentiation and is produced by a variety of cells including macrophages, synovial cells, endothelial cells, glia cells, karatinocytes, B-cells, T-cells and fibroblasts [84]. IL- 6 expression is induced by a variety of cytokines such as IL-1, tumor necrosis factor (TNF) and platelet-derived growth factor (PDGF). In addition, bacterial and viral infections and microbial components such as lipopolysaccharide have been known to induce IL-6 [85]. A knockout mouse model of IL-6 revealed that IL-6 is essential for antiviral antibody response [86].

IL-6 has been shown to serve as a growth factor in a number of cancers including multiple myeloma, prostrate cancer and cholangiocarcinoma [87]. IL-6 is associated with different features of tumor biology, including metastasis, higher stages of disease progression and decreased survival [88]. In breast cancer, IL-6 appears to be both tumor promoting and tumor degrading. In MCF-7 cells, pretreatment with IL-6 led to an eightfold increase in resistance to doxorubicin indicating that the presence of endogenous IL-6 increased the resistance of breast cancer cells to doxorubicin treatment [89]. Chiu et al. [90] demonstrated that in ERα positive breast cancer cell lines, IL-6 inhibited proliferation by inducing apoptosis. Treatment of ERα positive cells with IL-6 induced DNA fragmentation in MCF-7's and ZR-75's. *In vitro*, IL-6 has been shown to decrease cell adhesions of three breast cancer cell lines, which has been linked to decreasing E-cadherin expression [91]. High circulating levels of IL-6 have been shown to directly correlate with disease staging and unfavorable clinical outcomes in women with metastatic breast cancer and a variety of cancers, including prostrate, colorectal and myeloma[92-104]. IL-6 has also been shown to promote breast cancer

cell motility, which suggests that it may have a role in metastasis [105]. IL-6 is also hypothesized as a vulnerability factor that may contribute to the ethnic disparities in breast cancer mortality [106].

IL-6 signaling

IL-6 signals by binding to the specific membrane-bound receptor gp80 (IL-6R) to form the IL-6/IL-6R complex[107]. This complex then associates with two gp130 molecules and induces signal transduction through the intracellular domains of gp130 (Figure 1.1). Although the IL-6 receptor is not directly involved in signaling it is required in other to present ligand IL-6 to gp130, which leads to activation of the receptor complex [107]. IL-6 family; including leukemia inhibitory factor (LIF) and oncostatin M (OSM), as well as IL-11 and cardiotropin-1 (CT-1) all signal through the gp130 receptor also known as CD130 [83, 108-111].

Targeting IL-6 signaling pathway

IL-6 mediated activation of STAT3 is a principal pathway involved in promoting tumorigenesis [112]. STAT3 has been shown to be critical in tumor formation and metastatic progression, therefore targeting this signaling pathway is important to the treatment and management of breast cancer [113]. Given the importance of IL-6 signaling, in driving STAT3 activation, IL-6 blockade using IL-6 ligand binding antibodies such as (CNTO-328) and IL-6R blocking antibodies such as (tocilizumab) currently in clinical trials may prove as effective therapeutics [114-116]. In addition, inhibition of Jak signaling, which is currently in clinical testing for myeloproliferative models may also prove effective in the treatment of breast cancers with activated IL-6/Jak/STAT3 signaling [117]. STAT3 inhibitors, such as STAT3 decoy and targets of the SH2 domain which prevent STAT3 phosphorylation is an additional therapeutic that may be successful in inhibiting this signaling pathway *in vivo* [118].

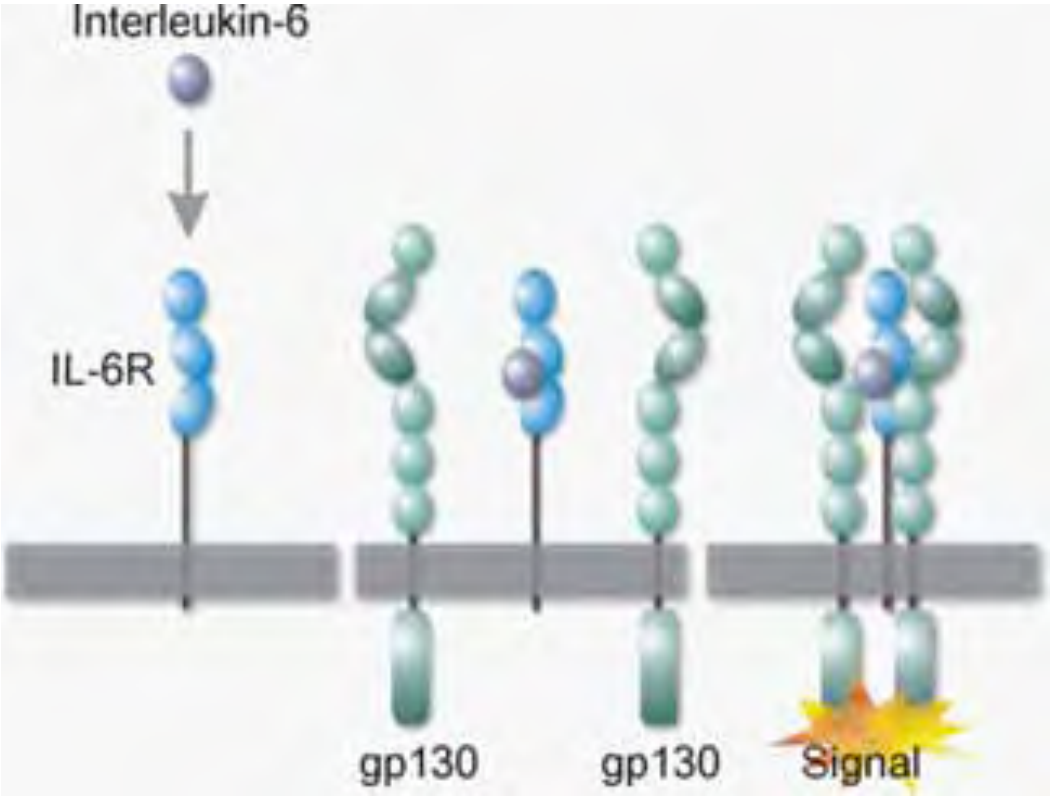


Figure 1.2. IL-6 signaling and receptor complex. [107]
Rose-John S, Waetzig GH, Scheller J, Grotzinger J, Seegert D: The IL-6/sIL-6R complex as a novel target for therapeutic approaches. Expert opinion on therapeutic targets, 2007, 11(5):613-624, copyright © 2007 Informa Healthcare. Reproduced with permission of Informa Healthcare.

Signal Transducer and Activator of Transcription 3 (STAT3)

STAT3 is a member of the STAT transcription factor family that plays critical roles in cytokine signaling, mediating cell proliferation, survival, as well as tumorigenesis. STAT3 is one of the primary intracellular targets activated after exposure to IL-6. As previously stated, IL-6 signals through the soluble IL-6R (gp80) coupled with the common signal-transducing receptor β subunit gp130, a 130kDa transmembrane signaling glycoprotein (Figure 1.1) [119, 120]. Signal transduction involves gp130 dimerization and activation of receptor-associated Janus kinases (JAKs), leading to the recruitment and phosphorylation of a number of signaling molecules including the Stat3 on tyrosine residue 705 ($pTyr^{705}$) [119, 121] [122] (Figure 1.2). STAT3 is generally maintained in the cytoplasm in its un-phosphorylated/inactive manner; following its phosphorylation, STAT3 forms homodimers, and enters the nucleus, where is activates several pro-growth and pro-survival genes [119].

Studies performed by the Bromberg lab and others provide strong evidence for a critical role of Stat3 in mammary tumorigenesis [112, 113, 123-126]. They've shown that Stat3 is constitutively active (tyrosine phosphorylated) in more than 50% of primary breast tumors and tumor-derived cell lines [112, 124, 126]. Side-population breast cancer stem-like cells express and require persistently activated Sat3 for viability and maintenance [127]. In its canonical role, STAT3 mediates its effects primarily through its ability to regulate gene transcription, targeting genes like vascular endothelial growth factor (VEGF), survivin, matrix metalloproteinase-9 (MMP-9) and twist [123, 124, 128-131]. The principal mechanism of STAT3 activation in breast cancer derived cell lines and primary breast tumors is through the IL6/gp130/Jak2 pathway [112]. Inhibition or removal of STAT3, via knockdown approaches, led to increased apoptosis, chemosensitivity, and decreased angiogenesis and metastatic spread both in cell culture and in xenograft models [125, 132-134].

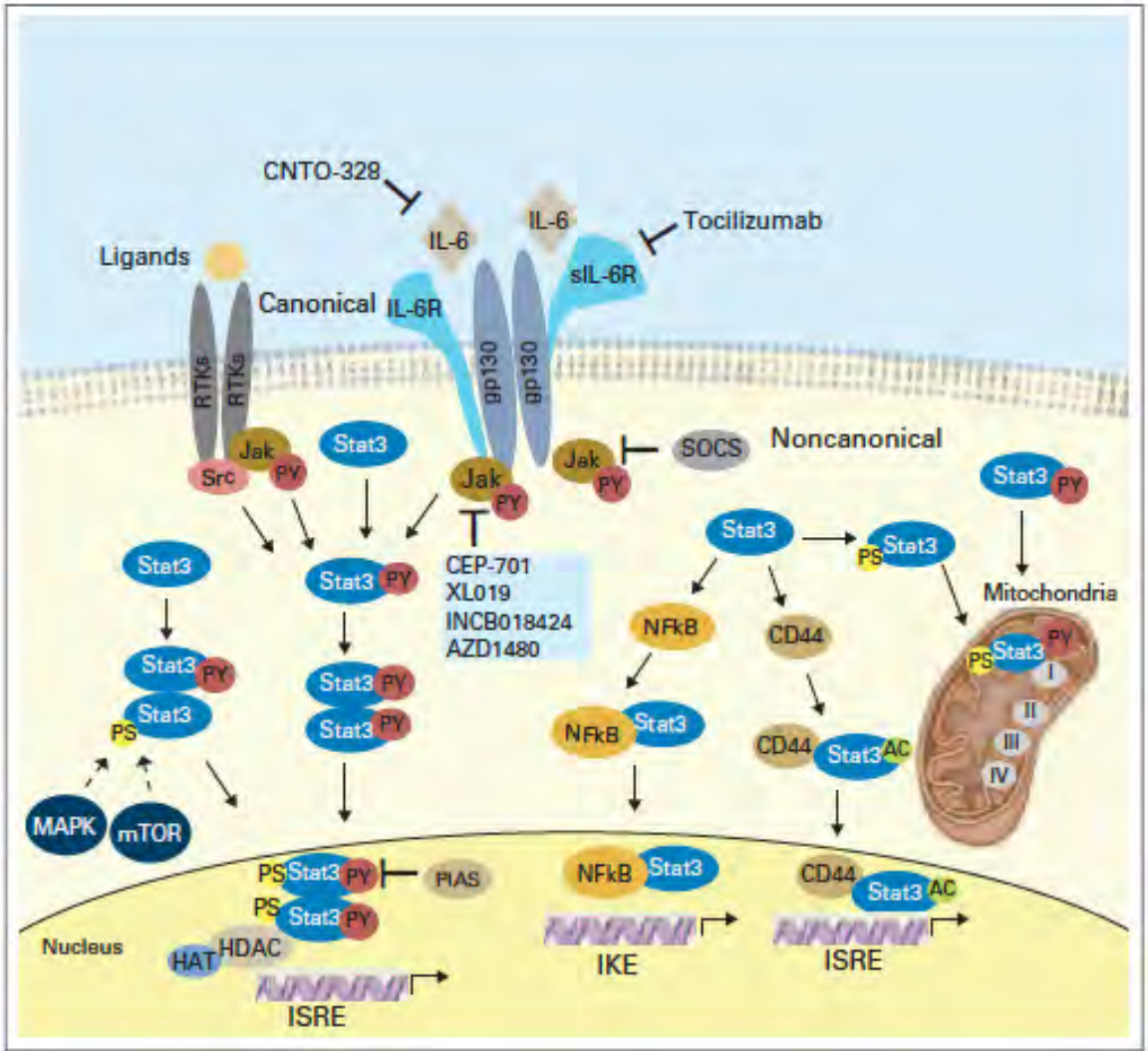


Figure 1.3. Signal transducer and activator of transcription (STAT3) signaling. Canonical: Stat3 is tyrosine phosphorylated by Janus kinase (Jak) kinases in response to cytokine/growth factor activation of cell surface receptors (ex., receptor tyrosine kinases [RTKs], glycoprotein 130 [gp130] with either interleukin-6 receptor [IL-6R] or soluble IL-6R [sIL-6R]). On tyrosine phosphorylation (PY), Stat3 dimerizes and localizes to the nucleus, where it binds to Stat3 responsive elements. Stat3 is also serine phosphorylated (PS). Soluble factors that activate Stat3 include the IL-6 family of cytokines. **Non-canonical**: Unphosphorylated Stat3 can bind to either nuclear factor _B (NF_B) or CD44 in the cytoplasm; the complexes translocate into the nucleus, where they bind NF_B (IKE) and Stat3 DNA-binding elements. Acetylated Stat3 is required for association with CD44. PS Stat3 and PY Stat3 can also localize into the mitochondria, where they modulate ATP production [135].
Reprinted with permission Sansone P and Bromberg J. J Clin Oncol 30(9), 2012.

Although a number of studies have shown that STAT3 is activated in epithelial tumors and have emphasized the necessity of IL-6 and the inflammatory response, the question about what regulates a continuous activation of STAT3 still remains largely unanswered. A paper by Zucman-Rossi identified in-frame somatic deletions of gp130, which activate gp130 in inflammatory hepatocellular tumors [136]. These deletions target the binding site of gp130 for IL-6; other mutations identified in gp130 led to constitutive activation of STAT3 in the absence of ligand. The identification of gain-of function gp130 mutations in human hepatocellular tumors elaborates on the acute inflammatory phase observed in these tumors, and suggests to us that similar mutations may be present in other inflammatory epithelial tumors with STAT3 activation, such as inflammatory breast cancer [136].

Negative regulator of STAT3; Suppressor of Cytokine Signaling 3 (SOCS3)

Suppressor of Cytokine Signaling 3 (SOCS3) is an important feedback inhibitor of several cytokines including IL-6, LIF, IL-11, ciliary neurotrophic factor (CNTF) and granulocyte colony stimulating factor (G-CSF). It was identified in a screen of murine thymus cDNA library after a STAT3 pull-down. Further experiments revealed that dominant negative STAT3 could inhibit the IL-6 or LIF-induced SOCS3 expression, indicating that it was one of the target genes of STAT3. It was also shown that over-expression of SOCS3 inhibits LIF and IL-6 induced murine monocyctic leukemic M1 cell line differentiation [137]. SOCS3 expression is induced by JAK/STAT3 signaling. SOCS3 inhibits JAK/STAT3 signaling by directly binding to the JAK1, JAK2 or TYK2 while they are bound to the gp130 receptor or by recruiting elongins B/C and Cullin5 to generate and E3 ligase that leads to ubiquitination of both JAK and gp130 receptor, thereby targeting them for degradation [138].

STAT3 as a transcriptional repressor

In addition to its role as a transcription factor, STAT3 has been described as a transcriptional repressor for a number of genes. Niu et al. [139] showed that STAT3 binds to the p53 promoter in vitro and in vivo and mediates down regulation of p53. Also, work by the Wasik group showed that STAT3 interacts with histone deacetylase (HDAC1) and DNA methyltransferase 1 (DNMT1) [140]. Specifically, STAT3 binds to the SIE/GAS binding sites on the SHP-1 promoter and in association with DNMT1, promotes epigenetic silencing of SHP-1 tyrosine phosphatase gene in lymphomas [140]. In addition, STAT3 has been also shown to negatively regulate a number of genes. It negatively regulates IL-6, IL-17 [141] Interferon beta and gamma (IFNβ, IFNψ) [142] and C-X-C motif ligand (CXCL10) [143].

Estrogen receptor and STAT3

Estrogen receptor alpha (ERα) is the gold standard biomarker for predicting response to therapy thus fully understanding the mechanism by which it is regulated is important to the management of breast cancers. Although most acquired resistance to tamoxifen occur despite continue expression of ERα, about 20% of resistance occurs due decrease/lack of ERα expression. Examination of approximately 50 patient tumor samples revealed that an inverse relationship exists between ERα expression and IL-6 (Figure 1.3). Furthermore it's been reported that ERα negatively regulates IL-6 [144]. A few published data have also revealed a correlation between IL-6/STAT3 signaling and ERα expression in breast cancers. IL-6/STAT3 signaling has been linked to modulating GREB1 (an ERα target gene) functions in breast cancer [69]. My thesis will further the relationship between IL-6/STAT3 signaling and breast cancer. I will reveal a novel role for IL-6/STAT3 signaling pathway in regulating ERα.

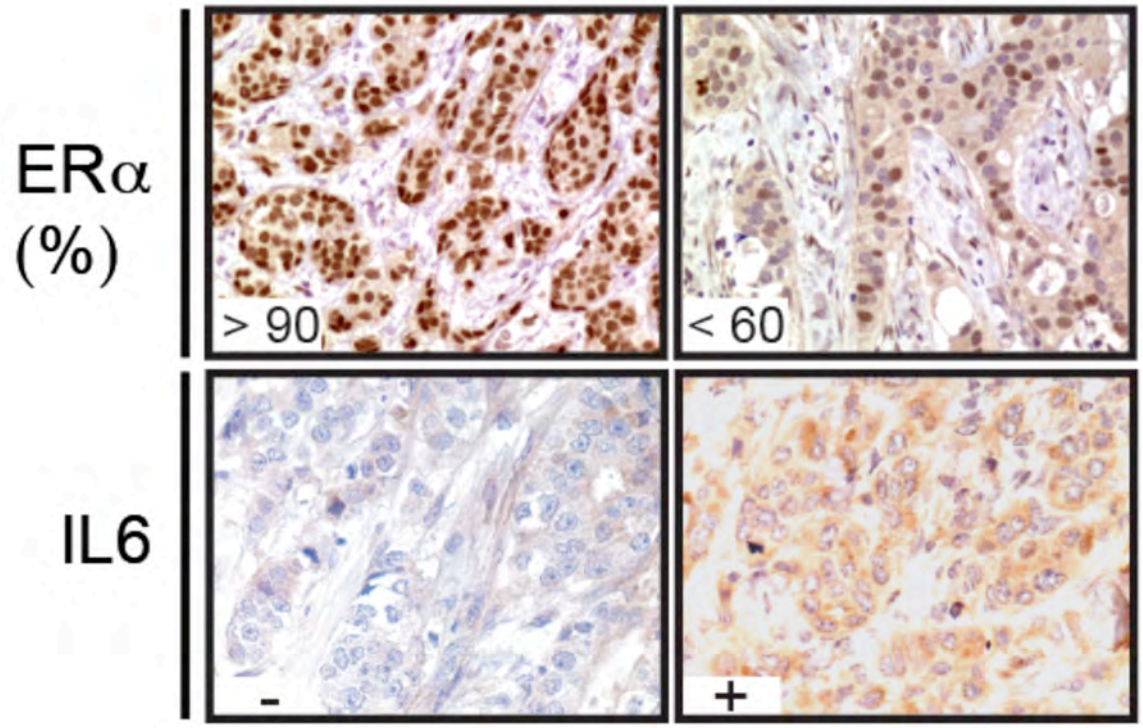


Figure 1.4. Representative IHC showing ERα and IL-6 expression in luminal breast cancer.

Introduction to Thesis

My research is focused on understanding the role of the IL-6/Jak/STAT3 pathway in regulating estrogen receptor alpha (ERα) positive breast cancers. ERα positive tumors occur in approximately 70 percent of breast cancers. Currently, one of the known mechanisms for resistance is via down-regulation of ERα. Understanding the mechanisms by which the IL-6/Jak/STAT3 pathway plays a role in modulating ERα expression may lead to targeted therapies for ERα positive breast cancers.

In chapter 3 of my research I investigated the role of IL-6/STAT3 mediated ERα regulation. Initial examination of ERα positive and triple negative breast cancer cell lines revealed an inverse relationship between ERα expression and IL-6/phospho-STAT3-Y705 expression. Furthermore, treatment of ERα positive breast cancer cell lines (T47D, MCF-7 and BT-474) led to a decrease in ERα mRNA and protein expression. We also observed that IL-6 signaling led to decrease in ERα positive cell growth *in vitro*.

We also showed that treatment of ERα positive breast cancer cells with IL-6 led to the recruitment of STAT3 to the ERα promoter; STAT3 bound to the putative STAT3 binding sites identified on the ERα promoter. We show that exogenous IL-6 treatment led to the recruitment of HDAC1, a transcriptional repressor to the ERα promoter, around the same sites we immunoprecipitated STAT3. The IL-6/STAT3 signaling pathway also led to changes in ERα promoter histone acetylation and methylation. Essentially, IL-6/STAT3 led to a decrease in active histone H3K9Ac and H3K4Me1 and an increase in repressive histone H3K9Me3.

Interestingly, we did not find STAT3 bound to the ERα promoter of triple negative breast cancer cell lines, MDA-MB-231 and MDA-MB-468. We hypothesize that is because the

CpG island located in exon 1 of ERα gene is hyper-methylated in triple negative breast cancer cell lines. In addition, only repressive histone marks are present on the ERα promoter of triple negative breast cancer cell lines. This suggests that the ERα DNA of triple negative breast cancer cell lines is in the heterochromatin state. Thus transcription factors like STAT3 cannot bind to the promoter. The research done here has revealed a novel role for the IL-6/Jak/STAT3 pathway in down-regulating ERα expression by epigenetically modifying of the ERα promoter.

CHAPTER TWO
MATERIALS AND METHODS

Generation and infection of lenti-viruses

To study the effects of Stat3 knockdown in breast cancer cell lines, 293T cells were transfected with shSTAT3 or shCTRL, using a previously established protocol (ref). Viral supernatant is collected and precipitated using PEG-it virus precipitation solution protocol. The resulting viral pellet is re-suspended in PBS and used immediately or stored at -80°C.

Cells seeded at 60% density in six-well plates a day before infection were infected with high-tither virus in 1ml of serum free media, in the presence of 8ug/mL polybrene. Four hours after infection, serum-containing media is added to cells and cells are placed in the incubator for 48 hours.

Genomic DNA extraction

Pelleted cells were lysed using a 10:1 ratio of tail lysis buffer: proteinase K and incubated at 56°C for 4 hours. After cells were lysed, phenol-chloroform extraction was carried out by adding 500ul of phenol-chloroform to each tube and spinning down for 5 minutes at the highest speed (13,200 rpm). The aqueous layer was washed twice with chloroform and then re-suspended in 500ul of isopropanol and 50ul of 5M Sodium Acetate. DNA was precipitated by spinning at the highest speed for 10 mins at 4°C. The resulting supernatant was discarded and pellet was washed with 70% ethanol. The final pellet was resuspended in H20 and final DNA concentration was determined using a nano-drop. Genomic DNA was given to the Geoffrey Beene Translational Oncology Core for EpiTYPER quantitative DNA methylation analysis.

EpiTYPER quantitative DNA methylation analysis

Genomic DNA obtained from cells was sent to the Geoffrey Beene Translational Oncology Core for EpiTYPER DNA methylation studies. EpiTYPER uses base specific cleavage and matrix-assisted later desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Like other DNA methylation protocols, EpiTYPER involves bisulfite treatment of genomic DNA, which converts un-methylated cytosine to uracil. PCR amplification involving the use of a T7 promoter tag is followed by in vitro RNA transcription on the reserve strand and base specific cleavage. MALDI-TOF-MS analysis of the cleavage product results in distinct signal pair patter from the methylated and non-methylated DNA template.

Chromatin Immunoprecipitation (ChIP).

Cross linking

Chromatin immunoprecipitation (ChIP) assays were performed on control and IL-6 treated cells at various time points. Cells (1x107) grown to 90% confluency are cross-linked with formaldehyde (1%) and incubated at room temperature. Cross-linking reaction is stopped by the addition of glycine, a final concentration of 0.125M and incubation of cells at room temperature of 5 mins. Media is aspirated and cells are rinsed twice with 10mL of cold PBS containing (1mM PMSF, 1uM DTT, 1mM Roche protease inhibitor). Adherent cells are scraped from dishes with 1mL cold PBS containing (1mM protease inhibitor and 1mM PMSF). Scraped cells are spun at 800xg for 5 mins at 4°C and supernatant is carefully removed. Cell pellet is resuspended in 1mL of cell lysis buffer containing protease inhibitors and incubated on ice for 10mins with occasional vortexing. Cell is spun down using a micro-centrifuge at 800xg for 5 mins at 4°C to pellet the nuclei.

Sonication/Chromatin Shearing

Nuclei is resuspended in 200ul of SDS lysis buffer plus protease inhibitors and incubated on ice for 10mins. Nuclei are sonicated to average chromatin length of about 600bp while keep the samples cold. Sonicated chromatin is spun at highest speed (13200 rpm) for 10mins at 4C. Supernatant (chromatin) is used immediately for immune-precipitation or snap frozen and stored at -80C for several months.

Immunoprecipitation

Samples can be pre-cleared by adding 80ul of salmon sperm DNA/protein A agarose beads (50% slurry, with PBS containing 1mM PMSF, 1uM DTT, 1uM protease inhibitor) for 1hr at 4C with agitation. After pre-clearing, 10% of sample is saved at input and the

rest are divided into necessary fractions immunoprecipated using antibodies of choice. Antibodies used in this study include: anti-STAT3 – (cat. # SC-482X, Santa Cruz, CA), anti-HDAC1 (cat. # ab51825, Abcam), anti-Histone 3 (tri-methyl K9) antibody (cat. # ab8898, Abcam), anti-Histone 3 (mono-methyl K4) antibody (cat. # ab8895, Abcam), anti-Histone 3 (acetyl K9) (cat. #ab4441), RNA pol II (positive control and IgG (negative control).

Wash and reserve crosslink

After immunoprecipitation, magnetic beads are separated and washed with each of the following buffers, Low salt wash buffer 1: (0.1 SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1, 150mM NaCl), low salt wash buffer 2: (0.1 SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1, 500mM NaCl), high salt wash buffer: (0.25 LiCl, 1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8.1. and TE. Immunocomplexes are eluted using 250ul of 1% SDS in 0,1M NaHCO3 and reverse cross-linked at 65C for 4 hours. DNA is extracted via phenol-chloroform or via DNA purification column.

PCR and qRT-PCR

Eluted and purified DNA for Stat3 IP was analyzed via PCR using primers:

Forward: 5' GCAGGTTGCATTCTCCTGAT 3'
Reverse: 5' ACTGGTCTCCCGAGCTCATA 3'

Eluted and purified DNA for Stat3-IP, HDAC1-IP, anti-histone 3 (acetyl K9) –IP, anti-histone 3 (mono-methyl K4) –IP and anti-histone 3 (tri-methyl K9) –IP was analyzed via qRT-PCR with ViiA7 Real Time PCR System from Life Technologies using primers:

Forward: 5' TCGCTCCAAATCGAGTTGTGCCTG 3'
Reverse: 5' ACTGGTCTCCCGAGCTCATATGCA 3'

Protein extractions, Western blotting and Antibodies

Protein extracts were obtained by lysing cells with radio-immunoprecipitation assay (RIPA) buffer and protein concentrations were determined using Bradford assay (BioRad, Hercules, CA, USA). Western blots were carried out using a previously described method [145]. In summary, 25ug of protein is loaded onto an SDS-PAGE gel, and transferred to PVDF membranes for immunoblotting. A variety of antibodies were used in this study. They include, phospho-STAT3-Y705 (Cell signaling – #9135B), total STAT3 (Cell signaling – #9139S), estrogen receptor alpha (Santa Cruz Biotechnology – SC-543), actin (Santa Cruz Biotechnology – SC-1615), and acetyl-STAT3 (Cell signaling – #2523).

ELISA

Breast cancer cell lines used in this study were seeded in a six well dish (200,000cells/well). After 24, cell media was aspirated. Seeded cells were subsequently washed with PBS and fresh serum free media was added. 24 hours later, conditioned media was analyzed for IL-6 production using a human IL-6 ELISA kit (catalogue # 850.030.096; Cell science, Canton, MA) according to manufacturer's instructions. Absorbance level was read at 450nm using a spectrophotometer.

RNA isolation and quantitative real time PCR (qRT-PCR)

RNA was isolated using Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). One microgram of total RNA was converted to cDNA using iScript conversion kit (Bio-Rad) with oligo (dT) primers according to the manufacturers instructions. Real time PCR reactions were performed using the ViiA 7 Real Time PCR System from Life Technologies. qRT-PCR reactions were performed using Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and pre-designed Taqman probes; ESR1 – HS00174860_m1, GAPDH – HS02758991_m1, EGR3 – HS00231780_m1,

PGR – HS01556702_m1, RET – HS01120030_m1, GREB1 – HS00536409_m1. To obtain normalized qPCR values for listed genes, triplicate threshold values were averaged amounts of targets were subtracted from GAPDH CT values and normalized to untreated samples.

Cell culture, plasmids and reagents

All human breast cancer cell lines were obtained from American Type Culture Collection (ATCC). Estrogen receptor positive breast cancer cells (T47D and ZR-75-1) were maintained in RPMI 1640, supplemented with glucose, hepes, 10% FBS, 1% Penicillin-Streptomycin. BT-474 were cultured in Hybri-Care media supplemented with 10%FBS, 1% Penicillin-Streptomycin. MCF-7 cells were maintained in EMEM media, containing 10% FBS and 1% Penicillin-Streptomycin. Estrogen receptor negative cell lines, (MDA-MB-231 and MDA-MB-468) were maintained in DMEM, supplemented with high glucose, sodium pyruvate and1% Penicillin-Streptomycin. HCC 1806, HCC 1143, HCC 38, were cultured in RPMI 1640, supplemented with glucose, 10% FBS, and 1% Penicillin-Streptomycin. Stat3 shRNA lentiviral and scrambled control ShRNA consturcts were previously described[146]. PEG-it Virus Precipitation Solution (Catalogue # LV810A-1/LV825A-1; system biosciences, Mountain View, CA). Other reagents used include: recombinant human IL-6 – 10n/mL (Cat. # 206-IL-050; R&D systems, Minneapolis, MN), 1uM Jak inhibitor 1480 (AztraZeneca), lipofectamine 2000 (Cat. # 11668-027; Life Technologies, Carlsbad, CA) and 4-Hydroxytamoxifen (cat. # 7904; Sigma-Aldrich, Saint Louis, MO).

MTT Assay

Cells were seeded in a 96-well plate in 5 replicates for 24 hours (10,000 cells/well). Cells were then serum starved overnight prior to treatment with tamoxifen, IL-6 or a combination. After 5 days of treatment, with replenishment media at day 3, cell

proliferation was measured using MTT Cell Proliferation Assay Kit (ATCC). MTT was added to cells and incubated at 37°C for 4 hours (visible purple precipitate), it was then solubilized by detergent and quantified by absorbance at 570nm with a reference wavelength of 670nm.

Electro-mobility Shift Assay (EMSA)

EMSA's were performed as previously described [147]. Briefly, nuclear proteins were extracted from T47D cells treated with IL-6 and incubated with 14 base long gamma p-32 labeled DNA oligonucleotides. In the supershift EMSA, antii-Stat3 antibody and cold M67 probe (positive control) were added to the cell lysates before their incubation with probes.

Statistical Analysis

Statistical analysis conducted throughout this study, mean +/- SD (standard deviation) and group comparisons using students t-tests were carried out in Prism 6.0. Results were considered statistically significant when $p < 0.05$. (*, $p < 0.05$, **, $p < 0.01$, *, $p < 0.001$).

CHAPTER THREE
RESULTS

IL-6 levels in breast cancer cell lines

Given the preliminary data in human breast cancer samples, where we observed an inverse relationship between IL-6 levels and ERα (Figure 1.3). We investigated whether a similar relationship exists in breast cancer derived cell lines. We examine 4 ERα positive breast cancer cell lines and 5 ERα negative breast cancer cell lines (Table 1). We performed an ELISA assay on conditioned media to measure how much IL-6 is produced by each cell line. We determined by ELISA that while ERα positive cell lines (T47D, BT-474, MCF-7, ZR-75) produce little to no IL-6 (0-10pg/mL), ERα negative cell lines (HCC 38, HCC 1143, HCC 1806, MDA-MB-468 and MDA-MB-231) produce significant amount of IL-6 (100-900pg/ml) with MDA-MB-231 producing the most highest amount – 900pg/mL (Figure 3.1).

Table 3.1. Characteristitcs of breast cancer cell lines used in this study. The name, subtype (determined by previously published data), tissued of origin, ER, PR, HER2 TP53, PIK3CA and PTEN status are shown for the nine cell lines used or mentioned in this study.

Cell line	Subtype	Tissue of Origin	ER Status	PR Status	HER2 Status	TP53	PIK3CA	PTEN
MCF-7	luminal	pleural effusion	positive	positive	negative	wt	mut	wt/pos
T47D	luminal	pleural effusion	positive	positive	negative	mut	mut	wt/pos
BT-474	luminal	primary tumor	positive	positive	positive	mut	wt	wt/pos
ZR-75-1	luminal	ascites	positive	positive	positive	wt	wt	323T>G(L108R)
HCC-38	basal-like	primary tumor	negative	negative	negative	wt	non-detectable	no expression
HCC-1143	basal-like	primary tumor	negative	negative	negative	wt		
HCC 1806	basal-like	primary tumor	negative	negative	negative	neg		
MDA-MB-468	basal-like	pleural effusion	negative	negative	negative	mut	mut	IVS4+1G>T (A71fsX5)/null
MDA-MB-231	basal-like	pleural effusion	negative	negative	negative	mut		wt/pos

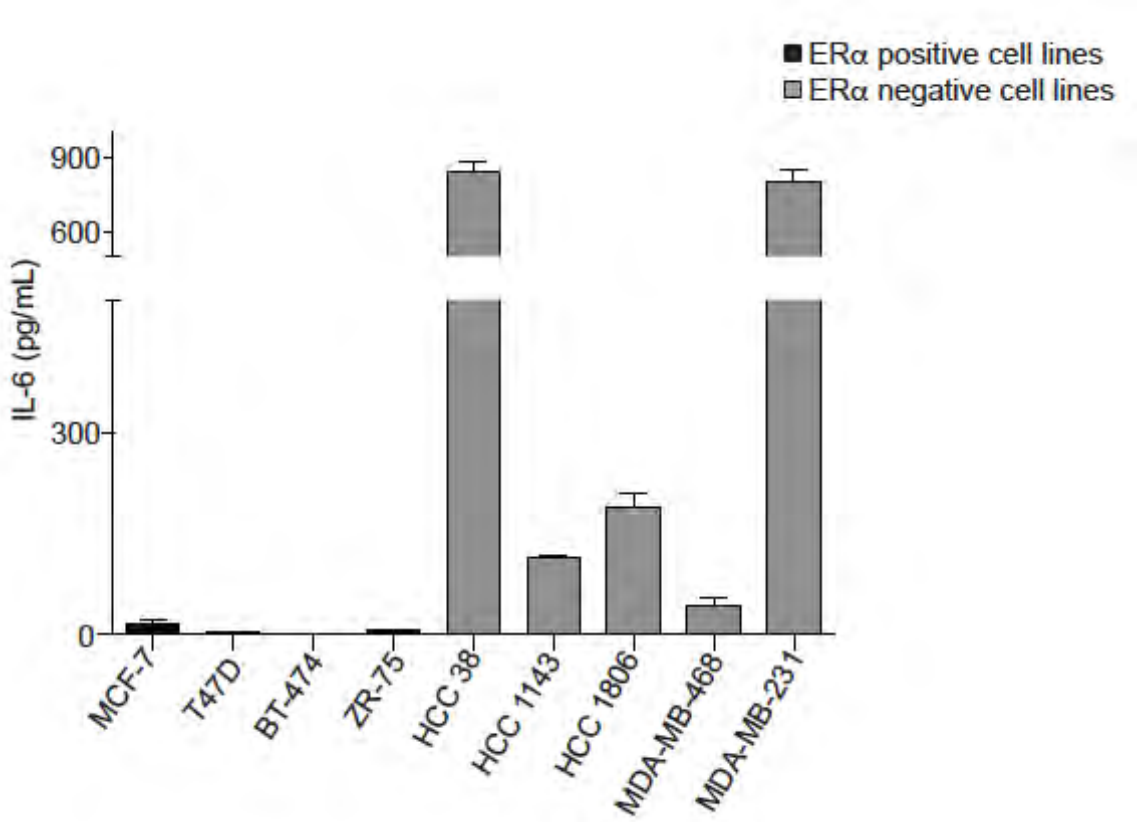


Figure 3.1. IL-6 levels by ELISA in breast cancer cell lines. Conditioned media was collected from cell lines listed above and analyzed for production of IL-6 by Elisa. Cell lines were normalized by cell number (250000 cells /well). Error bars show SD of triplicates.

Inverse relationship between IL-6 signaling and ERα in breast cancer cell lines

We further characterized the inverse relationship between ERα positive and triple negative cell lines by determining the expression levels of ERα mRNA in each cell line. Using qRT-PCR, we show that ERα positive cell lines express varying amounts of ERα mRNA, and that MCF-7 cells express the highest amount of ERα. As expected ERα negative cell lines lack expression of ERα mRNA (Figure 3.2A). Furthermore, western blot analysis confirmed that ERα positive cell lines make ERα protein and triple negative do not.

ERα negative breast cancer cell lines produce IL-6, which activates and phosphorylates Stat3 at tyrosine 705 [112] . Western blot analysis revealed that ERα negative cell lines express phosphor-Stat3-Y705 and ERα positive cell lines which do not produce IL-6, lacked expression of phospho-Stat3-Y705. (Figure 3.2B).

We examined MCF-7 xenografts to determine if what we observed *in vitro* occurred in vivo. We show that sections of the tumor that express ERα, were low/negative for phospho-Stat3 expression, while ERα low/negative sections of the tumor expressed high levels of phospho-Stat3-Y705. Our initial analysis and comparison of these 2 molecular subsets of breast cancer cell lines revealed the existence of an inverse relationship in breast cancer cell lines *in vitro* and *in vivo*. This inverse relationship between these two subsets of breast cancers led us to question whether IL-6/phosphor-Stat3 signaling, regulates ERα expression.

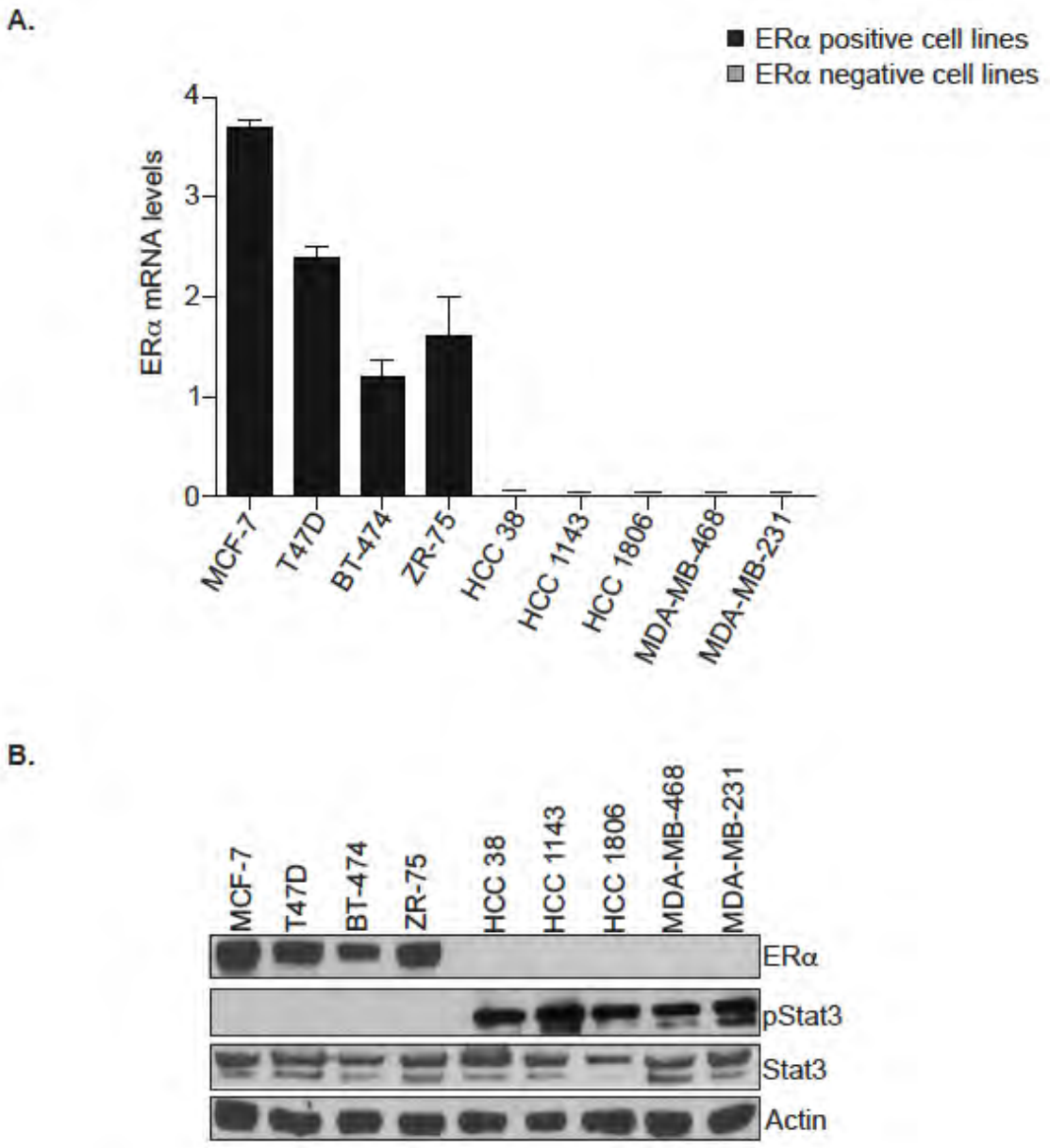


Figure 3.2. ERα expression in breast cancer cell lines. (A) mRNA was extracted from breast cancer cell lines listed above, ERα expression was detected by Q-PCR, the levels of ERα were normalized by comparison to GAPDH. Error bars show SD of 4 experiments. (B) Cell lysates from breast cancer cell lines were prepared and Western blot was carried out using ERα, pStat3 and Stat3 and actin antibodies. p-Stat3 and ERα were normalized against total Stat3 and actin, respectively.

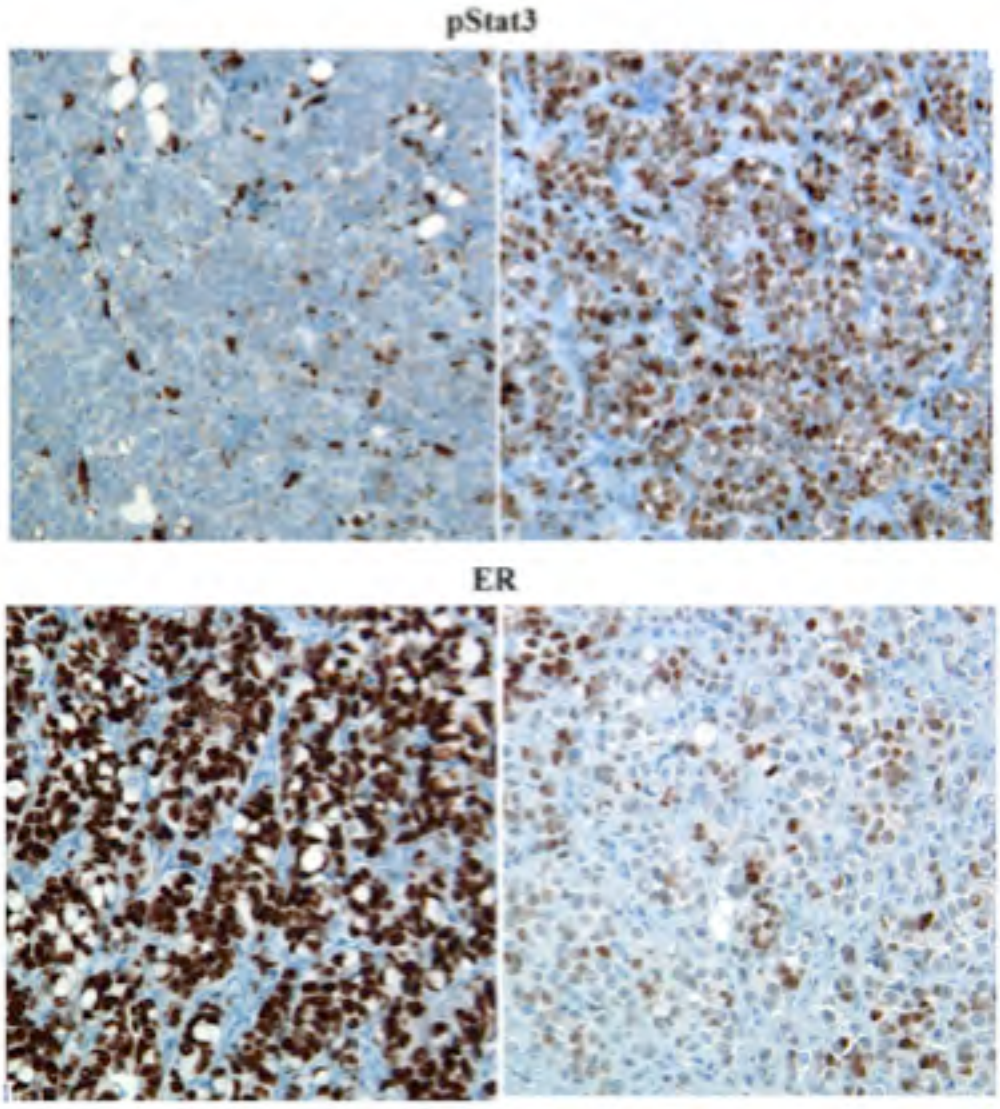


Figure 3.3. Representative IHC showing Inverse relationship between phospho-Stat3-Y705 and ERα in MCF-7 xenografts. IHC staining of pStat3 and ERα from tumor sections of MCF-7 xenografts.

IL-6 leads to a decrease in ERα mRNA and protein in ERα positive breast cancer cell lines

Previous report by Yang et al [144] showed that ERα negatively regulates IL-6 and we observed an inverse relationship between IL-6/pStat3 signaling pathway and ERα expression in human breast cancers and breast cancer cell lines. We therefore wanted to determine whether IL-6/pStat3 signaling could regulate ERα expression. Exogenous treatment of ERα positive breast cancer cell lines T47D, BT-474 and MCF-7 with 10ng/mL of IL-6 led to tyrosine phosphorylation of Stat3 (phospho-Stat3-Y705. Stat3 remained constitutively active in these cells even at 48hours of IL-6 signaling. We also observed that ERα protein expression began decreasing at 24hours, with the lowest amount of ERα expression observed at 48hours in all three cell lines. (Figure 3.4A-C).

As seen in Figure 3.4A-B, ERα mRNA levels slightly increased 3 hours (3hours = 1.2, $p<0.01$) after exogenous treatment with IL-6 compared to control. After 12 hours of treating these ERα positive cell lines with IL-6, ERα mRNA began decreasing, with the most significant decrease observed in BT-474 cells (~40% decrease). 24 and 48 hours post IL-6 treatment, there was at least a 50% decrease in ERα mRNA expression compared to control in all three cell lines, with the most significant in BT-474 cells (60-80% decrease). Interesting, MCF-7 cells which express the highest levels of ERα mRNA and protein, when compared to all other ERα positive breast cancer cell lines decreased by only 50% while T47D and BT-474 showed ~60 and 80 percent decreases (Figure 3.5A-C).

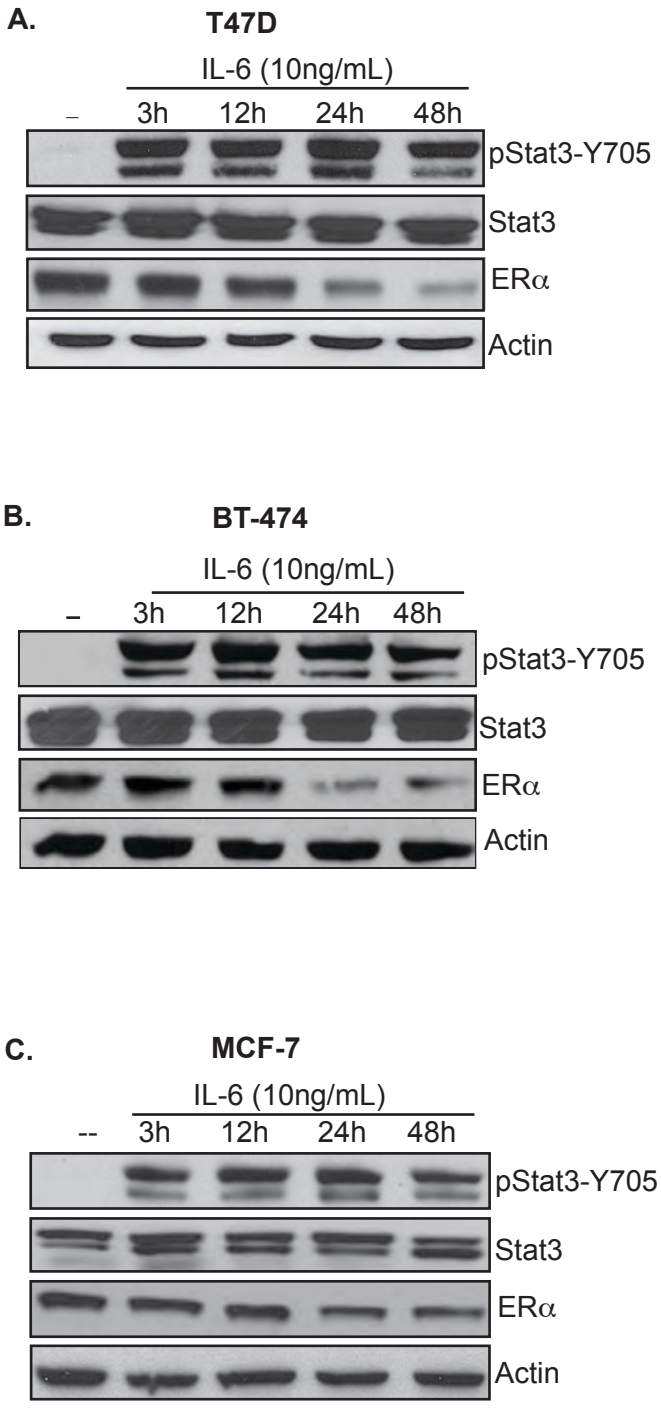


Figure 3.4. IL-6 leads to a decrease in ERα protein. (A) T47D cells were subjected to serum starvation overnight followed by treatment with IL-6 (10ng/mL) at indicated time points. Cell lysates were prepared and subject to western blotting with antibodies against p-Stat3-Y705, Stat3, ERα and actin. phospho-Stat3-Y705 and ERα were normalized against total Stat3 and actin respectively. (B) BT-474 cells. (C) MCF-7.

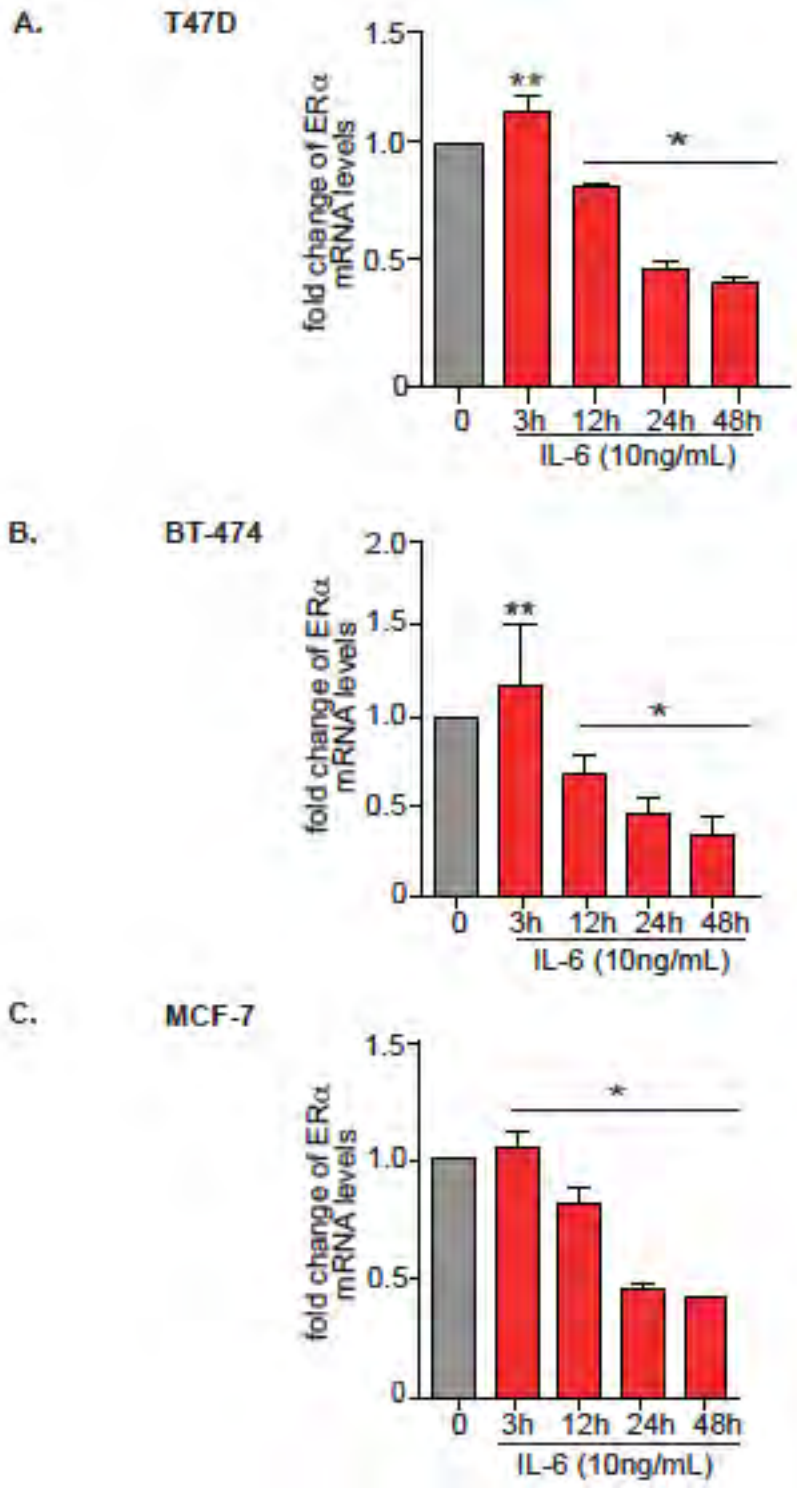


Figure 3.5. IL-6 leads to a decrease in ERα mRNA. (A) T47D cells were subjected to serum starvation overnight and then IL-6 treatment at 10ng/mL at time points indicated. RNA samples were collected and ER⟨ mRNA expression was detected by Q-PCR. The levels of ERα mRNA were normalized by comparison to GAPDH content. The error bars show an SD of 4 experiments. **, P < 0.01; *, P < 0.05 vs. 0h. (B) BT-747. (C) MCF-7.

ERα target genes expression and down-regulation by IL-6

The resulting decrease in ERα mRNA and protein levels after treatment with IL-6 led us to question the effects of IL-6 on ERα signaling. Essentially, we wanted to determine the functionality of the observed ERα decreases. ERα is the defining and driving transcription factor in most breast cancers and in the breast cancer cell lines used in this study [148]. ERα target genes determine cell proliferation, and other endocrine responses in breast cancer cells [63]. We therefore hypothesized that the decrease in ERα mRNA and protein expression would lead to a decrease in expression of ERα target genes. Examined ERα target genes were identified by ChIP-Seq in primary breast cancers from patients with different clinical outcomes and in distant ER-positive metastases [64, 149] and in breast cancer cell lines. We selected 4 ERα target genes – EGR3, PGR, GREB1 and RET and examined changes in these genes after treatment with IL-6. Prior to determining what changes occurred in these genes after treatment with IL-6, we observed the expression of these genes at baseline. We confirmed that, EGR3, PGR, GREB1 and RET are variably expressed in MCF-7, T47D and BT-474 (ERα positive cell lines) and not in MDA-MB-231 and MDA-MB-468 cells (ERα negative cells) (Figure 3.6).

We then treated T74D, BT-474 and MCF-7 cells with IL-6 and via qRT-PCR we determined the expression levels of ERα target genes – PGR, GREB1, RET and EGR3 levels after treatment with IL-6. We show that in all three cell lines, the levels of PGR, GREB1, RET and EGR3 increased slightly after 3 hours of treatment with IL-6, this increase mirrors the increase we see in ERα after 3 hours of IL-6 exposure. At 24 hours, we observe decreases in mRNA levels of these ERα target genes with the most significant decrease at 48 hours (Figure 3.7). The changes observed here are in agreement with the changes observed in ERα after treatment with IL-6 suggesting that

decreases we observe in ERα after treatment with IL-6 is functional.

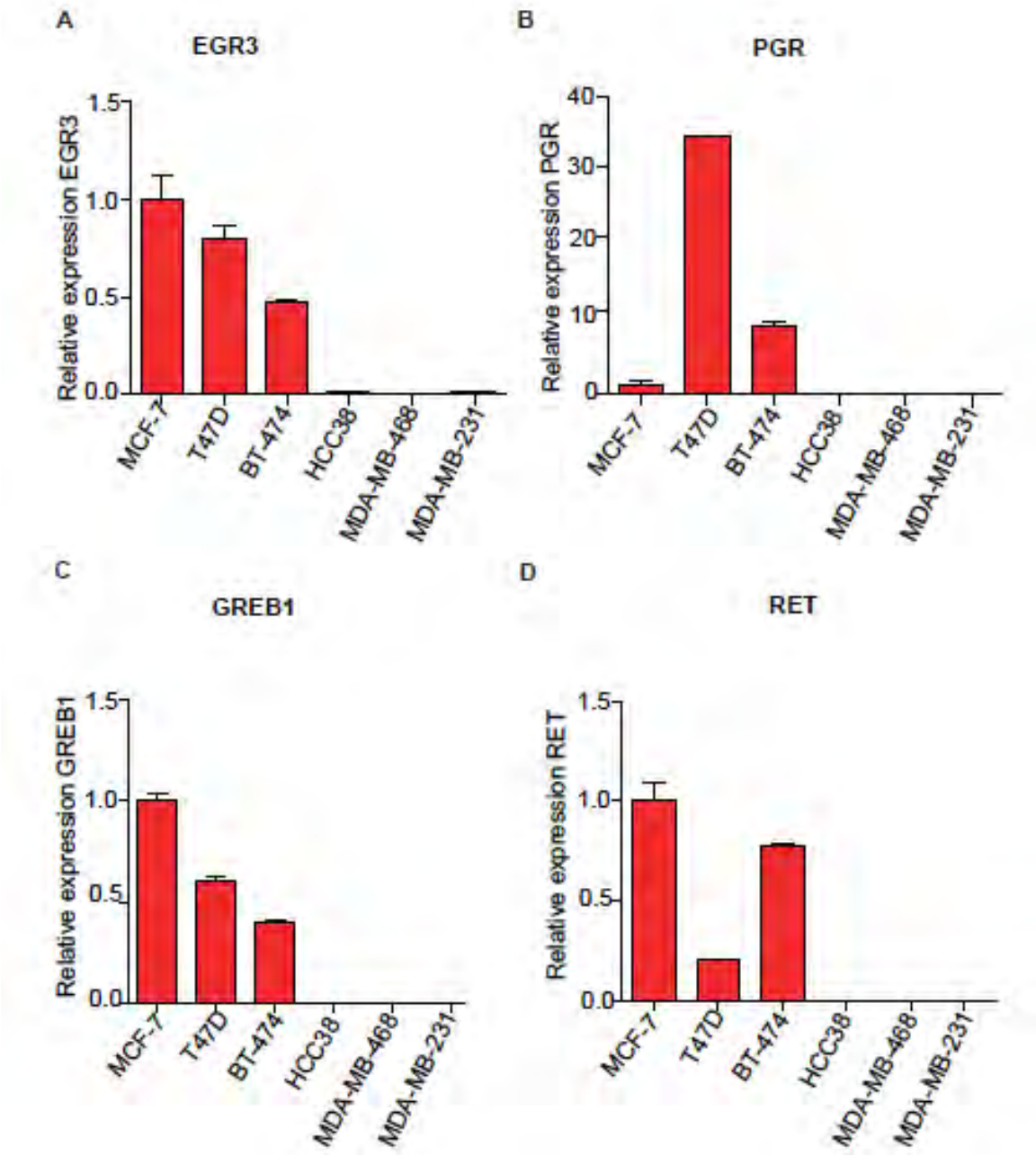


Figure 3.6. ERα target gene expression in breast cancer cell lines. (A) RNA samples were extracted from breast cancer cell lines and EGR3 mRNA expression was detected by Q-PCR. The level of EGR3 was normalized by comparison to GAPDH. (B) RNA samples extracted from breast cancer cell lines and PGR expression was detected by Q-PCR. PGR was normalized by comparison to GAPDH. (C) RNA samples were extracted from breast cancer cell lines and GREB1 expression detected by Q-PCR was normalized by comparison to GAPDH. (D) RNA samples were extracted from breast cancer cell lines and RET expression was detected by Q-PCR, which was normalized to GAPDH. Error bars represent an SD of 3 experiments.

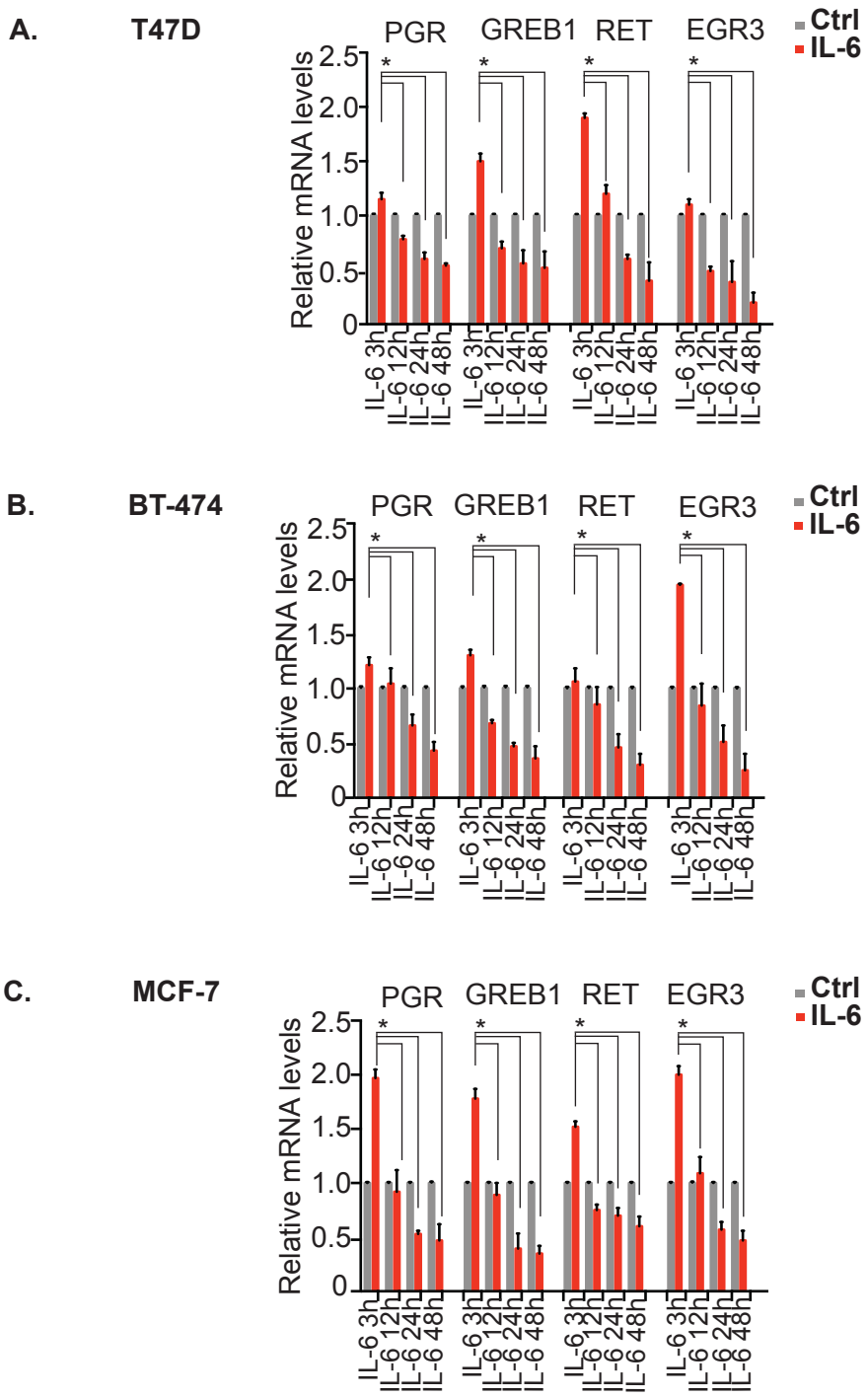


Figure 3.7. IL-6 leads to a decrease in ERα regulated genes in ERα positive breast cancer cell lines. (A) T47D cells were subjected to serum starvation overnight followed by treatment with IL-6 (10ng/mL) at indicated time-points. RNA samples were collected and PGR, GREB1, RET, EGR3 expression was detected by Q-PCR. The levels of PGR, GREB1, RET and EGR3 were independently normalized to GAPDH. The error bars show an SD of three different experiments. *, $P < 0.05$ vs. Ctrl (gray bars). (B) BT-474. (C). MCF-7.

Stat3 regulates ERα expression

IL-6 signaling is capable of activating 3 major proliferative pathways. IL-6 signaling activates not only the Jak/Stat3 pathway, but also the MEK/Erk and PI3K/Akt pathways [150]. Although our observation in patient breast samples and breast cancer cell lines suggested that the IL-6/Stat3 signaling pathway inversely correlates with ERα expression, we wanted to confirm that the observed decreases by IL-6 was mediated by Stat3 signaling.

We hypothesized that inhibiting activation of Stat3 with a Jaki while treating with IL-6 will help us determine if Jak/Stat3 signaling was required for IL-6 mediated down regulation of ERα. The Jaki inhibitor would inhibit the kinase activity of Jak2 and as a result Stat3 will not be phosphorylated. To do this, T47D cells were treated with Jak inhibitor – AZD 1480 in the presence of IL-6. As seen in figure 3.8, ERα expression was decreased with IL-6 alone but not in the presence of a Jaki. This suggests that Stat3 activation is necessary for the IL-6 induced down-regulation of ERα expression.

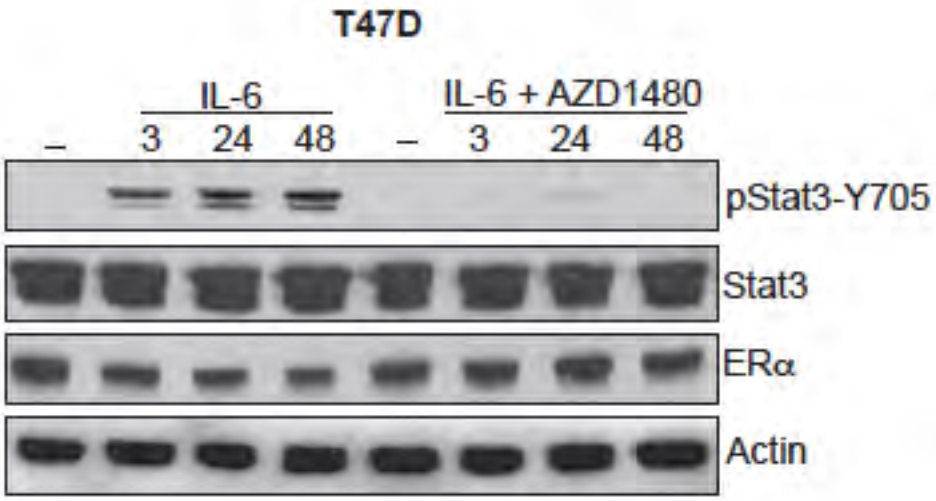


Figure 3.8. Stat3 a downstream signaling pathway of IL-6 regulates to ERα expression. T47D cells were serum starved overnight followed by pretreatment with IL-6 or IL-6 + Jak inhibitor (AZD1480, 1uM) at time points indicated. Whole cell lysates were subject to western blotting with indicated antibodies. Actin protein is used as loading control. The levels of ERα were normalized against actin.

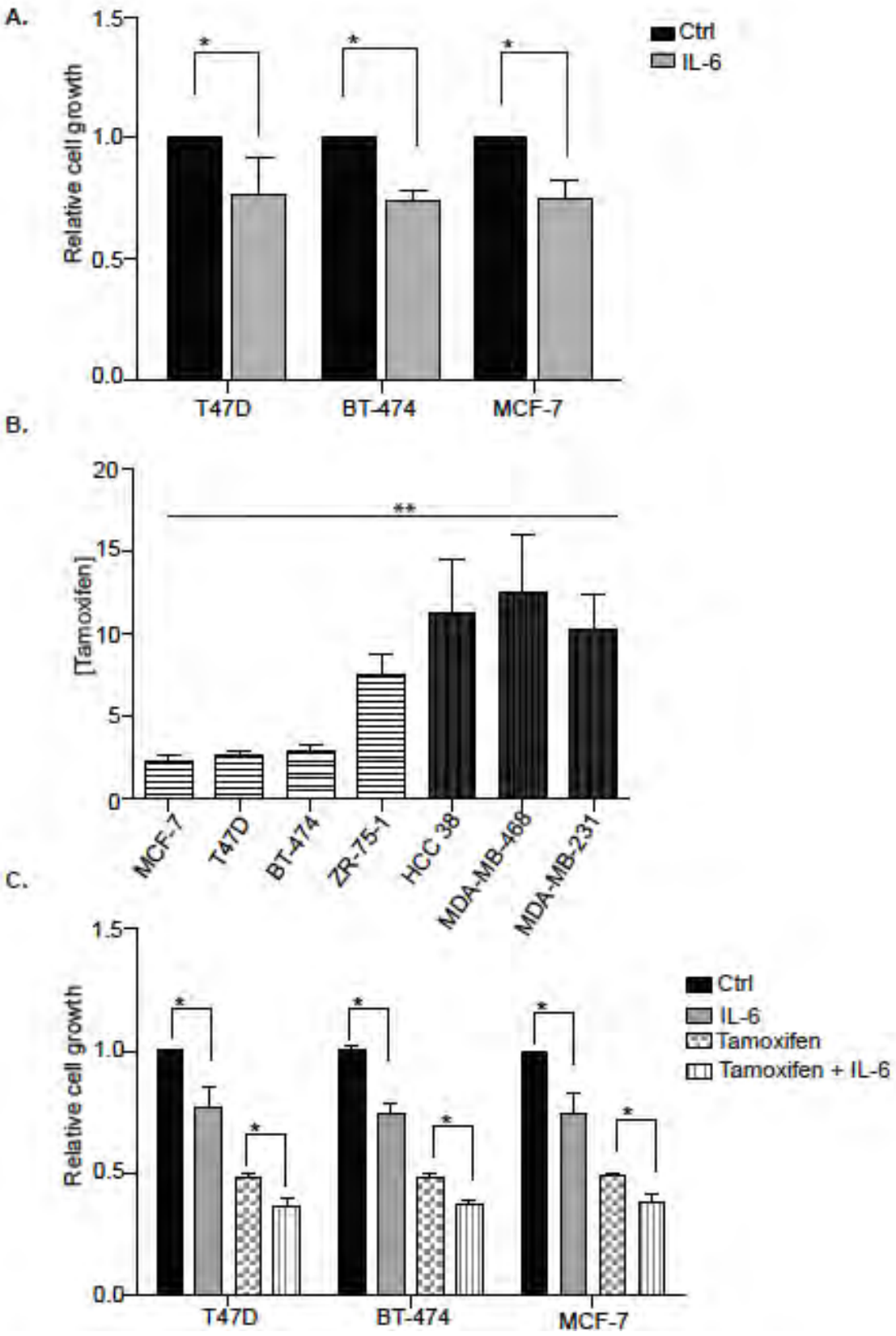
IL-6 leads to a decrease in cell growth *in vitro*

We hypothesized that IL-6 mediated decrease in ERα will lead to a decrease in proliferation since these cells are dependent on ERα signaling for growth. We treated T47D, BT-474 and MCF-7 cells with IL-6 and measured proliferation over 5 days using a metabolic assay (MTT). We observed IL-6 led to a 20 percent decrease in cell growth *in vitro* in all three cell lines (Figure 3.9A).

One of the most significant therapies in the treatment of in breast cancer therapy is the administration of tamoxifen [18]. Tamoxifen, a selective estrogen receptor modulator is used for the treatment of hormone receptor positive breast cancer. It works by competitively binding to the estrogen receptor thus inhibiting its ability to signal [151-154]. We hypothesized that in combination with tamoxifen, IL-6 will lead to a further decrease in proliferation of these ERα positive breast cancer cell lines. We first determined the IC50's of each ERα positive breast cancer cell line before treating with IL-6. Our results show that ERα positive cell lines have a low IC50 of 2-3uM compared to ERα negative cell lines, which have a higher IC50 of 7-10uM (Figure 3.9B).

We then treated T47D, BT-474 and MCF-7 cells with IL-6 alone, tamoxifen alone or a combination of IL-6 and tamoxifen and assayed for cell proliferation via MTT. We observed that the combination of IL-6 and tamoxifen led to a further decrease in proliferation compared to treatment with tamoxifen alone. IL-6 and tamoxifen decreased cell proliferation by 60 percent at a concentration where tamoxifen decreased growth by 50% (Figure 3.9C).

Figure 3.9. IL-6 leads to a decrease in cell growth in vitro. (A) T47D, BT-474 and MCF-7 cells were grown in 96-well plates for 24hours. The cells were then serum starved overnight followed by treatment with IL-6 for 96 hours under low serum (0.5%) condition. Cell proliferation was measured by MTT assay. Error bars represent an SD of 5 wells and triplicate experiments. (B) Cells were grown in 96-well plates for 24hours. Cells were then serum starved overnight followed by treatment with tamoxifen. Cell proliferation was measured by MTT assay. Error bars represent SD of 5 identical wells and quadruple experiments. ***, $p < 0.001$. (C) T47D, BT-474 and MCF-7 cells were grown in 96 well plates for 24 hours. The cell were then serum starved overnight followed by treatment with either IL-6, tamoxifen or a combination of IL-6 and tamoxifen for 96 hours under low serum conditions. Cell proliferation was measured by MTT assay.


A.
Relative cell growth
1.5
1.0
0.5
0.0
Ctrl
IL-6
T47D
BT-474
MCF-7
B.
[Tamoxifen]
20
15
10
5
0
MCF-7
T47D
BT-474
ZR-75-1
HCC 38
MDA-MB-468
MDA-MB-231
C.
Relative cell growth
Ctrl
IL-6
Tamoxifen
Tamoxifen + IL-6
T47D
BT-474
MCF-7

Stat3 binds to the ERα promoter

To investigate whether the observed down-regulation of ERα mRNA and protein after treatment with IL-6 was induced by Stat3's activity on the ERα promoter, we examined the ERα gene including its promoter for Stat3 binding sites. We looked for Stat3 binding sites within the promoter region and around exon1 (location of CpG islands). Proximal to exon 1 were previously identified ERα promoters A and B, shown in purple and yellow boxes respectively. Within and around the ERα promoter region, we identified 4 putative Stat3, binding sites (TTN5AA) shown in red triangles (Figure 3.10).

Next, we were interested in determining whether Stat3 can bind to the putative Stat3 binding sites identified on the ERα promoter. Using electro-mobility shift assay (EMSA), we tested the ability of each putative site to bind Stat3. Our results revealed that lysates from IL-6 treated T47D cells bound to all four probes *in vitro.* (Figure 3.11B). To provide additional evidence on specificity of the binding, we performed EMSA-supershift by using a Stat3-specific antibody and a cold M67 probe. M67 is the optimal Stat3 binding site (ttcccgtaa). As shown in Figure 3.11C, antibody treatment led to diminished density of the basal band and to the formation of a "supershifted" band.

The confirmation that Stat3 can bind *in vitro* to the putative Stat3 binding sites identified on the ERα promoter led us to question whether Stat3 can also bind to the ERα promoter *in vivo*. We designed primers that encompassed all 4 of the putative Stat3 binding site identified on the ERα promoter (Figure 3.12) for a ChIP assay. Then, we carried out ChIP assays on T47D and MCF-7 cells treated with IL-6 for 3 and 24 hours to determine whether Stat3 binds to the ERα promoter. Our results revealed that Stat3 binds to the ERα promoter in an IL-6 dependent manner. We observed Stat3 on the ERα promoter after 3 hours of treating with IL-6. By 24 there was a there a significant increase in the amount of Stat3 on the ERα promoter (Figure 3.13).

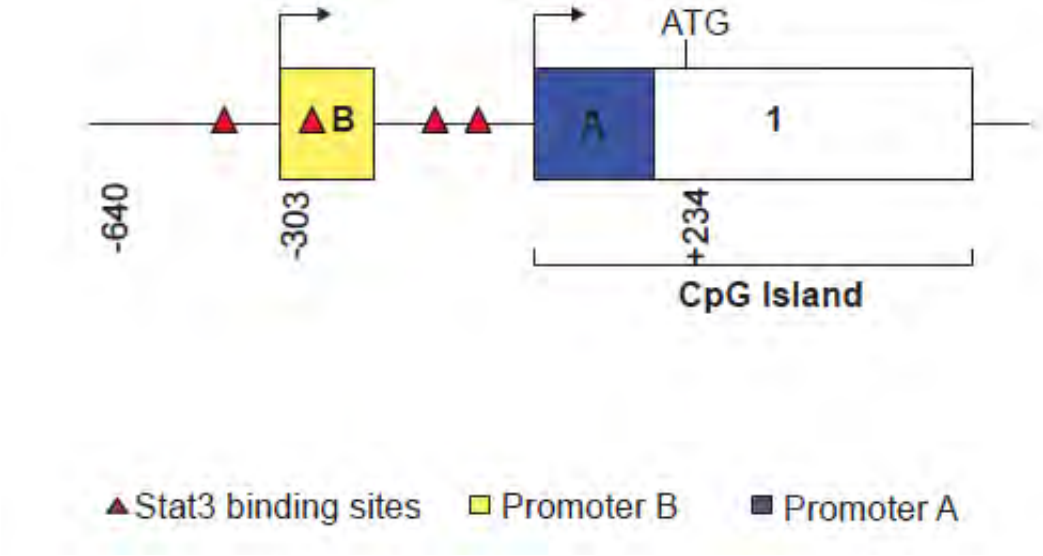


Figure 3.10. Organization of the promoter region of the human ERα gene. Schematic of the ERα promoter region. Red triangles represent putative stat3 binding sites yellow box shows promoter B, blue box shows promoter A. CpG island is located in exon 1, which also contains the first ATG. RefSeq accession: NM_000125.3

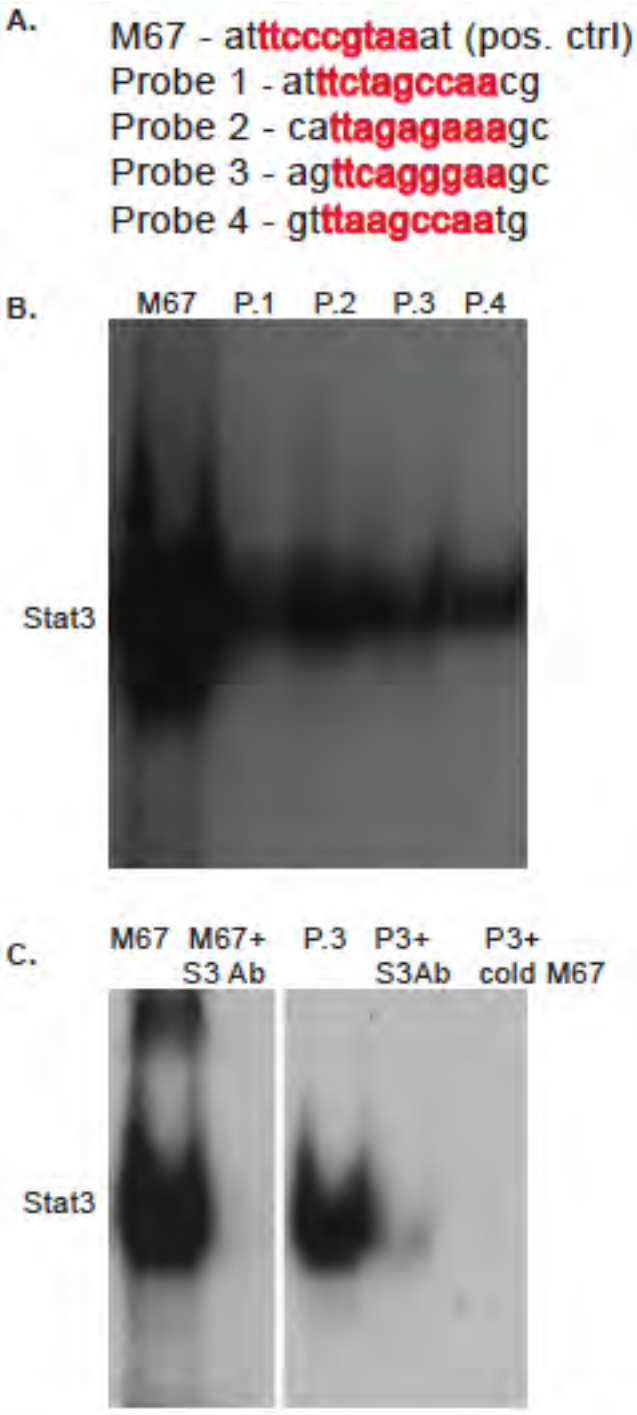


Figure 3.11. Stat3 binds in vitro to the putative Stat3 binding sites identified on the ERα promoter. (A) Probes with Stat3 binding sites used in EMSA. (B) EMSA with 4 probes including positive control M67. (C) Supershift EMSA using anti-Stat3 antibody and cold M67 probe.

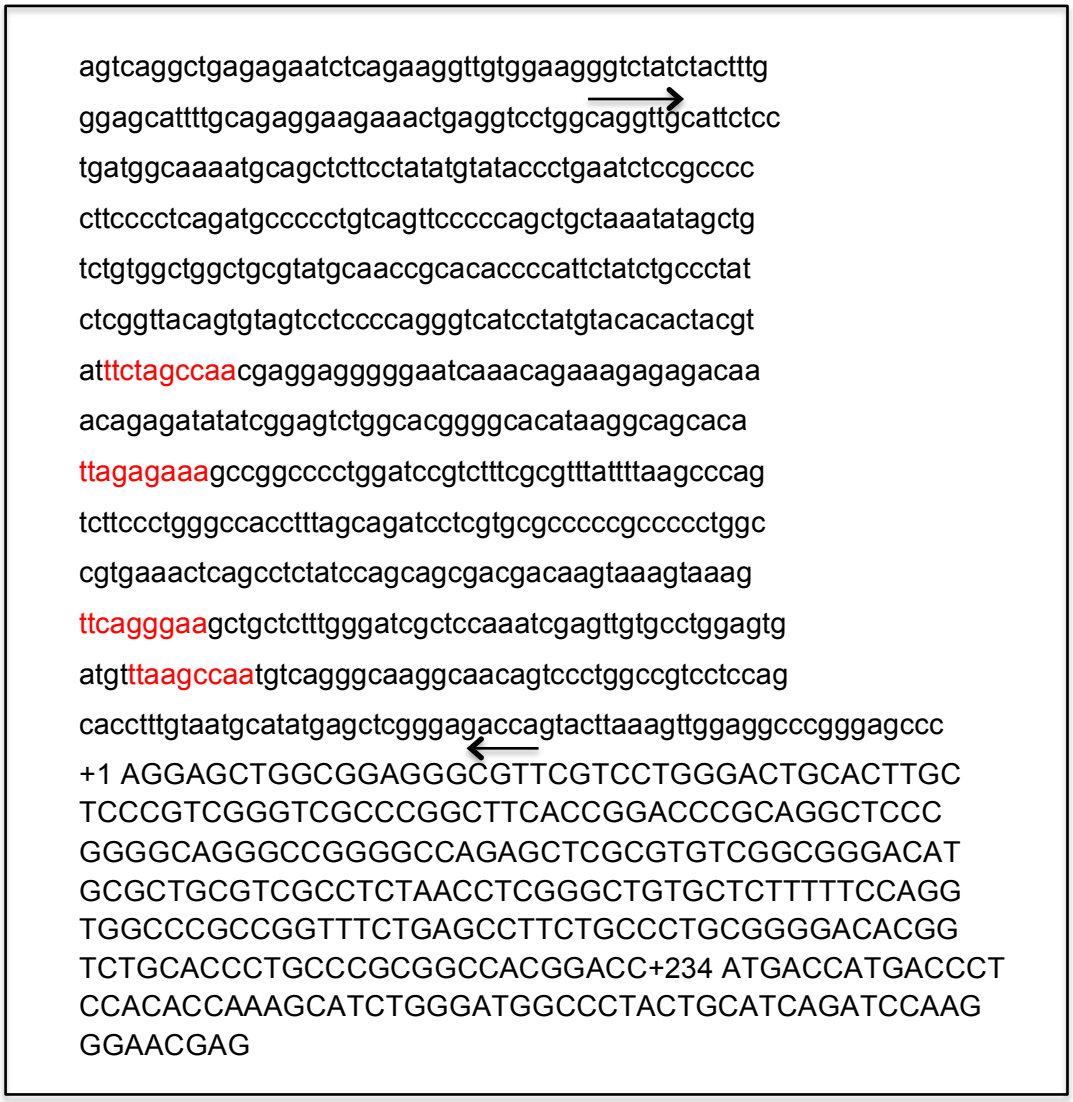


Figure 3.12. Putative Stat3 binding sites identified on the ERα promoter. The red letters represent putative Stat3 binding sites identified on the ERα promoter. Arrows represent primers used in ChIP assays. RefSeq accession: NM_000125.3

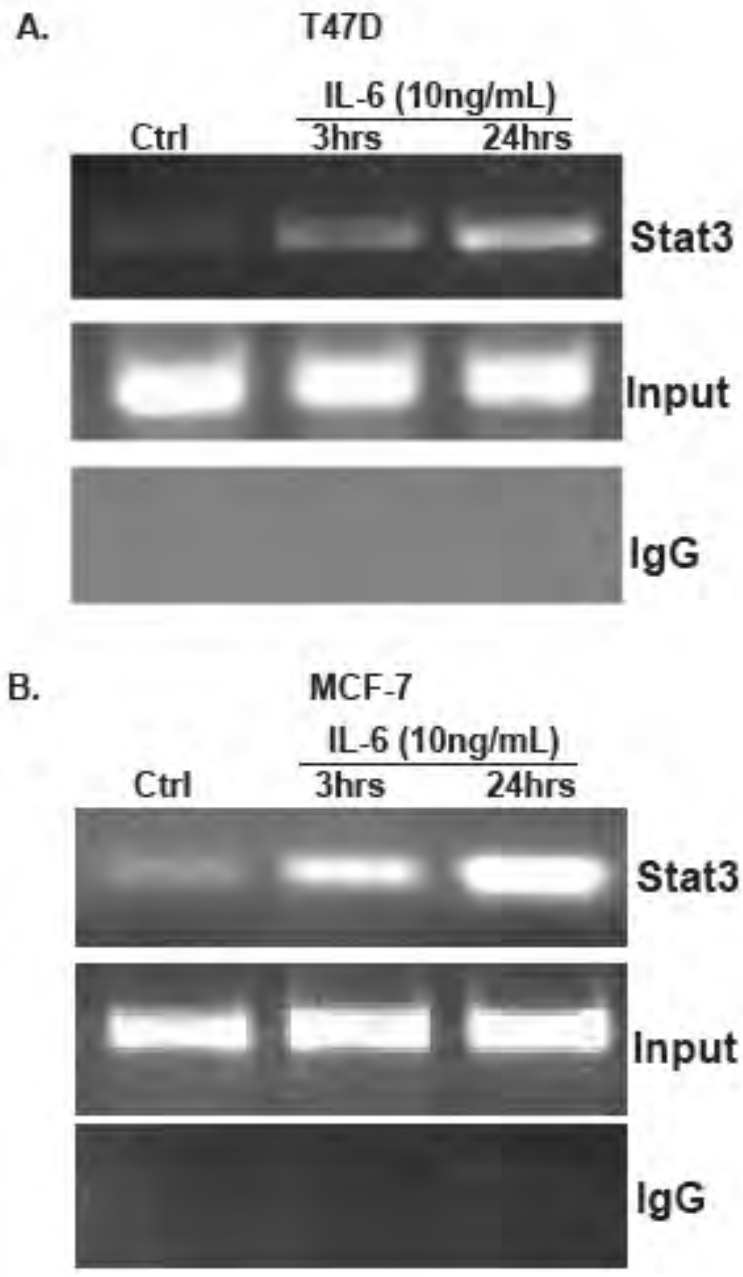


Figure 3.13. Binding of Stat3 to ERα promoter in vivo. (A and B) Cell lysates obtained from listed breast cancer cell lines at indicated time points were examined by ChIP assay using antibodies against Stat3 and PCR primer pairs corresponding to ERα promoter.

Since we observed Stat3 binding to the ERα promoter in an IL-6 dependent manner using a primer that encompassed all 4 putative Stat3 binding sites identified within and around the ERα promoter, we were curious to determine whether Stat3 bound to each site or if it bound to only one particular site. We designed primers for each Stat3 binding site identified on the ERα promoter and then carried out a ChIP assay followed by qRT-PCR using primers for each putative Stat3 binding site.

We observed that the presence of Stat3 on the ERα promoter was not significantly increased after IL-6 treatment when we used primers P-1 to P-3 (Figure 3.14A). However, using primer P-4 (Figure 3.14B), we observed a 2-fold increase in the presence of Stat3 on the ERα promoter in T47D and MCF-7 cells, 3 hours after treatment with IL-6 (Figure 3.15). 24 hours after IL-6 treatment, we observed a 4-7 fold increase of Stat3 on the ERα promoter (Figure 3.15). Interestingly, Primer P-4 is encompasses not only the Stat3 binding site (that contains the CAAT box) but also the TATA box.

To confirm that Stat3 bound to the ER promoter wasn't an artifact, we looked for the presence of Stat3 on the SOCS3 promoter after IL-6 treatment. SOCS3, a bonafied Stat3 target is also a negative regulator of Stat3[155, 156]. We confirmed that Stat3 is also bound to the SOCS3 promoter in T47D and MCF-7 as a consequence of IL-6 signaling, confirming that the presence of Stat3 on the ERα promoter is not an artifact (Figure 3.15).

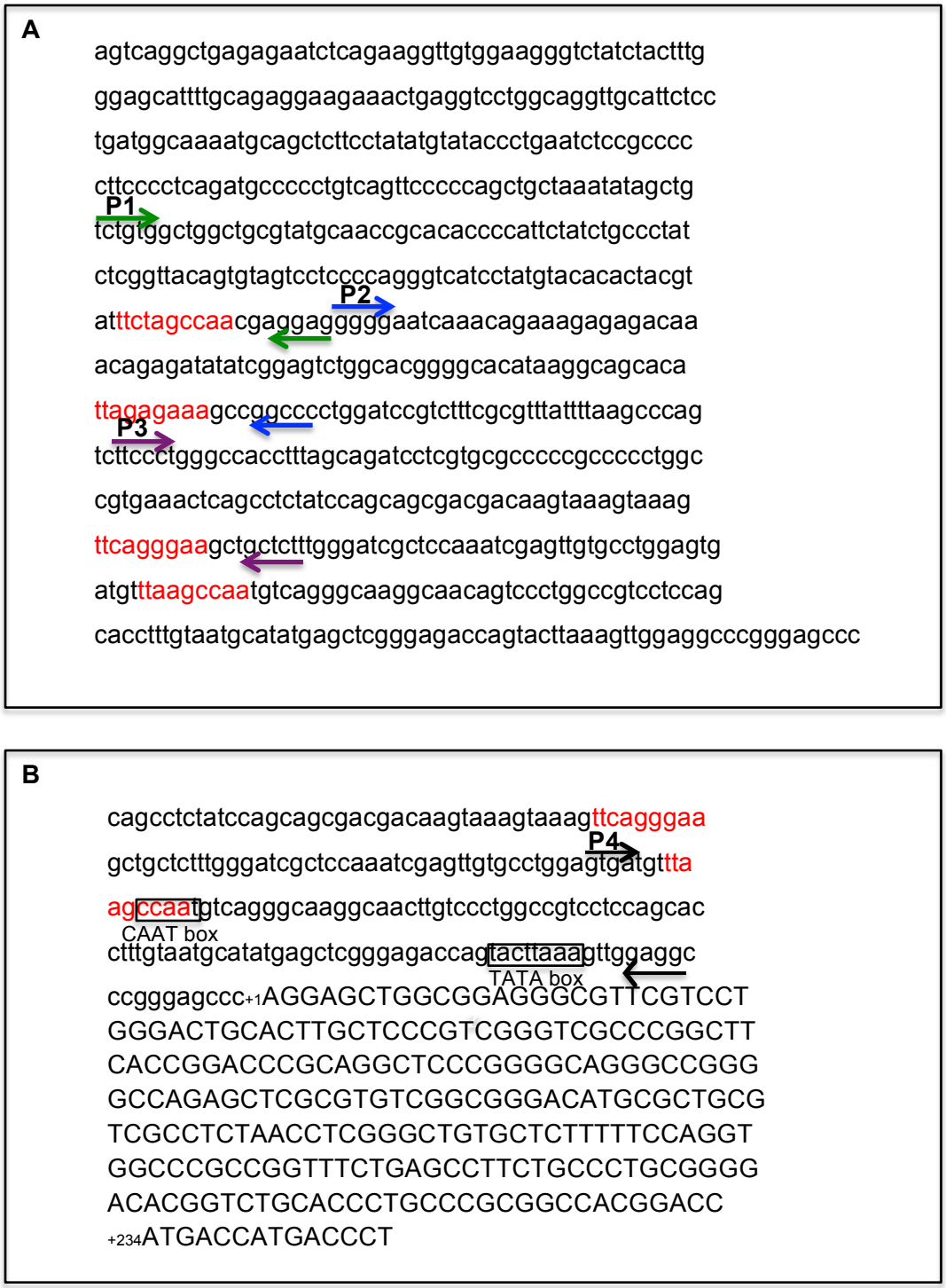


Figure 3.14. Sub-section of ERα promoter region, showing CAAT and TATA box in proximity to Stat3 binding sites. The red letters represent a putative Stat3 binding site identified in proximity to the CAAT and TATA box. Arrows represent primers used for qRT-PCR analysis after immuno-precipitation. RefSeq accession: NM_000125.3

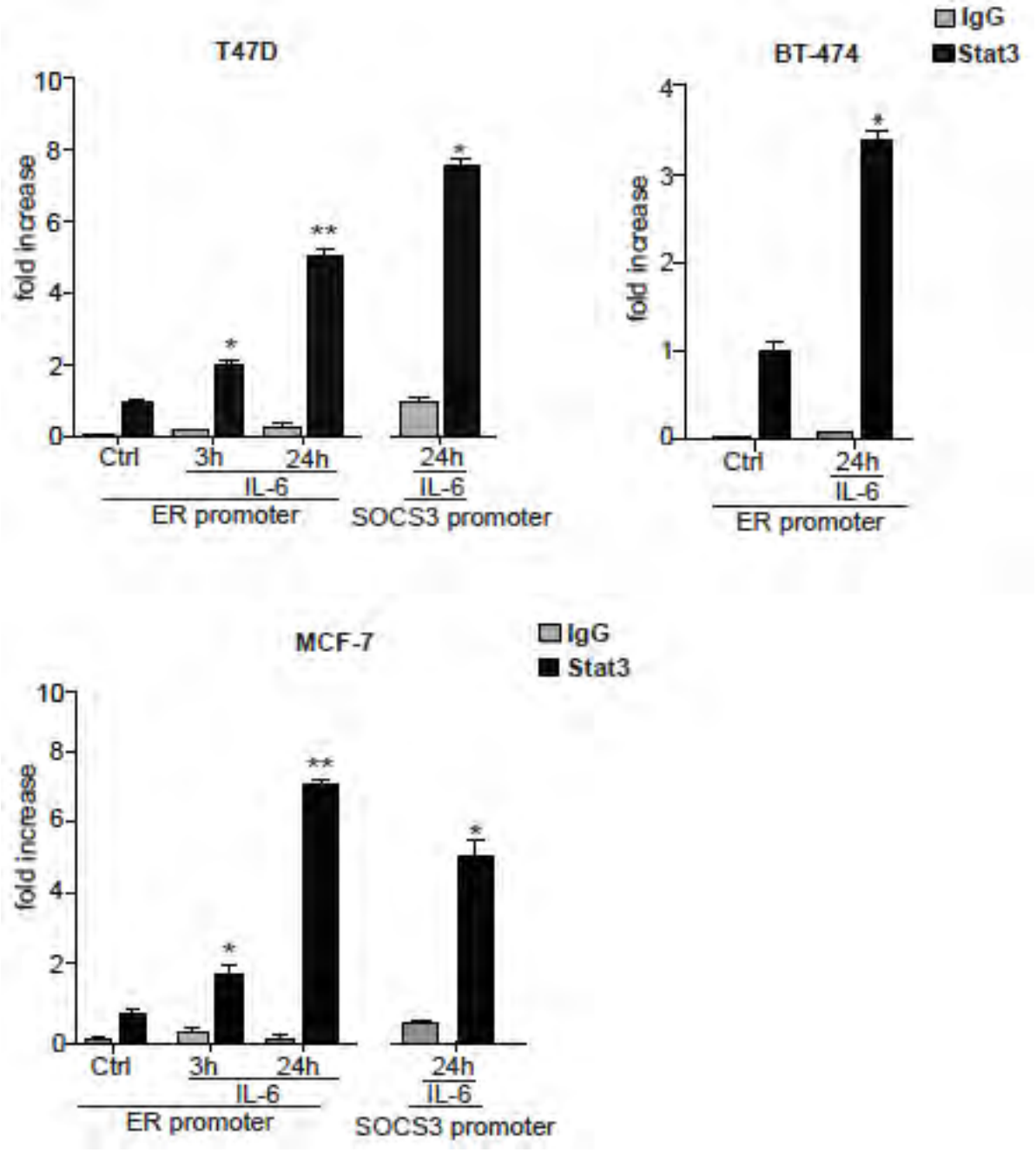


Figure 3.15 Stat3 binds by ChIP to the estrogen receptor alpha (ERα) promoter. ERα positive breast cancer cell lines were serum starved overnight, followed by treatment with IL-6 (10ng/mL) at indicated time-points. Cross-linked cell lysates were examined by ChIP assay using antibodies against Stat3 and PCR primer pairs corresponding to ER⟨ promoter and SOCS3 promoter (positive control). Error bars show SD for triplicate experiments. **, P < 0.01, *, P < 0 .05 vs. control.

Triple negative breast cancer cell lines produce IL-6 and constitutively express Stat3. We hypothesized that because triple negative cell lines MDA-MB-231 and MDA-MB-468, constitutively express Stat3, Stat3 will be bound to the ERα. We carried out ChIP on these cells by immunoprecipitating Sta3 and using primers P-4 to see whether Stat3 was bound to the ERα promoter. Our ChIP assay revealed that Stat3 is not bound to the ERα promoter of triple negative breast cancer cell lines (Figure 3.16).

We hypothesized that Stat3 is not bound to the ERα promoter of ERα negative cell lines, because the ERα chromatin in triple negative cell lines is modified and is in a repressed state. To confirm this, we used ChIP to assay for active ERα transcription via histone mark (H3K4Me1) and a repressed chromatin state via histone mark (H3K9Me3). Our analysis revealed that active transcription mark H3K4Me1 is expressed on the ERα promoter of ERα positive cell lines but not on the ERα promoter of triple negative cell lines. However, triple negative cell lines express H3K9Me3 – a repressed chromatin marker, but ERα positive breast cancer cell lines do not (Figure 3.17).

In other to further understand the differences in the chromatin structure of ERα positive cell lines and ERα negative cell lines, we extensively examined CpG islands methylation of the ERα gene. Examination of the CpG islands of the ERα promoter revealed that methylation patterns in vary cell lines. Most importantly, we observed that ERα positive cell lines have much lower levels of methylation compared to ERα negative cell lines (Figure 3.18). The hypermethylation observed within the CpG island of ERα negative cell lines suggest that the gene is in an inactive/heterochromatin state hence transcription factors such as Stat3 are unable to bind. Deaton et al. [43] demonstrated that DNA methylation can lead to the inhibition of transcription by hindering the binding of transcription factors to the promoter.

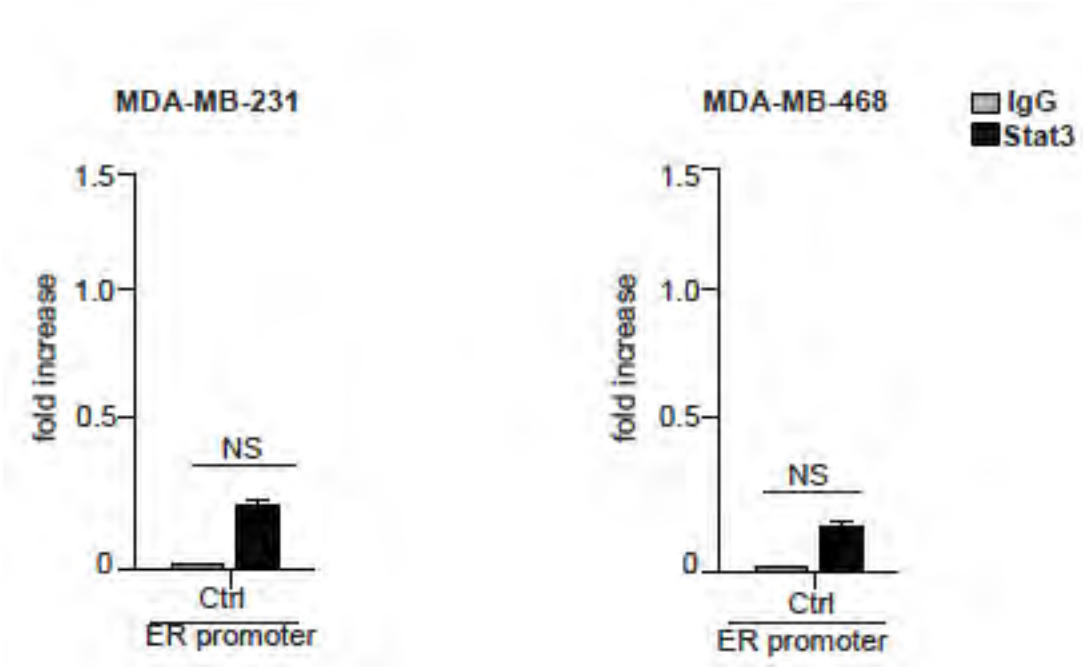


Figure 3.16. Stat3 does not associate with ERα promoter in triple negative breast cancer cells. Cross linked cell lysates from MDA-MB-231 and MDA-MB-468 cells were examined by ChIP assay using antibodies against Stat3 and PCR primer pairs corresponding to ERα promoter. Error bars show SD for triplicate experiments. NS compared to IgG control.

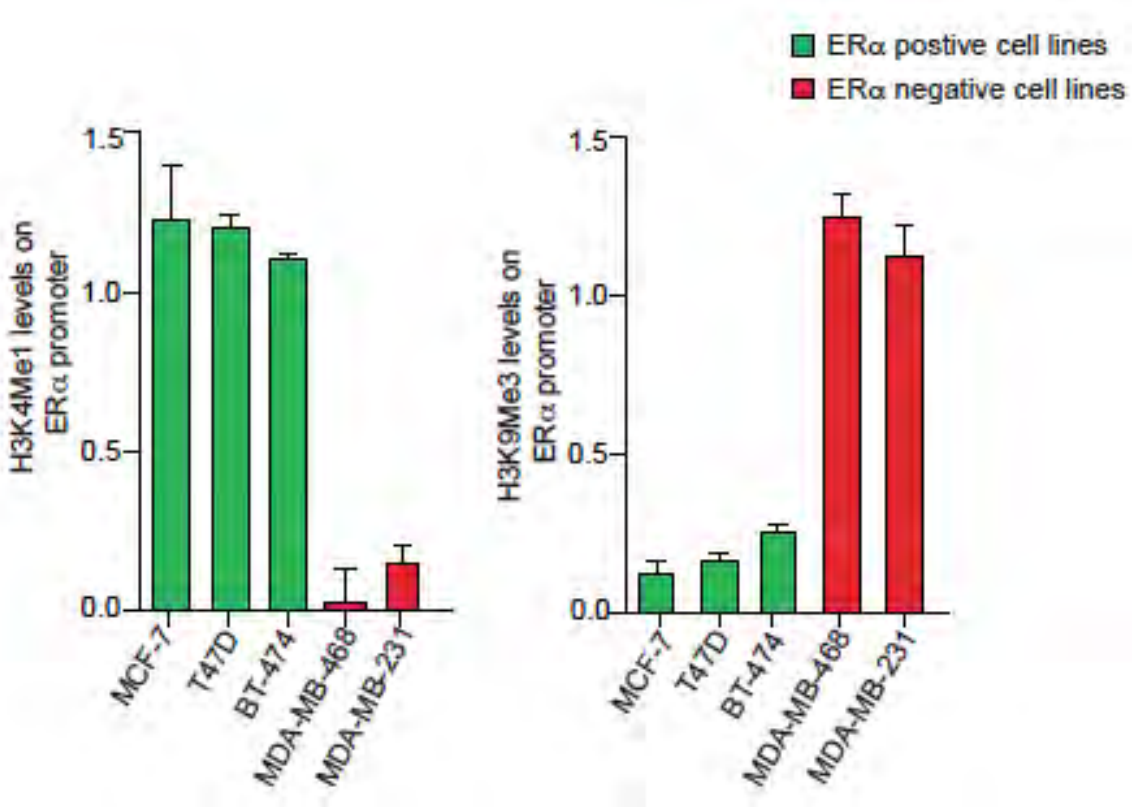
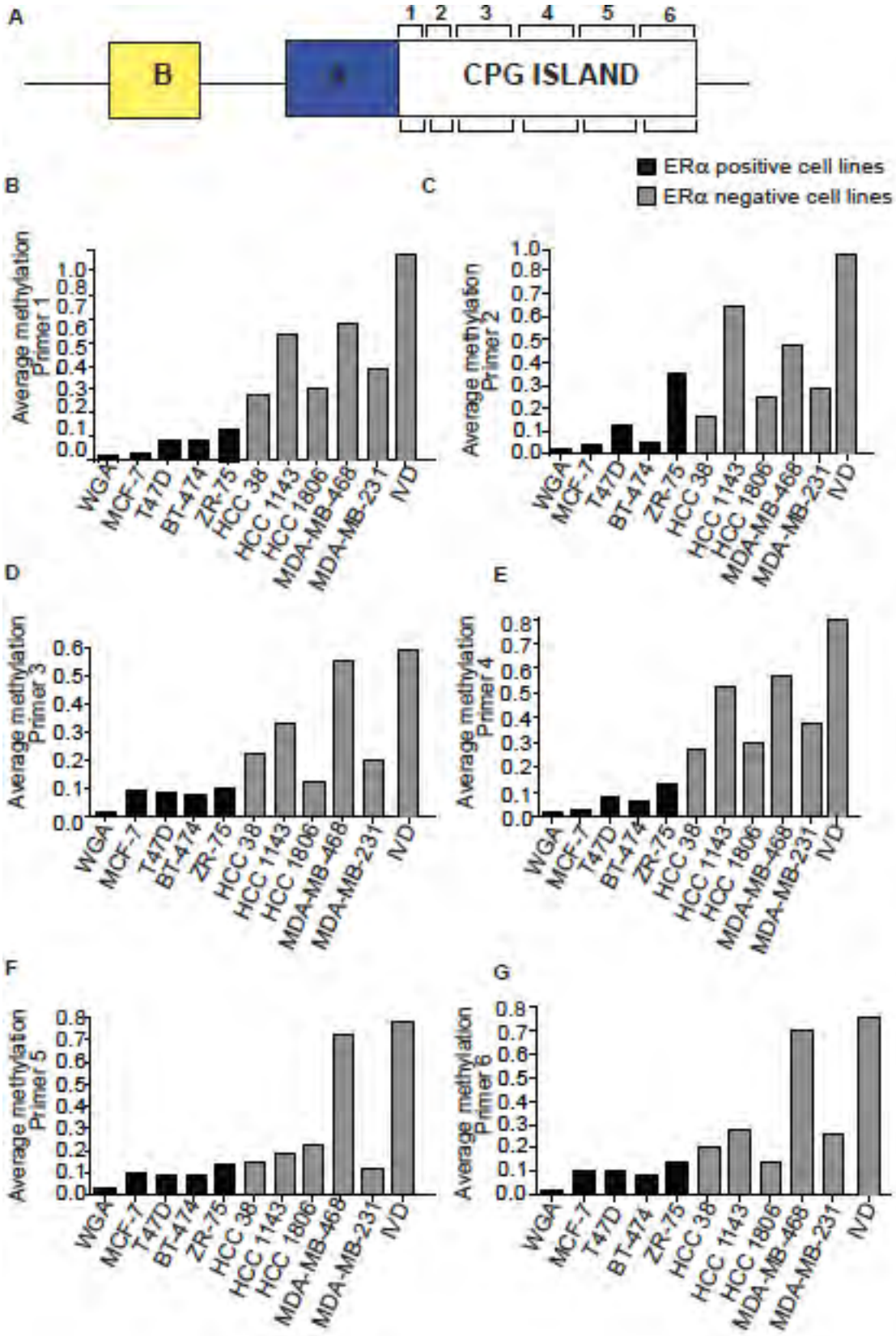


Figure 3.17. ERα promoter of ERα positive cell lines express activating histone mark (H3K4Me1) and ERα negative cell lines express repressive histone mark (H3K9Me3). (A) Cross-linked cell lysates from listed cell lines are assayed by ChIP using an antibody against H3K4Me1 and PCR primer pairs corresponding to the ERα promoter. (B) Cross-linked cell lysate from each cell line is assayed by ChIP using an antibody against H3K9Me3 and PCR primer pairs corresponding to the ERα promoter. Error bars represent show SD for triplicates, normalized to input DNA.

Figure 3.18. Average ERα promoter methylation in breast cancer cell lines. (A) Schematic of ERα promoter and exon 1, which contains CpG islands. Brackets represent different primer pairs used in EpiTYPER methylation assay. (B) Average methylation of CpG islands within primer section 1 in indicated breast cancer cell lines. (C) Average methylation of CpG islands within primer section 2 in indicated breast cancer cell lines. (D) Average methylation of CpG islands within primer section 3 in indicated breast cancer cell lines. (E) Average methylation of CpG islands within primer section 4 in indicated breast cancer cell lines. (F) Average methylation of CpG islands within primer section 5 in indicated breast cancer cell lines. (G) Average methylation of CpG islands within primer section 6 in indicated breast cancer cell lines.


A
1 2 3 4 5 6
B
CPG ISLAND
ERα positive cell lines
ERα negative cell lines
B
C
D
E
F
G
Average methylation Primer 1
Average methylation Primer 2
Average methylation Primer 3
Average methylation Primer 4
Average methylation Primer 5
Average methylation Primer 6
WGA
MCF-7
T47D
BT-474
ZR-75
HCC 38
HCC 1143
HCC 1806
MDA-MB-468
MDA-MB-231
IVD

IL-6 induces HDAC1 binding to the ERα promoter

Stat3 has been shown to act as a transcriptional repressor by recruiting HDAC1 and DNMT1 to the SHP-1 promoter [140]. It's also been shown to bind to the p53 promoter in vitro and in vivo and mediates p53 down-regulation [139]. We hypothesized the down-regulation of ERα after treatment with IL-6 was due in part by the recruitment to transcriptional repressors such as HDAC1 to the ERα promoter.

To test whether IL-6 signaling in ERα positive breast cancer cell lines leads to the recruitment of HDAC1 to the ERα promoter, we performed ChIP. We immuno-precipitated HDAC1 using an anti-HDAC1 antibody from control and IL-6 treated cells, and then carried out a qRT-PCR using primer P-4, to determined whether HDAC1 was bound to the ERα promoter in the same region we observed Stat3 binding. Our results revealed that HDAC1 was detected at the ERα promoter of T47D, BT-474 and MCF-7's cells 24 hours after exogenous addition of cells with IL-6 (Figure 3.19).

Ray, S et al. [157] showed that IL-6 dependent acetylation of Stat3 at it's NH2-terminal acetylation domain is required for the ability of Stat3 binding to HDAC1 and that a. Acetylation of Stat3 has also been linked with its ability to recruit DNMT1 to the promoter of tumor suppressor genes [158, 159]. We therefore wanted to determine whether IL-6 signaling in ERα positive cell lines led to the acetylation of Stat3. Western blot analysis carried out on cell lysates from control and IL-6 treated cells at 3 and 24 hours revealed that IL-6 leads to the acetylation of Stat3 at 24 hours (Figure 3.20). The observance of acetyl-Stat3 at 24 hours is a likely explanation for the recruitment of HDAC1 at 24hours. Overall, the results suggest that IL-6 mediated down-regulation of ERα is observed at 24 hours after Stat3 acetylation and HDAC1 recruitment to the ERα promoter to the ERα promoter.

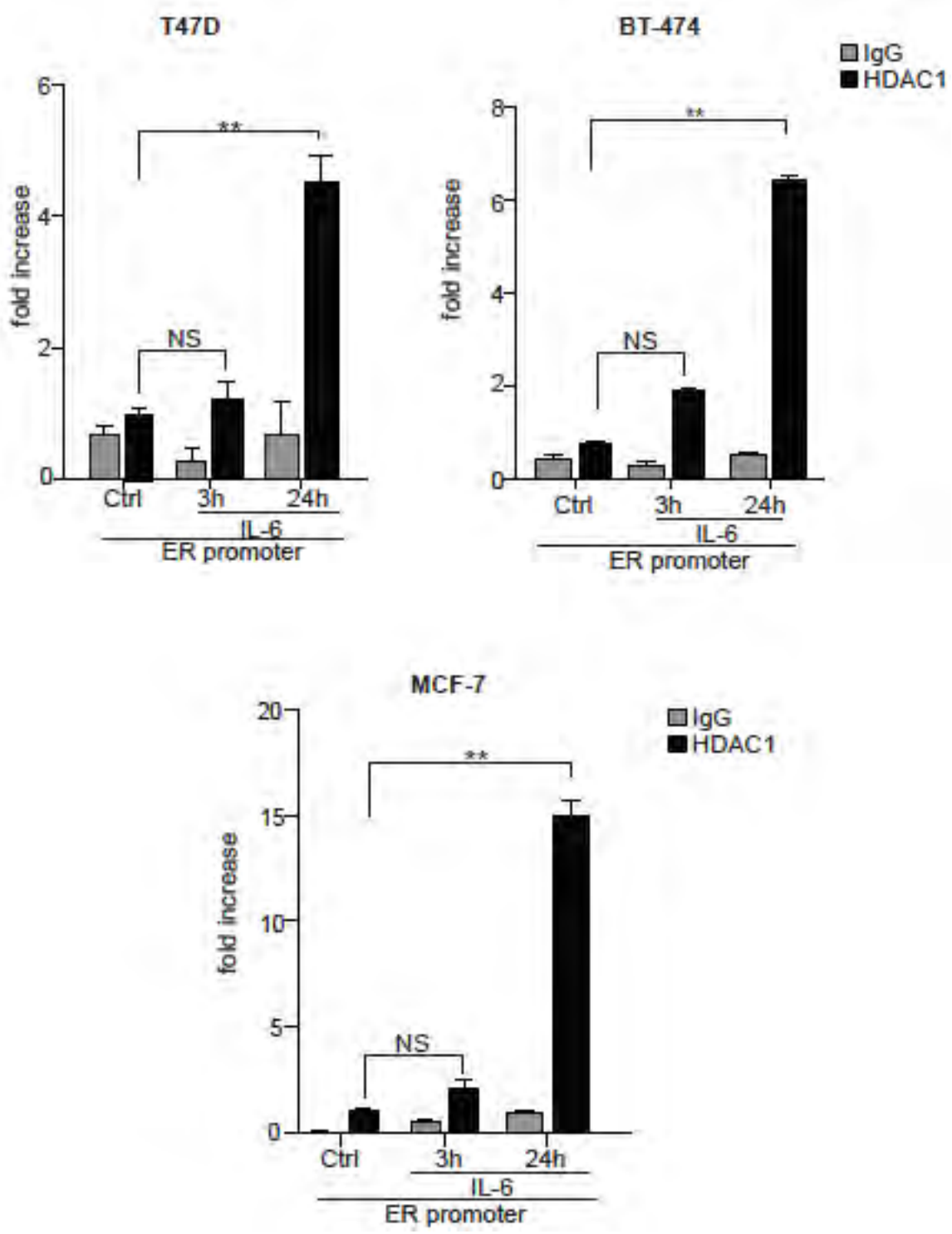


Figure 3.19. IL-6 induces HDAC1 binding to the ERα promoter. Listed breast cancer cell lines were serum starved overnight and then treated with IL-6 at indicated time points. After treatment, cell lysates were examined by ChIP assay using antibody against HDAC1 and PCR primer pairs corresponding to ERα promoter. CT values were normalized to input DNA. Error bars show an SD of triplicates. **, P < 0.01 vs. Ctrl.

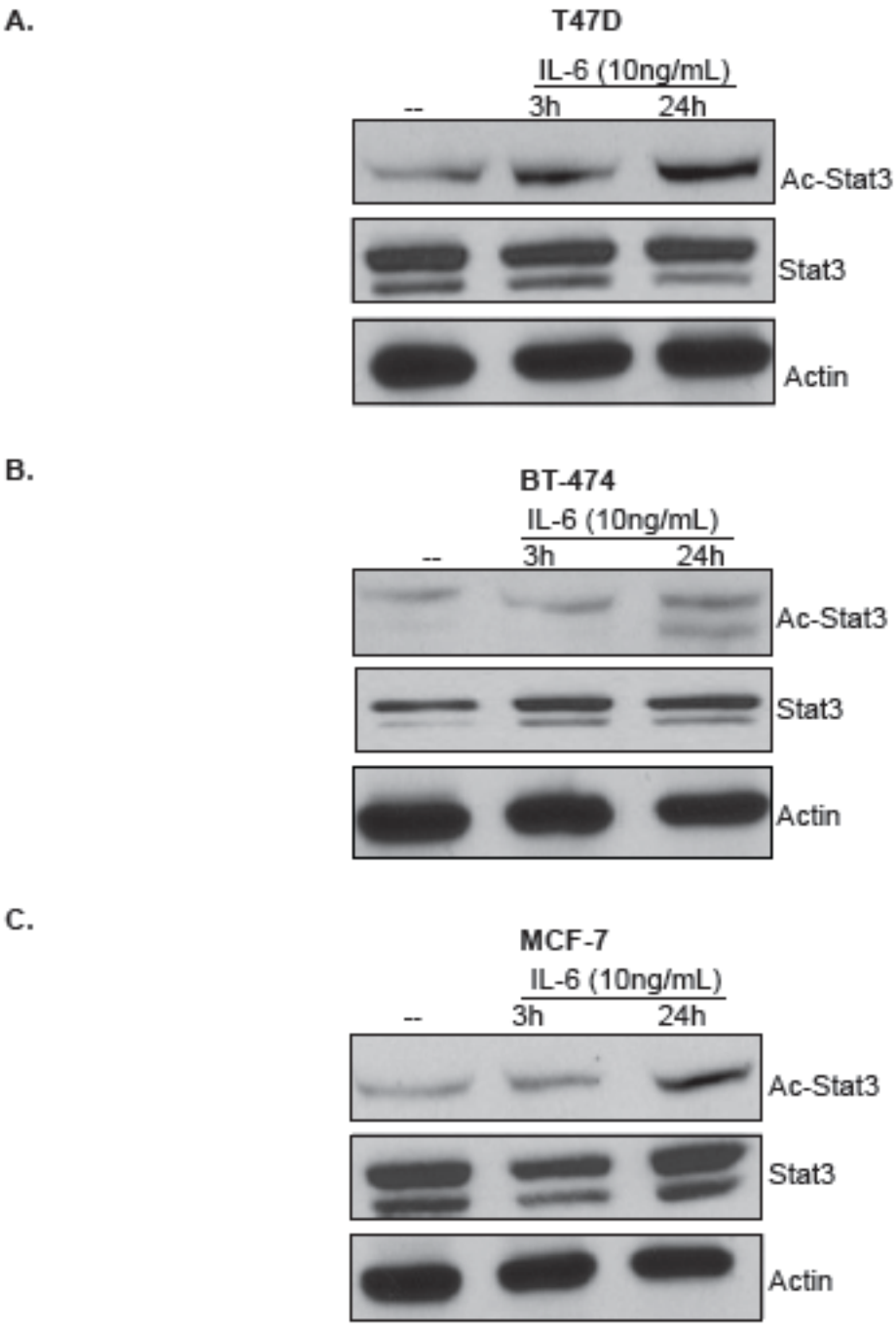


Figure 3.20. IL-6 leads to the acetylation of Stat3 in ERα positive breast cancer cells. (A) T47D cells are serum starved overnight followed by treatment with IL-6 (10ng/mL) at indicated time-points. Total lysates were prepared and subjected to western-blotting with Ac-Stat3, total Stat3 and actin. Ac-Stat3 was normalized against total Stat3 and actin.

IL-6 leads to changes in histone marks on the ERα promoter.

The presence of HDAC1 on the ERα promoter in response to IL-6 (Figure 3.19) and a subsequent decrease in ERα expression (Figure 3.4-3.5) led us to hypothesize that IL-6 signaling in ERα positive cell lines leads to changes in histone marks.

As shown in figure 3.17, the ERα promoter of ERα positive breast cancer cell lines T47D, MCF-7 and BT-474 are enriched for histone H3K4 mono-methylation (H3K4Me1), however after treatment with IL-6, we observed a 2-fold decrease in the level of H3K4 mono-methylation (Figure 3.21). In addition, ChIP on H3K9 acetylation, another marker of an actively transcribed promoter revealed a 2-fold decrease in H3K9 acetylation (H3K9Ac) after treatment of ERα positive breast cancer cell lines with IL-6 (Figure 3.22).

We also examined whether any changes occurred in the expression of H3K9 tri-methylation (H3K9Me3). H3K9Me3 is a marker of gene repression found in constitutively repressed genes [160]. As observed in figure 3.17, the ERα promoter of T47D, BT-474 and MCF-7's cells does not express H3K9 tri-methylation at baseline. However, after treatment with IL-6, we observed approximately 2-fold increase in H3K9 tri-methylation at the ERα promoter compared to control (Figure 3. 23).

Our result suggests that IL-6/pStat3 pathway down-regulates ERα expression by recruiting acetylated and phosphorylated Stat3 to the ERα promoter, which leads to the recruitment of HDAC1. The presence of HDAC1 and other co-repressors likely DNMT1 leads to the de-acetylation of histones and also leads to an increase in histone trimethylation.

Figure 3.21. IL-6 alters activating histone mark (H3K4Me1) expression on the ERα promoter. ERα positive breast cancer cell lines were serum starved overnight and then treated with IL-6 (10ng/mL) for 24 hours. Cell lysates from each cell line were examined by ChIP using antibody against H3K4Me1 and PCR primer pairs corresponding to the ERα promoter. Values were normalized to input DNA. Error bars show SD of triplicates. **, P < 0.01 vs. Ctrl.

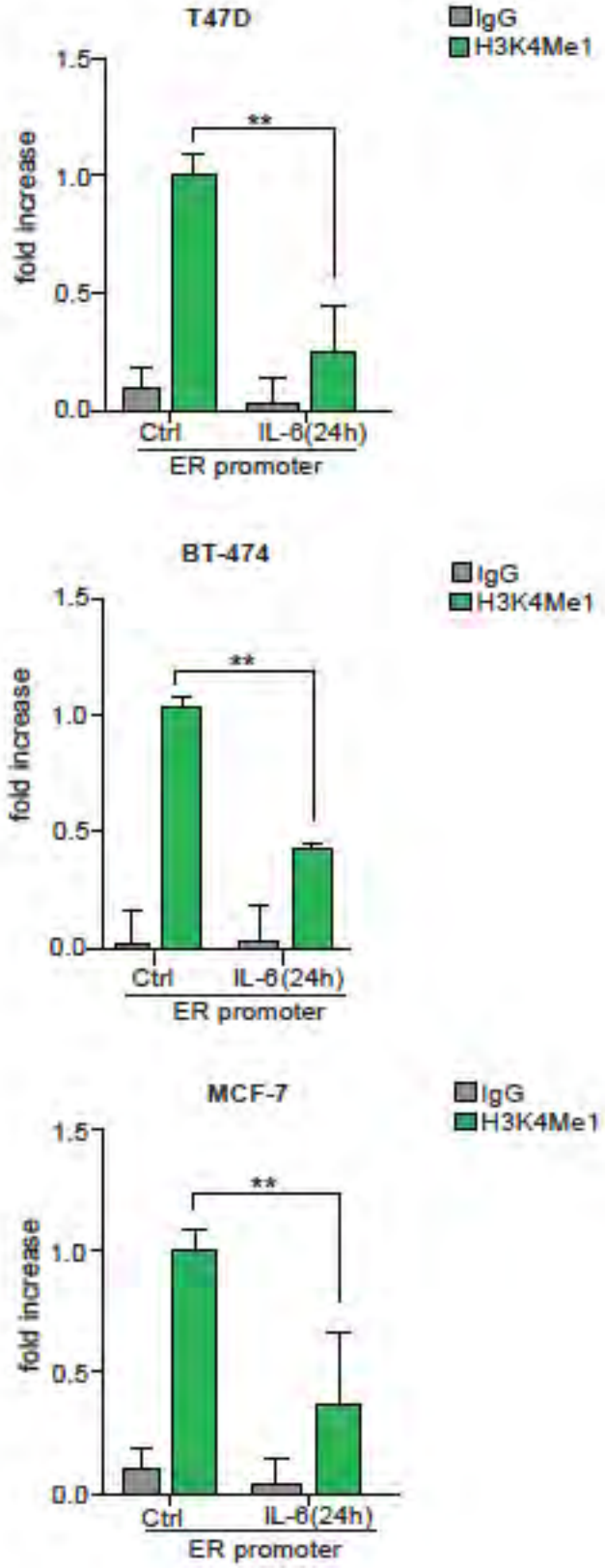

T47D
IgG
H3K4Me1
fold increase
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter
BT-474
IgG
H3K4Me1
fold increase
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter
MCF-7
IgG
H3K4Me1
fold increase
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter

Figure 3.22. IL-6 alters activating histone mark (H3K9Ac) expression on the ERα promoter in ERα positive breast cancer cell lines. ERα positive breast cancer cell lines were serum starved overnight and then treated with IL-6 (10ng/mL) for 24 hours. Cell lysates from each cell line were examined by ChIP using antibody against H3K9Ac and PCR primer pairs corresponding to the ERα promoter. Values were normalized to input DNA. Error bars show SD of triplicates. **, $P < 0.01$ vs. Ctrl.

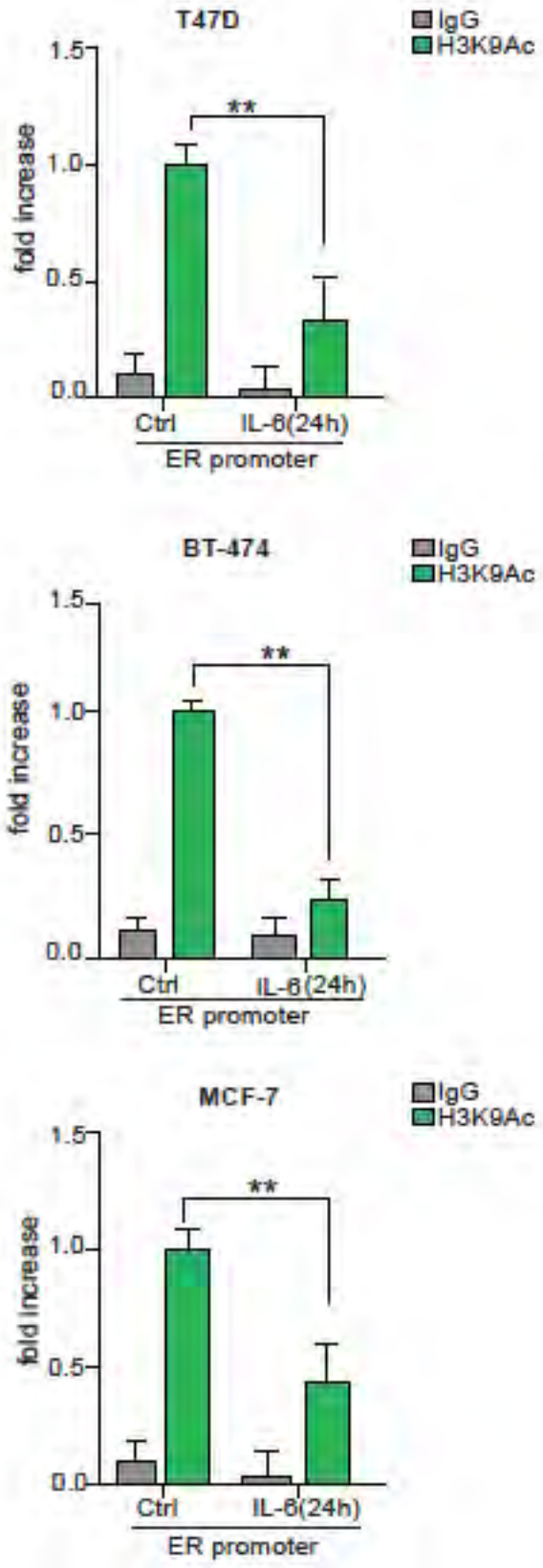
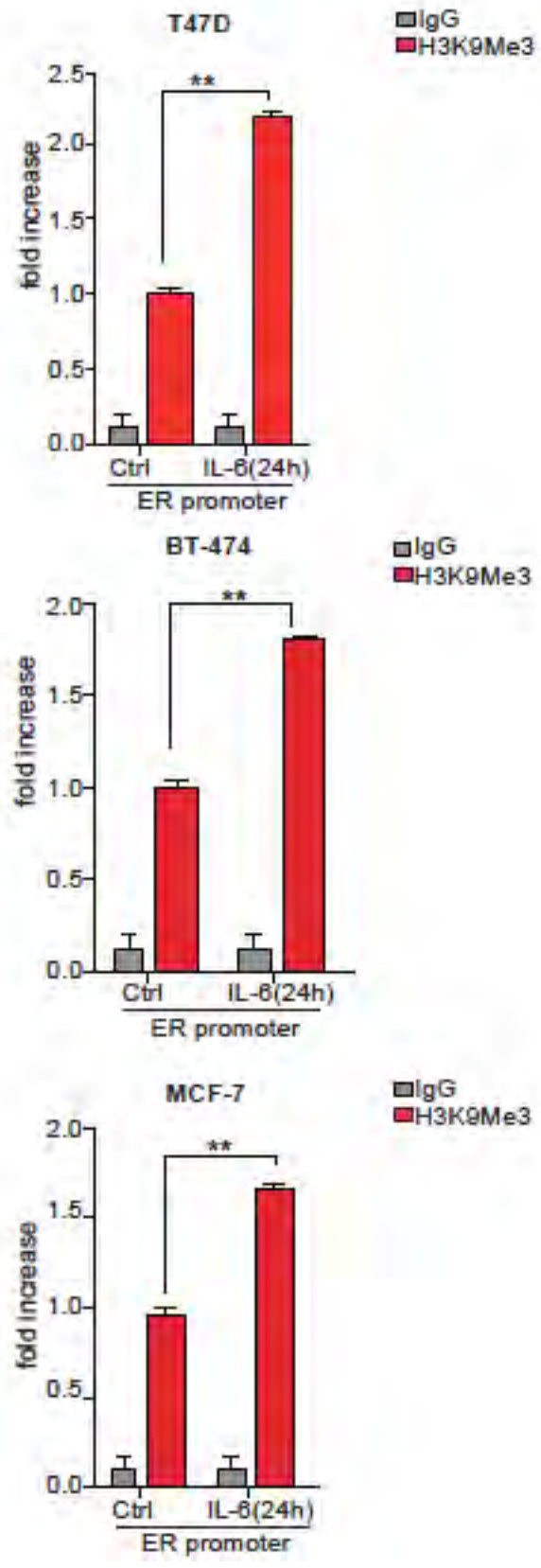

T47D
IgG
H3K9Ac
1.5
1.0
0.5
0.0
fold increase
**
Ctrl
IL-6(24h)
ER promoter
BT-474
IgG
H3K9Ac
1.5
1.0
0.5
0.0
fold increase
**
Ctrl
IL-6(24h)
ER promoter
MCF-7
IgG
H3K9Ac
1.5
1.0
0.5
0.0
fold increase
**
Ctrl
IL-6(24h)
ER promoter

Figure 3.23. IL-6 led to an increase in repressive histone mark (H3K9Me3) expression on the ERα promoter. ERα positive breast cancer cell lines were serum starved overnight and then treated with IL-6 (10ng/mL) for 24 hours. Cell lysates from each cell line were examined by ChIP using antibody against H3K9Me3 and PCR primer pairs corresponding to the ERα promoter. Values were normalized to input DNA. Error bars show SD of triplicates. **, $P < 0.01$ vs. Ctrl.


T47D
IgG
H3K9Me3
fold increase
2.5
2.0
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter
BT-474
IgG
H3K9Me3
fold increase
2.0
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter
MCF-7
IgG
H3K9Me3
fold increase
2.0
1.5
1.0
0.5
0.0
**
Ctrl
IL-6(24h)
ER promoter

Knockdown of Stat3 in triple negative breast cancer cell lines does not lead to re-expression of ERα

We revealed a novel role for the IL-6/phospho-Stat3 signaling pathway in mediating ERα expression. Essentially, we showed that exogenous treatment of ERα positive breast cancer cell lines with IL-6 led to the down-regulation of ERα via the recruitment of Stat3 HDAC1 to the ERα promoter. We then hypothesized that since Stat3 mediated ERα down-regulation in ERα positive cell lines, knocking down Stat3 ERα negative breast cancer cell lines could lead to ERα re-expression. Zhang et al. [140] showed that knockdown of Stat3 with a siRNA Stat3 construct led to re-expression of the SHP-1 gene.

Using a previously described shStat3 construct [146], we knockdown Stat3 in two triple negative cell lines, (MDA-MB-468 and MDA-MB-231). Our results revealed a Stat3 knockdown of about 70- 90% (Figure 3. 24) and no increase in ERα mRNA (not shown) or ERα protein expression (Figure 3.24). However, these results were not surprising because we have shown that ERα is in the heterochromatin state in triple negative breast cancer cell lines thus transcription factors such as Stat3 are not bound to the ERα promoter. ERα repression has been induced in triple negative breast cancer cell lines by treating with DNMT inhibitors such as 5-aza-dC and HDAC inhibitors such as TSA [161]. We hypothesize that knocking down Stat3 in conjunction with 5-aza-dC and TSA will lead to an even greater expression of ERα than using the inhibitors alone.

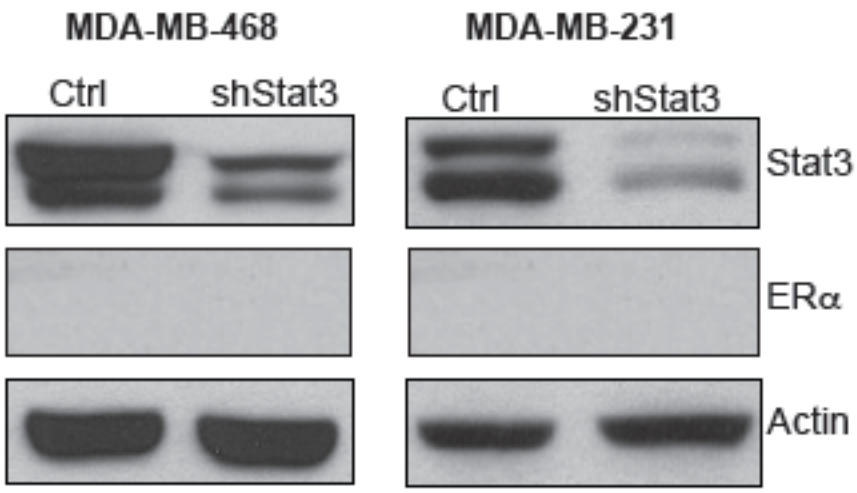


Figure 3.24. Knocking-down Stat3 in ERα negative breast cancer cell lines does not lead to ERα re-expression. After transfecting MDA-MB-468 and MDA-MDA-231 cells with shStat3-GFP virus, cells were sorted for GFP expression. Cell lysates from ctrl and shStat3 cell lines were prepared and subjected to western blotting using antibodies against Stat3, ERα and actin. Stat3 and ERα were normalized to actin.

CHAPTER FOUR
DISCUSSION

Summary

The estrogen receptor signaling is a central regulator of mammary tumors with approximately 70% of breast cancers expressing ERα [23]. However, about 20% of breast cancers lose ERα expression and acquire estrogen-independent features during tumor progression [63]. Since ERα expression levels determine response to endocrine therapy with SERMS (such as tamoxifen [23]), a decrease in ERα expression results in resistance to therapy, which represents a daunting challenge in the treatment of breast cancer. Therefore, understanding the molecular mechanisms by which ERα expression is repressed is important for the management of breast cancer, since restoring ERα expression may lead to improved sensitivity to anti-estrogen therapies.

This study demonstrated that IL-6/phospho-Stat3-Y705 expression is inversely correlated with ERα expression in primary breast cancers and in breast cancer cell lines, suggesting a regulatory relationship between both pathways. Indeed, ERα has been shown to regulate IL-6 expression [144]. However, we hypothesized that there was more to this inverse relationship and that IL-6/Stat3 signaling pathway also regulates ERα signaling. Exogenous treatment of ERα positive breast cancer cell lines with IL-6 led to a significant decrease in ERα mRNA and protein levels, suggesting that the IL-6/Stat3 signaling pathway may directly repress ERα expression and contribute to the progression of breast cancer by altering the sensitivity of the cancer to endocrine therapy.

Indeed, our molecular analyses provided compelling evidence for direct repression of ERα by Stat3. We identified functional Stat3 binding sites within and adjacent to the transcriptional start sites of the ERα promoter. Validation of these sites using EMSA (*in vitro* assay) and ChIP (*in vivo* assay) revealed that indeed Stat3 binds directly to these sites on the ERα promoter. The IL-6 induced recruitment of Stat3, a known transcriptional activator, to the ERα promoter also led to the recruitment of HDAC1, a transcriptional repressor, to the ERα promoter. This result is consistent with the finding that Stat3 interacts with transcriptional repressors, HDAC1, DNMT1, NuRD complex and mediates repression of a number of genes [139, 140]. HDAC1 and DNMT1 recruitment to chromatin is linked to IL-6 induced acetylation of Stat3 [158, 159]. We also found that, IL-6 treatment of ERα positive cell lines also led to the acetylation of Stat3 at K-685.

Our results demonstrated that the recruitment of Stat3 and HDAC1 to the ERα promoter led to decreased histone H3K9 acetylation and H3K4 mono-methylation. We also observed an increase in histone H3K9 tri-methylation. Taken together, our results demonstrate that ERα can be regulated by the Stat3 and that Stat3 recruits HDAC1 to directly repress ERα expression in breast cancer cells (Figure 4.1).

The proposed model (Figure 4.1) demonstrates a mechanism by which ERα is regulated by the IL-6/Stat3 signaling pathway. However, IL-6/Stat3 signaling pathway is not sufficient in regulating ERα expression. In triple negative breast cancer cell lines (MDA-MB-231 and MBA-MB-468) where IL-6/pStat3 is expressed and ERα is not expressed, simply inhibiting Stat3 signaling using a shStat3 construct does not lead to ERα re-expression (Fig. 3.24). These data demonstrate that in conjunction with IL-6/Stat3 signaling, there are other mechanisms involved in regulating ERα expression.

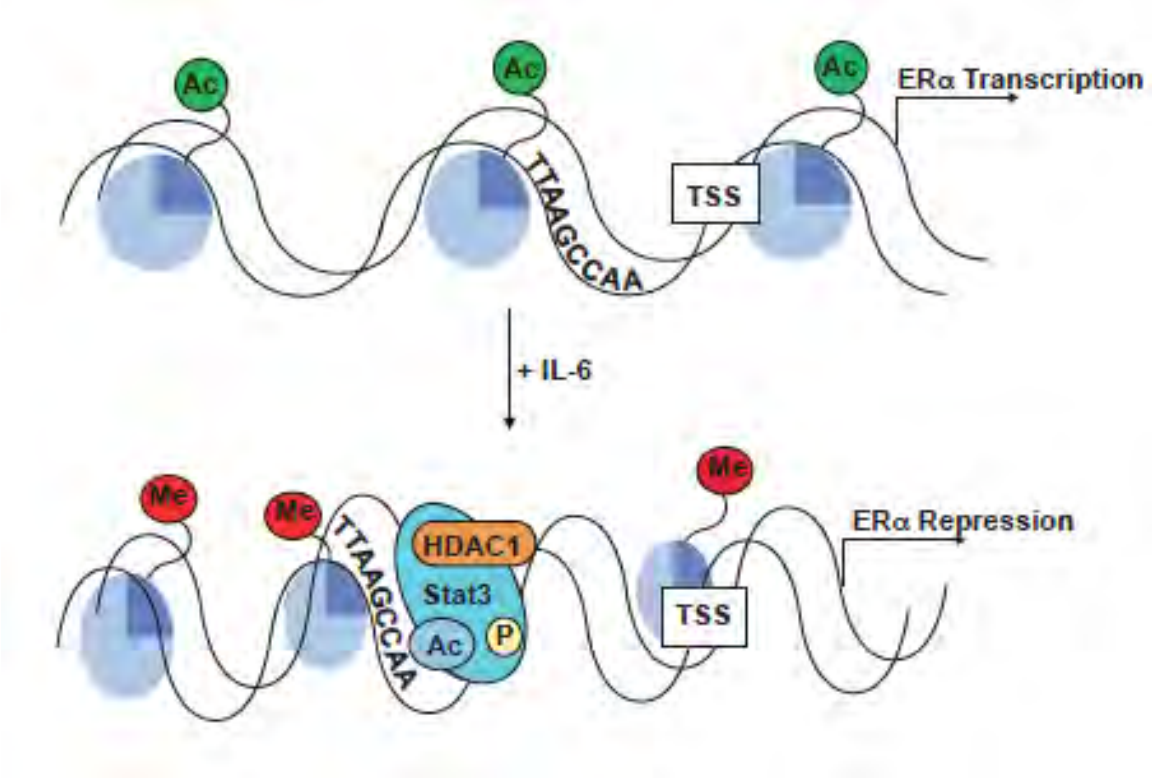


Figure 4.1. Model of ERα repression by the IL-6/Jak/pStat3 pathway. In its active state, estrogen receptor alpha promoter DNA is un-methylated and the histones are acetylated. After IL-6 exposure, Stat3 is recruited to the Stat3 binding site on the ERα promoter. This leads to the acetylation of Stat3, recruitment of HDAC1 and other transcriptional repressors, which results in changes in histone marks like de-acetylation of histones and as well as increased histone tri-methylation.

Stat3: Activator? or Repressor?

A fundamental aspect of transcriptional regulation is defining whether a protein or a transcription factor functions either as an activator or a repressor. We and others have shown that Stat3, a well known transcriptional activator, can function as a transcriptional repressor as well [139, 140]. Interestingly, we see evidence that activated Stat3 can increase the expression of ER. We show that 3 hours after treatment with IL-6, Stat3 is activated by phosphorylation at Y-705 and we observed an increase in ERα mRNA in all cell lines. We know this increase in ERα mRNA is functional because we also observe a similar increase in the expression of ERα regulated genes (PGR, GREB1, RET, EGR3). In addition, we observed by ChIP, binding of Stat3 to the ERα promoter at this time. Thus, we hypothesize that Stat3 is recruited to the promoter and acts as a transcriptional activator of ERα until transcriptional repressors that interact with Stat3 are also recruited to the ERα promoter.

Stat3 can assemble a variety of multi-protein complexes that affects its regulatory functions [162]. These complexes are regulated by post-transcriptional modifications. For example, Yuan et al [163] showed that Stat3 is acetylated on lysine residue 685, and this acetylation is critical for Stat3's ability to form dimers required for cytokine-stimulated DNA binding and transcriptional regulation. In our study, we show that acetylation of Stat3, which occurs much later than 3-hours after treatment with IL-6 enhances Stat3's ability to recruit and interact with HDAC1. We observed that Stat3 is acetylated approximately 24hours hours after treating with IL-6, which correlates with the recruitment of HDAC1 and a reduction in ERα expression. This has led to the hypothesis that sustained IL-6 signaling is necessary for Stat3's ability to act as a transcriptional repressor. Although we do not show what happens if IL-6 is withdrawn after 3 hours by

washing away ligand, we hypothesize that the activated Stat3 currently in the nucleus would not lead to repression of ERα because it needs to be acetylated in order to interact with HDAC1.

IL-6 signaling up-regulates a number of other signaling pathways including the MEK/Erk and PI3K/Akt pathways [150]. In addition IL-6 has been linked to induction of epithelial to mesenchymal transition (EMT) in breast cancer cells [164]. Induction of EMT involves the up-regulation of Vimentin, N-Cadherin, Snail and Twist, which occurs in a Stat3 dependent manner [164]. Interestingly, both Snail and Twist have been shown to negatively regulate ERα expression by binding to the intronic regions of the ERα gene [32-34]. Our results show that Stat3 binds within the promoter region of the ERα gene. We hypothesize that Stat3-mediated increases in Twist and Snail expression potentiate transcriptional repression of ERα. Stat3 binds to the promoter, followed by its acetylation via CBP and recruitment of HDAC1. Snail and Twist subsequently bind to the intronic regions, resulting in further recruitment of HDAC1 resulting in sustained repression. Although a decrease in ER levels was consistently observed in cell lines, the degree of repression was variable. We hypothesize that the expression levels of these transcription factors can fluctuate as a function of culturing conditions (e.g. density and pH) and thus alter the effective repression by IL6/Stat3.

We observed that IL-6 signaling led to a decrease in proliferation of ERα positive breast cancer cell lines. We attributed this decrease in proliferation to a decrease in ERα mRNA and protein expression. Although the attenuation of ER repression leads to decreased growth *in vitro*, these cells exhibit an EMT phenotype and are more migratory. ERα cells are highly dependent on ERα signaling, thus decreases in ERα

expression induced by IL-6/Stat3 signaling led to decrease in proliferation. However, we hypothesize that prolonged IL-6 signaling will lead to a sustained ERα repression and a likely shut down of ERα expression, allowing surviving cells to turn-on other signaling pathways for survival. Likely, prolonged IL-6 signaling will lead to up-regulations of genes involved in EMT, thus allowing these cells to proliferate faster than when they depended on ERα signaling.

We extensively analyzed estrogen receptor methylation patterns in five ERα positive breast cancer cell lines and six ERα negative breast cancer cell lines. First, we observed that methylation is extremely variable. Within ERα positive cell lines, the methylation pattern varied from cell line to cell line. For example, although ZR-75 is an ERα positive cell lines the level of methylation in particular sections of the CpG are comparable to ERα negative cell lines (appendix). In addition, the level of methylation in ERα negative cell lines such as MDA-MB-231, arguably the most common triple negative breast cancer cell line isn't significantly higher than the level of methylation in ERα positive breast cancer cell lines. These data suggests that there may be a threshold for how much methylation is sufficient to shut off gene expression.

A reduction in ERα expression as well as discrepancy in ERα levels between primary tumors and metastatic disease are commonly observed with disease progression and are associated with worse prognosis and resistance to tamoxifen [23, 64]. Here we have demonstrated that the IL-6/Jak/Stat3 signaling pathway leads to decreased ERα expression in luminal A and B breast cancers. We show that Stat3 in conjunction with HDAC1 directly regulates ERα expression.

Overall, we have described a novel role for the IL-6/Stat3 signaling pathway in regulating ERα expression in ERα positive breast cancers.

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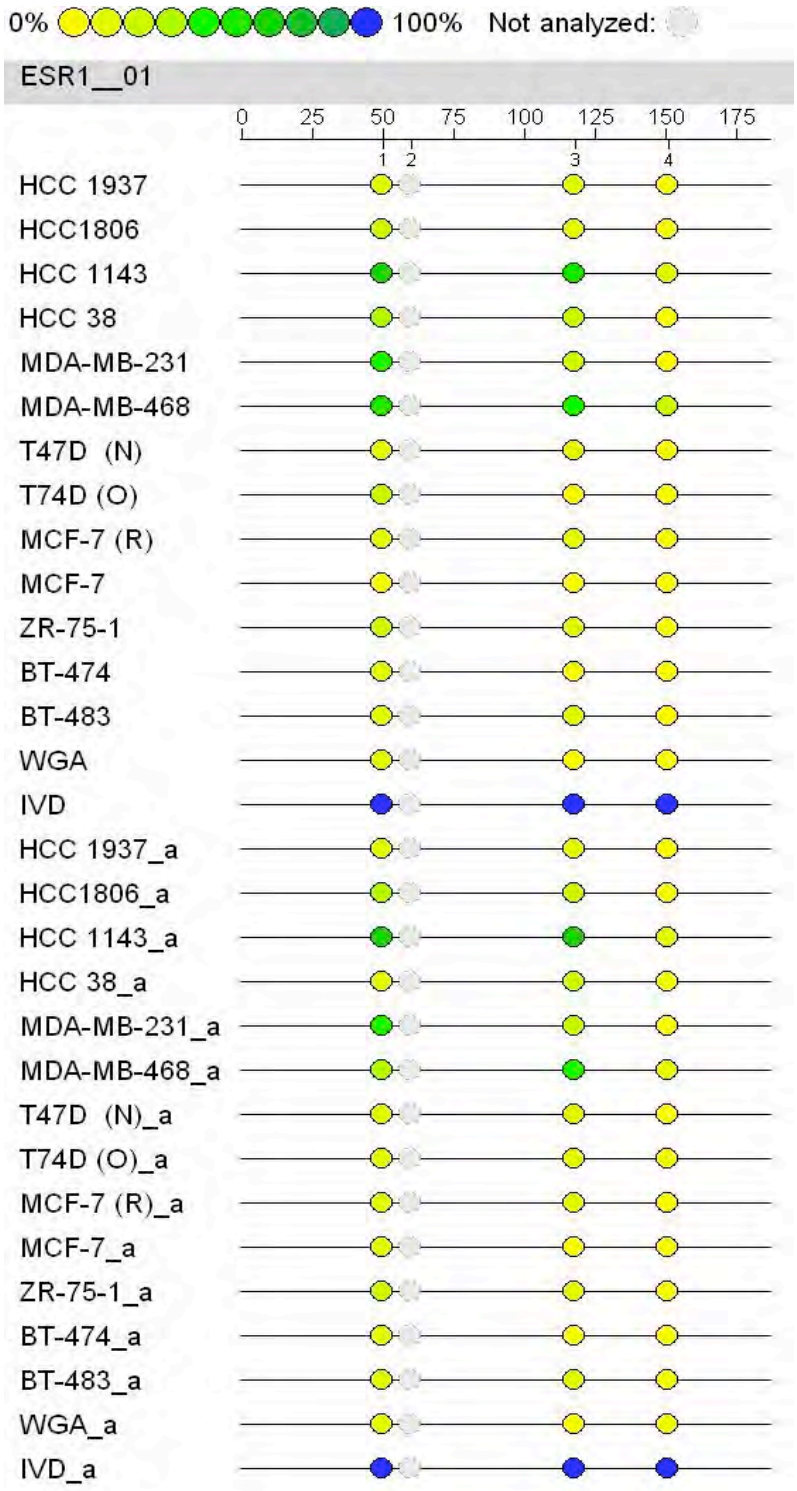
APPENDIX
EpiTYPER METHYLATION GRAPHS

Primer 1



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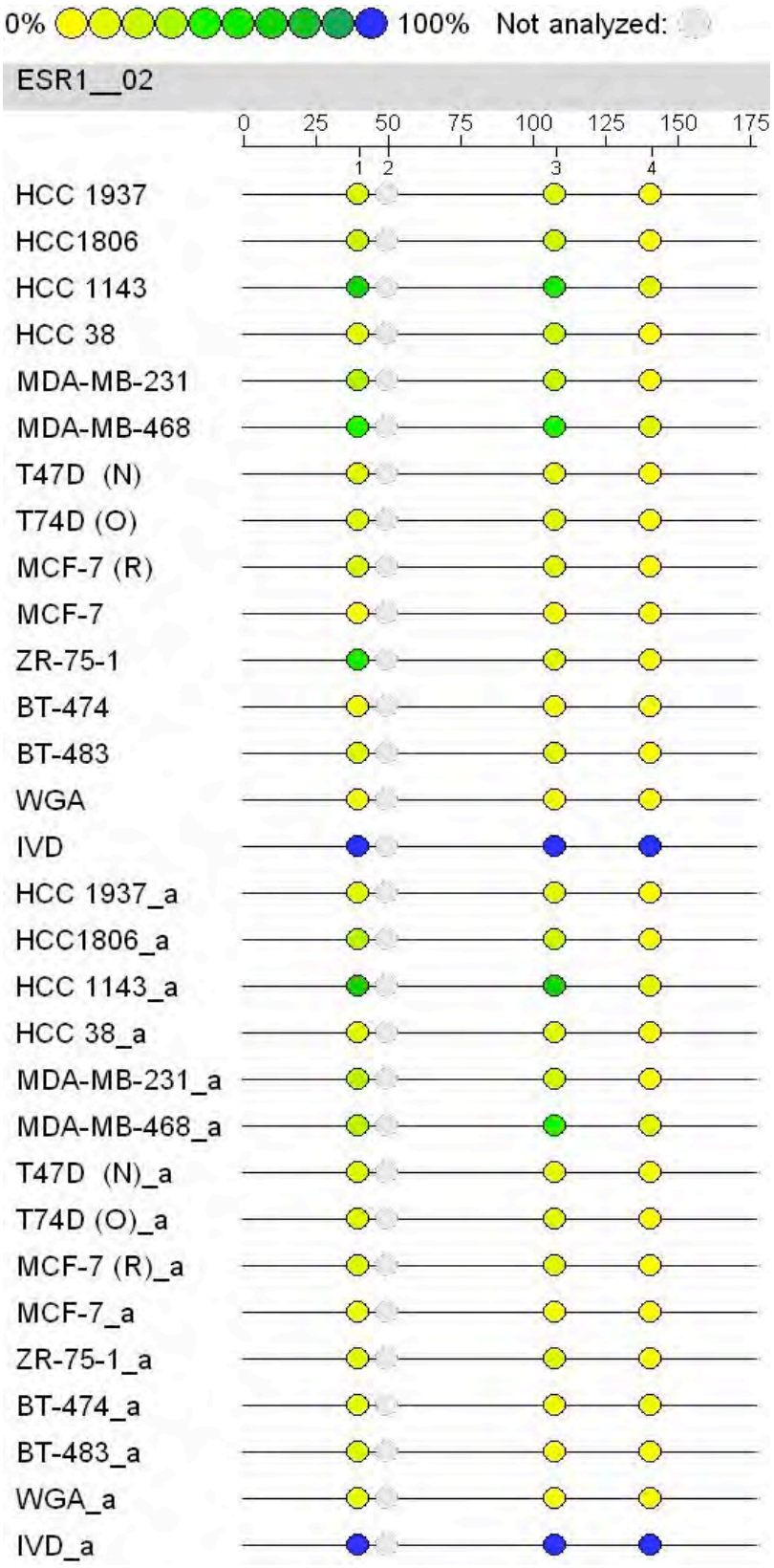
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25
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75
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1
2
3
4
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HCC 1143
HCC 38
MDA-MB-231
MDA-MB-468
T47D (N)
T74D (O)
MCF-7 (R)
MCF-7
ZR-75-1
BT-474
BT-483
WGA
IVD
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HCC1806_a
HCC 1143_a
HCC 38_a
MDA-MB-231_a
MDA-MB-468_a
T47D (N)_a
T74D (O)_a
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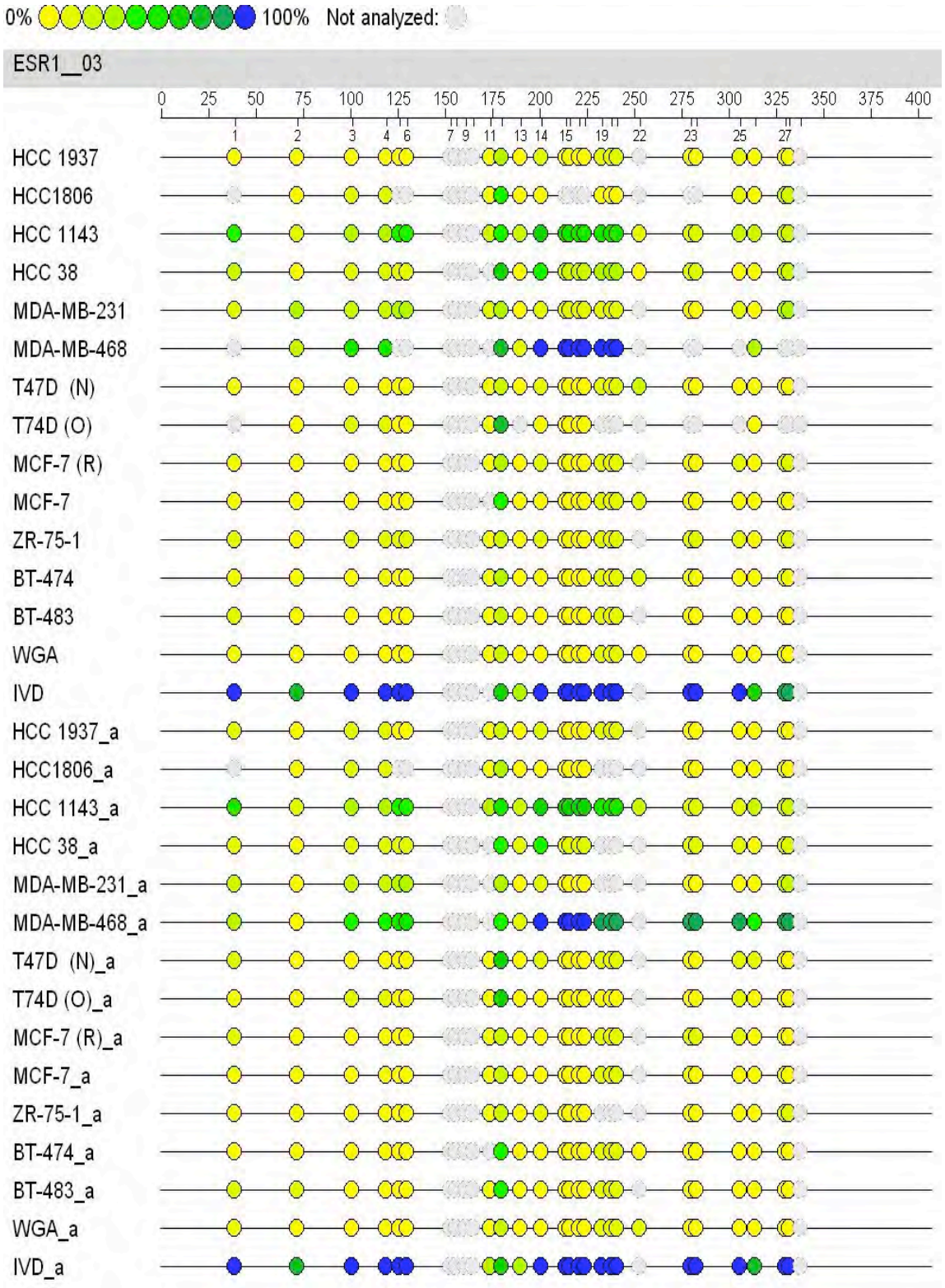
IVD_a

Primer 3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T74D (O)
MCF-7 (R)
MCF-7
ZR-75-1
BT-474
BT-483
WGA
IVD
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HCC1806_a
HCC 1143_a
HCC 38_a
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MDA-MB-468_a
T47D (N)_a
T74D (O)_a
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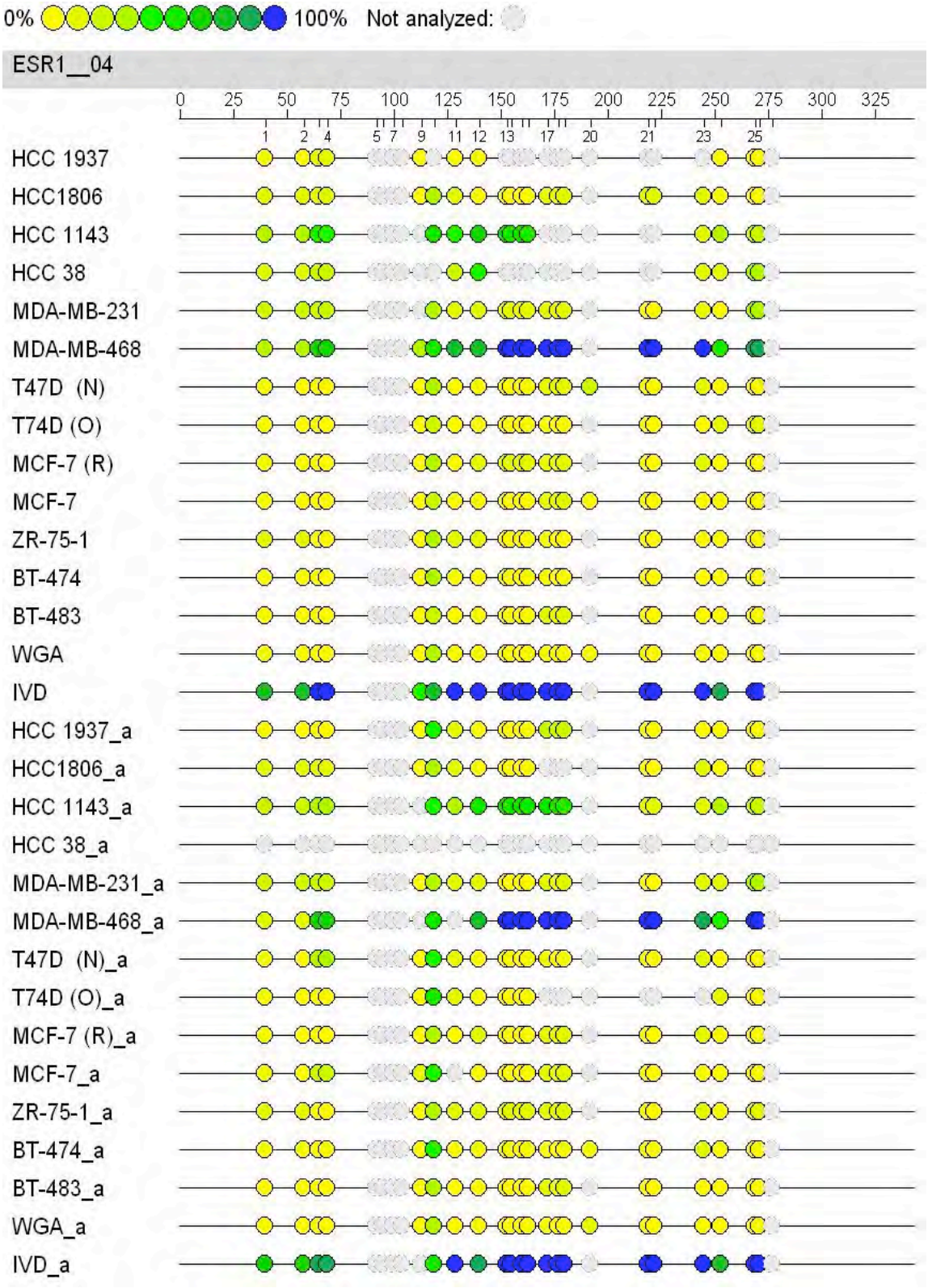
WGA_a
IVD_a

Primer 4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MDA-MB-468
T47D (N)
T74D (O)
MCF-7 (R)
MCF-7
ZR-75-1
BT-474
BT-483
WGA
IVD
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HCC1806_a
HCC 1143_a
HCC 38_a
MDA-MB-231_a
MDA-MB-468_a
T47D (N)_a
T74D (O)_a
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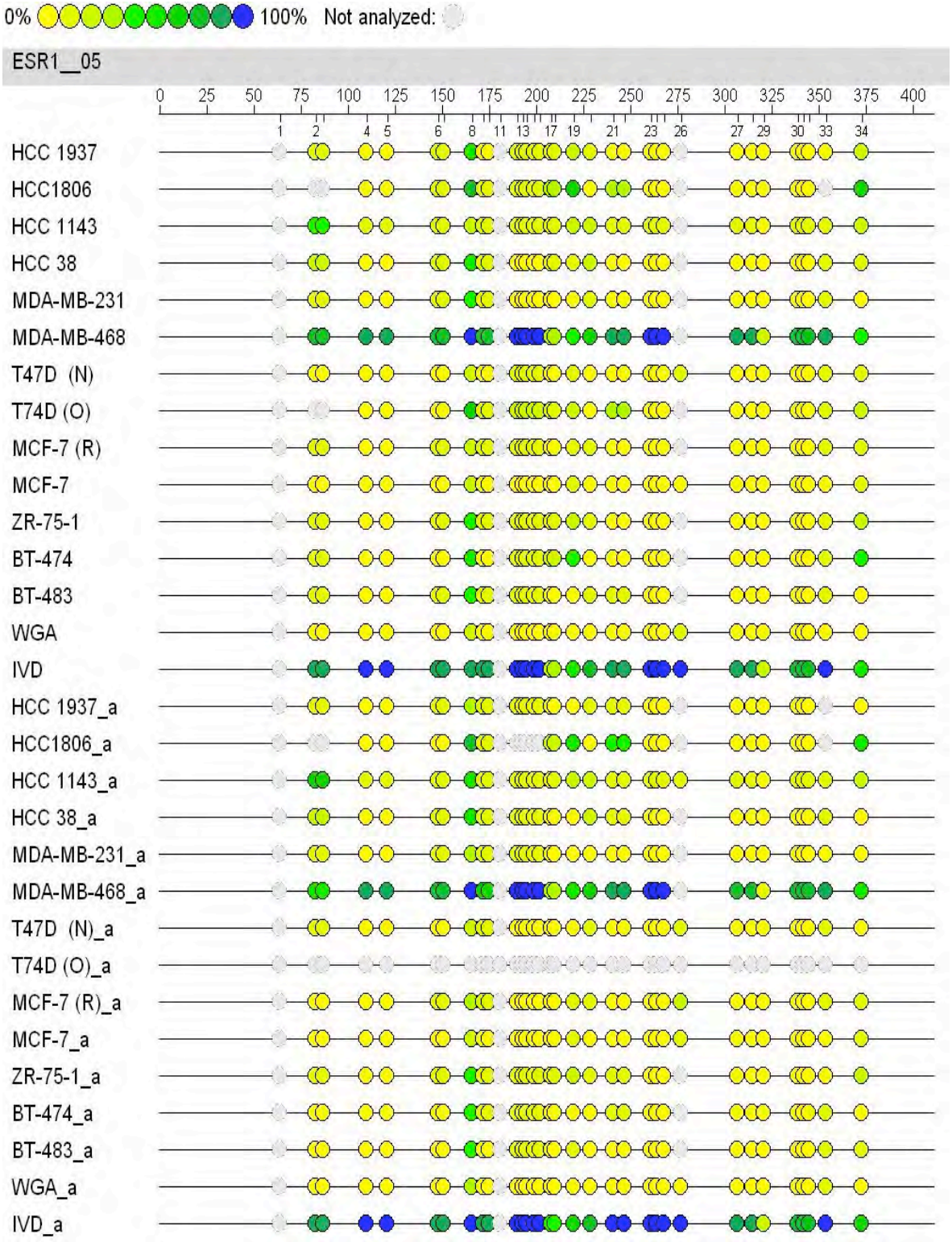
MCF-7_a
ZR-75-1_a
BT-474_a
BT-483_a
WGA_a
IVD_a

Primer 5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MDA-MB-468
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T74D (O)
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MCF-7
ZR-75-1
BT-474
BT-483
WGA
IVD
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HCC1806_a
HCC 1143_a
HCC 38_a
MDA-MB-231_a
MDA-MB-468_a
T47D (N)_a
T74D (O)_a
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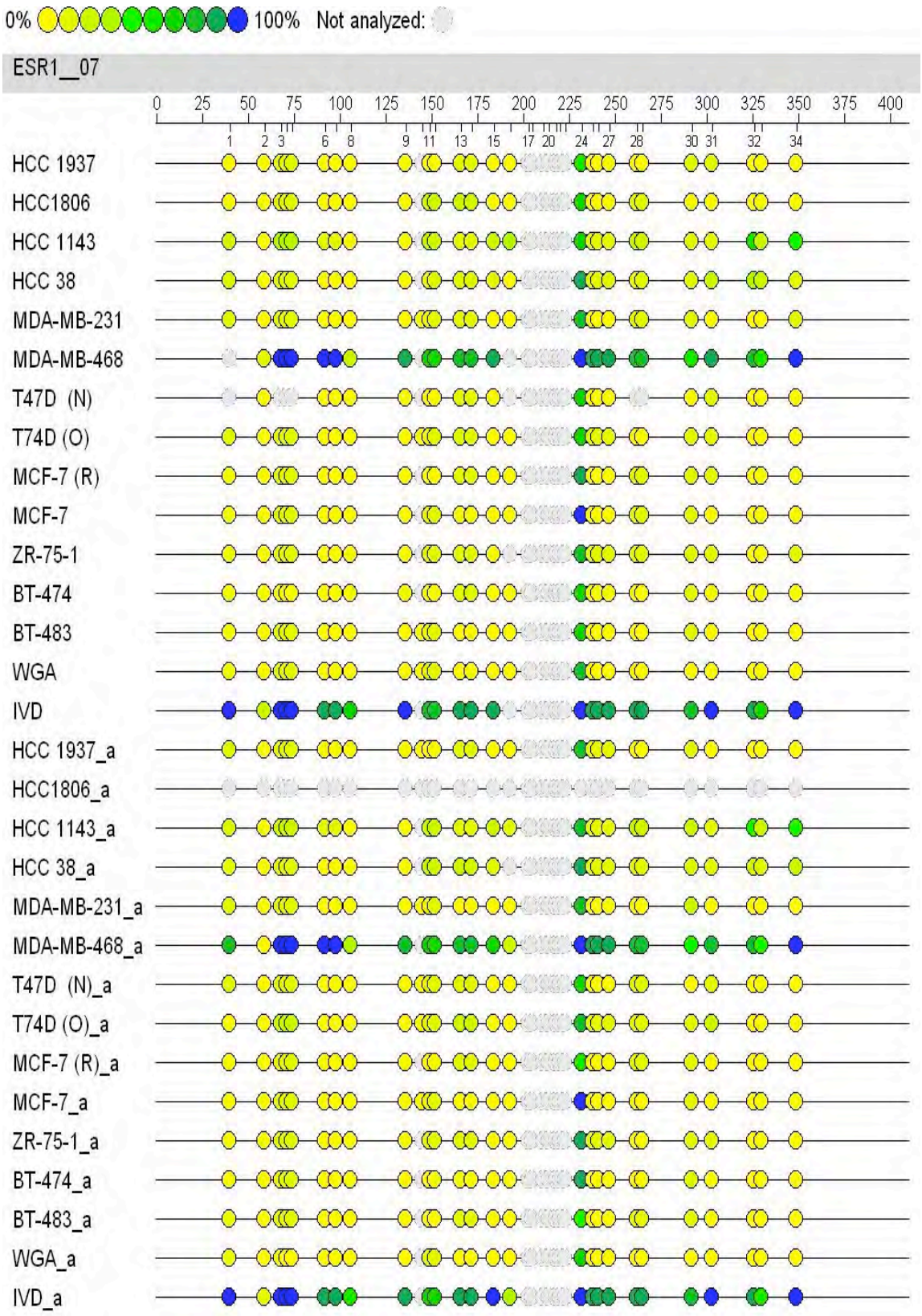
MCF-7_a
ZR-75-1_a
BT-474_a
BT-483_a
WGA_a
IVD_a

Primer 6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MCF-7 (R)
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ZR-75-1
BT-474
BT-483
WGA
IVD
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HCC 1143_a
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MDA-MB-231_a
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T47D (N)_a
T74D (O)_a
MCF-7 (R)_a
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ZR-75-1_a
BT-474_a
BT-483_a
WGA_a
IVD_a