Functional interaction between Estrogen Receptor α and epithelial-specific ETS transcription factor ELF3 in breast cancer cells

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
By

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CERTIFICATE

This is to certify that this thesis entitled **"Functional interaction between Estrogen Receptor α and epithelial-specific ETS transcription factor ELF3 in breast cancer cells"** submitted to the University of Hyderabad by **Mr. Vijaya Narasimha Reddy. G**, for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this has not been submitted earlier for the award or diploma from any other University or Institution.

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Dr. Bramanadam Manavathi, Ph.D.,** I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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DECLARATION

I, Vijaya Narasimha Reddy. G, hereby declare that this thesis entitled "**Functional interaction between Estrogen Receptor α and epithelial-specific ETS transcription factor ELF3 in breast cancer cells"** submitted by me under the guidance and supervision of **Dr. Bramanadam Manavathi, Ph.D.,** is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in shodganga/INFLIBNET.

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…….Vijaya Narasimha Reddy Gajulapalli

ACRONYMS

AF1	Activation function 1
AF2	Activation function 2
AIB1	Amplified in breast cancer 1
AKT	A serine/threonine protein kinase
ALDH1	Aldehyde dehydrogenase 1
AP-1	Activation protein 1
BRCA1	Breast cancer amplified 1
BSA	Bovine serum albumin
CA12	Carbonic anhydrase 12
CBP	CREB-binding protein
cDNA	Complementary DNA
CoRNR	Corepressor nuclear receptor
CREB	cAMP responsive element binding protein
DAPI	4’, 6-diamidino-2-phenylindole
DBD	DNA-binding motif
DCC serum	Dextran-charcoal stripped serum
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
E2	Estrogen or 17β-estradiol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor

EGTA	Ethylene glycol-bis (beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ErbB2	Avian erythroblastosis oncogene B2
ERE	Estrogen response element
ERE-luc	Estrogen response element –luciferase
ESR1	Estrogen Receptor 1
ETS	E26 transformation-specific sequence
FACS	Fluorescence-activated cell sorting
FSH	Follicle-stimulating hormone
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPR30	G protein-coupled receptor 30
GREB1	Gene regulated by estrogen in breast cancer 1
GST	Glutathione *S*-transferase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEK293T	Human Embryonic Kidney 293T cells
HER2	Human epidermal growth factor receptor 2
HRE	Hormone response element
IC_{50}	Half maximal inhibitory concentration
ICB	Inhibitors of coactivator binding

IDC	Invasive ductal carcinomas
IgG	Immunoglobulin G
IL-1β	Interleukin-1 beta
ILC	Invasive lobular carcinoma
IMEM	Improved minimum essential medium
IPTG	Isopropyl β-D-thiogalactopyranoside
kDa	Kilo Dalton
LCIS	Lobular carcinoma in situ
LHRH	Luteinising hormone-releasing hormone
M	Molar
$MgCl_2$	Magnesium chloride
MIP3-α	Macrophage inflammatory protein 3 *alpha*
MTA1s	Metastasis tumor-associated 1 short form
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NCoR	Nuclear receptor corepressor
NF-kB	Nuclear factor κ-light-chain-enhancer of activated B cells
NR3A1	Nuclear receptor subfamily 3, group A, member 1
NuRD	Nucleosome remodeling and histone deacetylation complex
OD	Optical density
CREB	cAMP response element-binding protein
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank

PELP1	Proline, glutamic acid, and leucine-rich protein
PI3K	Phosphatidylinositol 3 kinase
PKCα	Protein kinase C *alpha*
PMSF	Phenyl methylsulfonyl fluoride
PNT	Pointed domain
PR	Progesterone receptor
PTM	Posttranslational modification
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
SAR	Serine and aspartic acid-rich domain
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SP1	Specificity protein 1
SRA	Steroid RNA activator
TAD	Transactivation domain
Taq	Thermophilus aquaticus
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween20
TFF1	Trefoil factor 1
TGF-β RII	Transforming growth factor beta-type-II receptor
TNBC	Triple negative breast cancer
Tris	Tris-(Hydroxymethyl) aminoethane
UTR	Untranslated region
WHSC1	Wolf-Hirschhorn syndrome candidate 1

Y2H	Yeast two-hybrid
ZAC1	Zinc finger protein, a regulator of apoptosis and cell cycle arrest

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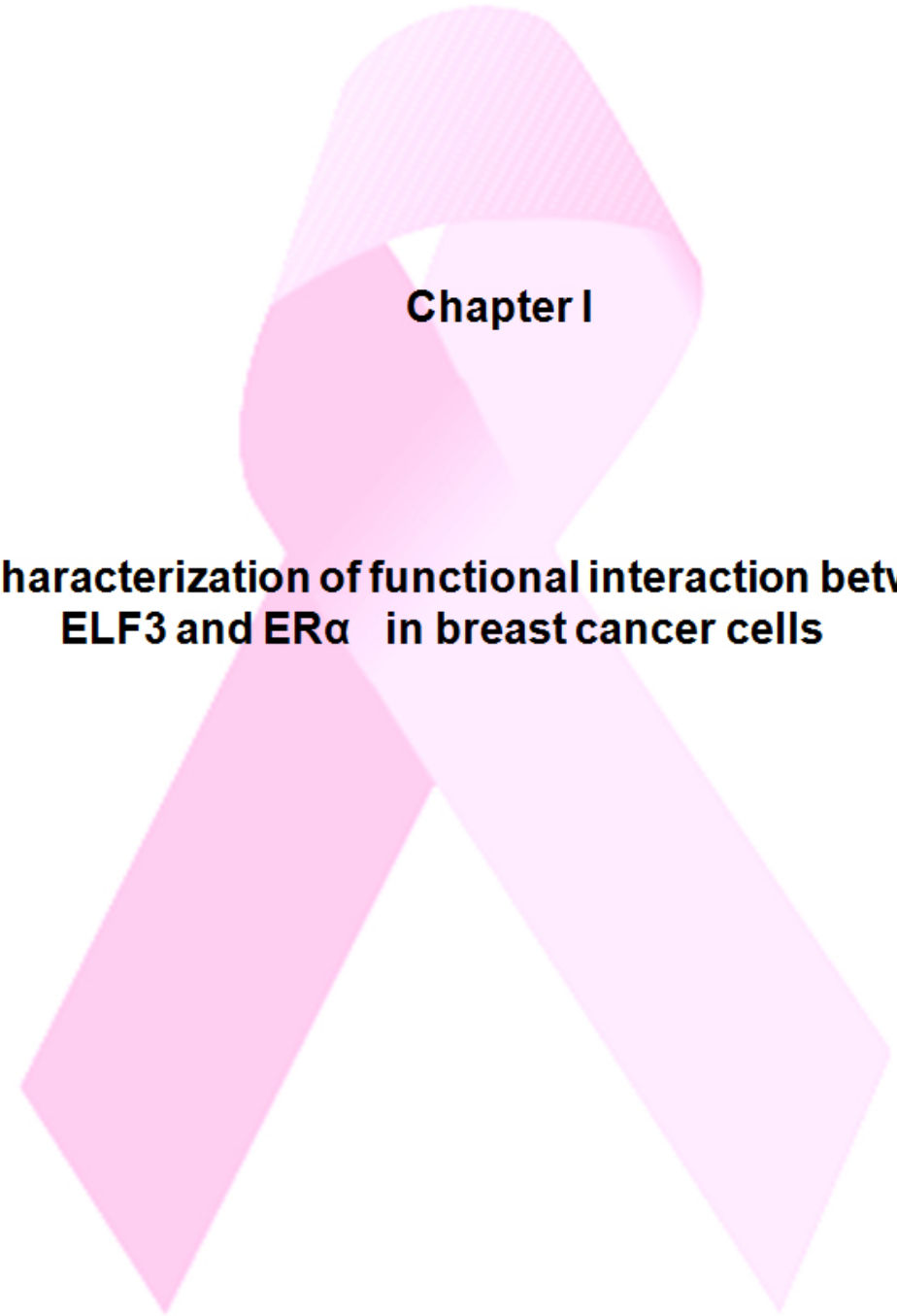


Chapter I

Characterization of functional interaction between ELF3 and ERα in breast cancer cells

1.1 INTRODUCTION

1.1.1 Estrogen signaling: Estrogen, a steroid hormone, exhibits a wide spectrum of physiological functions that range from regulation of the mammary gland development, menstrual cycle and reproduction to modulation of brain function, bone density, growth, proliferation, differentiation, cholesterol mobilization in wide range of tissues in human body and is also involved in breast tumor progression when it is over produced in the body (Koos, 2011; Liang and Shang, 2013). Estrogen exists in three forms, such as estrone, 17β-estradiol and estriol. 17β-estradiol (here after we refer as E2) is present in large amount and is more potent than either estrone or estriol, and play critical role in the development of mammary gland (Bjornstrom and Sjoberg, 2005).

1.1.2 Role of ER in mammary gland development: Unlike the other human organs, mammary gland development occurs postnatally (Russo and Russo, 2004). The ovarian hormones, 17β-estradiol and progesterone play a pivotal role in the development of the mammary gland. Although prenatal development of mammary gland occurs independently without these steroid hormones, marked growth occurs during puberty which requires E2. Therefore, the hormone-dependent mammary gland development occurs only after puberty. These ovarian hormones bring about profound morphogenetic changes in the glands by inducing ductal elongation, side branching, terminal end bud formation and alveologensis (Brisken and O'Malley, 2010).

E2 exerts its biological functions through specific ligand-inducible nuclear receptors. E2 binds to two distinct classical estrogen receptors (ERs) called ERα and ERβ, that are encoded by genes located on two different chromosomes and share considerable sequence homology (Nilsson *et al*., 2001). These proteins regulate the transcription of a variety of target genes during development in response to specific physiological and pathological signals (Klinge,

2000). The knockout approach in mice models has clearly demonstrated that ERα is a prerequisite for the postnatal development of mammary gland while ERβ is not.

1.1.3 Estrogen Receptor (ER) α: ERα (NR3A1 (nuclear receptor subfamily 3, group A, member 1) is the major ER in the mammary epithelium. It is encoded by *ESR1* (*Estrogen Receptor 1*) gene in humans, which is located on chromosome 6q25.1. It is a ligand-dependent transcription factor and regulates genes involved in cell proliferation, differentiation, and migration (Nilsson *et al*., 2001). Deregulated actions of ERα signaling are associated with breast cancer development (Barnes *et al*., 2004). This protein (595 amino acid length with M.W of 67 kDa) consists of 5 domains-N-terminal A/B, C, D, E and C-terminal F domains. N-terminal A/B domain also known as the activation function 1 (AF-1), is involved in ligand independent function of the receptor. The transcriptional activation of AF-1 is normally weak but is in synergy with AF-2 in the E-domain to regulate ERα target gene expression. This region of the receptor is involved in protein-protein interactions, whereas the ERα "C" domain contains DNA-binding motif (DBD). It is a highly conserved domain with two zinc fingers that bind to specific sequences of the DNA called hormone response elements (HRE) (Kumar and Chambon, 1988; Ascenzi *et al*., 2006). The "D" domain which follows DBD is known as a hinge region. It contains nuclear localization signal which gets exposed upon ligand binding to serve as a flexible region connecting DBD and LBD. The central hinge region is important for receptor dimerization (residues 248–314) (McKenna *et al*., 1999). The E domain of ERα is also called as Ligand binding domain (LBD), consists of 12 helices, and one hormone binding pocket, is responsible for the most of the functions activated by ligand binding such as coregulator binding to AF2 (Activation function 2) and dimerization interface. The dimer interface is primarily encompassed by helices 10 and 11 (Kumar *et al*., 2011). The C terminal "F" domain, containing 42 amino acids, followed by the

LBD and is known to impose a restraint on dimerization, and transactivation activity (De Vries-van Leeuwen *et al*., 2013).

1.1.4 Estrogen Receptor (ER) β: ERβ (NR3A2 (nuclear receptor subfamily 3, group A, member 2), is one of two main types of ER. In humans, ERβ is encoded by the *Estrogen Receptor 2* (*ESR2)* gene, which is located on chromosome 14q23.2. It shares common structural characteristics with the ERα, including five distinguishable domains denoted A to F (Mosselman *et al*., 1996; Ogawa *et al*., 1998). The full-length human ERβ protein includes 530 amino acids with an estimated molecular mass of 59.2 kDa (Ogawa *et al*., 1998). The main function of ERβ is to prevent undesired ERα-mediated actions of E2 (Hall and McDonnell, 2005). ERβ protein expression has been well documented in ovarian granulosa cells, brain, bone, ,heart, kidney, lungs, intestinal mucosa and prostate (Babiker *et al*., 2002).

Estrogen exerts its action either by genomic or nongenomic signaling pathways.

1.1.5 ERα genomic signaling: In the absence of estrogen, ERα is sequestered in complex with an inhibitory heat shock protein. Upon ligand binding, the receptor dissociates from the heat shock protein complex to undergo dimerization (Klein-Hitpass *et al*., 1986). ERα can interact with target gene promoters either directly, through specific estrogen response elements (ERE), or indirectly through other DNA-bound transcription factors such as specificity protein 1 (Sp1), activation protein 1 (AP-1), or nuclear factor κ-light-chain-enhancer of activated B cells (NF-kB). Upon ligand binding to LBD of ERα, receptor dimerizes and translocates to nucleus, where it recruits co-activators or co-repressors, and chromatin-remodelling factors also, to estrogen response elements (EREs), which is the 15-bp (AGGTCAnnnTGACCT) palindromic inverted repeat (IR) separated by any three nucleotides (nnn) sequence on target gene promoters (Mason *et al*., 2010). Once this complex tethered to DNA, the receptor can regulate the target gene transcription, either positively or negatively (O'Lone *et al*., 2004). ERα regulates the expression of several genes that are

involved in mammary gland development and their deregulated expression is therefore associated with breast cancer progression. In general, genomic actions induce biological responses more slowly than the nongenomic actions (Welboren *et al*., 2009).

1.1.6 ERα nongenomic signaling: Estrogen generates rapid cellular responses and takes place in seconds or minutes, which cannot be explained by its nuclear action and thus imply the existence of alternative mechanisms involving rapid cytoplasmic signaling. These responses can possibly be explained by the existence of signals generated from cytoplasmic and cell surface steroid receptors, generally known as nonclassical, or nongenomic or extra nuclear steroid signals (Losel and Wehling, 2003). ERα nongenomic signaling is divided into two major categories: classical receptor-mediated responses, which are mediated through ERs, and nonclassical, non-receptor-mediated responses, which are mediated through proteins other than ERs, such as GPR30. In classical receptor-mediated signaling, estrogen-stimulated nongenomic pathways are initiated at the plasma membrane. Serine 522 residue within the estrogen binding domain of ERα is critical in linking the receptor to the cell membrane through interaction with caveolin-1 (Razandi *et al*., 2003). Posttranslation modification like palmitoylation at cysteine 447 is shown to be required for ERα localization to plasma membrane and such modifications are responsible for the ligand-induced MAPK and PI3K/AKT pathways (Acconcia *et al*., 2005; Manavathi and Kumar, 2006). In nonclassical-receptor-mediated signaling, ER or ER-like proteins are likely candidates for the membrane ERs (mERs) that mediate these estrogen actions in a variety of target cells (Chen *et al*., 1999; Song *et al*., 2002). These are unrelated to nuclear steroid receptors, but show the characteristics of G protein-coupled receptors (GPCRs) suggesting the existence of other GPCRs with characteristics of steroid membrane receptors (Zhu *et al*., 2003). Two groups independently identified such a GPCR called GPR30 as a non-genomic signaling mediator of E2 in breast cancer cells. These novel membrane ERs mediate a pleiotropic action of

estrogens in breast and other estrogen target tissues (Revankar *et al*., 2005; Thomas *et al*., 2005).

1.1.7 The concept coregulator: Transcriptional activity of ERα is largely controlled by a special category of proteins called “coregulators”. These coregulators usually do not bind to DNA element, but are recruited to target gene promoters by protein–protein interactions with the NRs (McKenna *et al*., 1999; Shao *et al*., 2004). The concept of coregulator was introduced to the nuclear receptor field approximately two decades ago (Onate *et al*., 1995). Though initially there was confusion about classifying these molecules as cofactors based on their similarity to a cofactor for an enzyme, later they were referred as ‘coregulators’ (O'Malley and McKenna, 2008). Unlike to a cofactor to an enzyme, coregulators acts as bridging or helper molecules that help in forming large protein complexes to facilitate appropriate activity on the target gene chromatin. The steroid hormone receptors (SHRs) require these ‘transcription adaptors’ for their optimal activity. Although enzymes exhibit absolute cofactor specificity in a tissue-independent manner, steroid receptors utilize diverse tissue-specific coregulators for their activity in a spatio-temporal manner. Since SHRs are, in general, transcriptional factors, the coregulators may positively or negatively influences receptor’s transcriptional activity by modifying target gene chromatin and are termed as coactivators or corepressors, respectively. Though these coregulators form large protein complexes with SHRs, they are not just “bridging” molecules to link SHR and the transcription machinery, rather they often exhibit various enzymatic activities like acetylation, methylation, and ubiquitination to regulate the target gene transcription (Lonard and O'Malley B, 2007). In general, coactivators exhibit an intrinsic histone acetyl transferase activity (HATs). Alternatively, they may recruit HATs along with SHRs to enhance the transcriptional activity of the receptor. On the other hand, corepressors recruit histone deacetylases (HDACs) to keep the chromatin in closed conformation that shuts off the target

gene transcription **(Figure 1.1)** (Manavathi *et al*., 2013). These coregulators could involve in chromatin modification and remodelling, initiation of transcription, elongation of RNA chains, termination of the transcriptional response, mRNA splicing, mRNA translation, miRNA processing, and even in degradation of the activated NR-coregulator complexes (Lonard and O'Malley B, 2007). *In vivo* studies using knockout mouse models provided the evidence that several of these coregulators are indeed important for E2-dependent mammary growth (Manavathi *et al*., 2014).

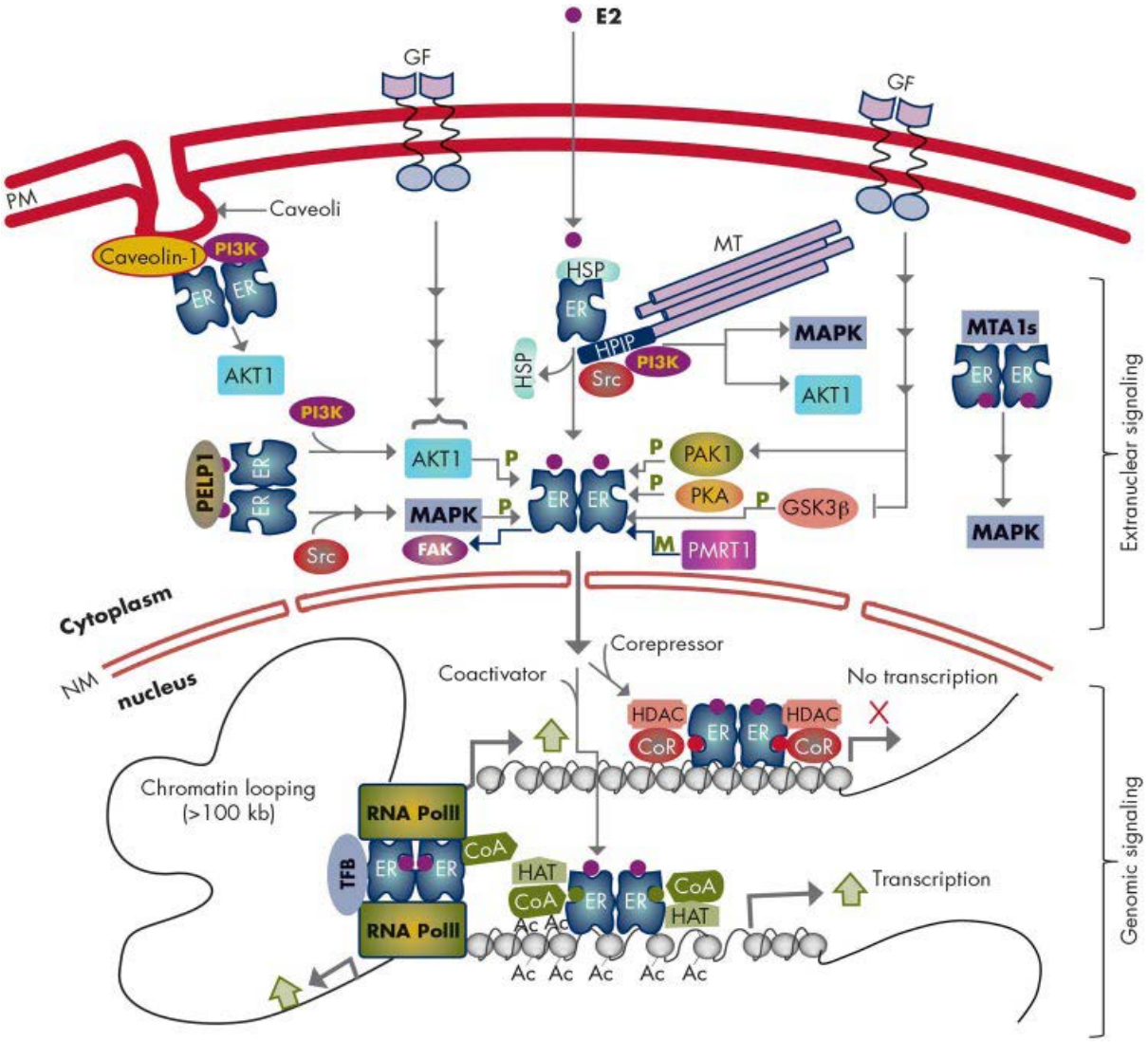


Figure 1.1 Hypothetical model illustrates the E2-ERα signaling pathway involving both genomic and extranuclear signaling pathways. *Extranuclear signaling of E2/ERα:* Several signaling proteins like PI3K, Src, HPIP, MTA1s, *etc.*, interact with ERα in the cytoplasm to activate E2 extranuclear signaling. Rapid E2

signaling promotes interaction of ERα with caveolin-1 in the caveoli and activates the PI3K/AKT pathway. Sequestration of ERα by HPIP in the cytoplasm through a microtubule scaffolding mechanism facilitates PI3K/Src recruitment, and activation of AKT/MAPK pathways occurs in response to rapid E2 signaling. MTA1s also activate E2 rapid signaling through cytoplasmic sequestration of the receptor. In response to rapid E2/ERα signaling, PELP1 could activate AKT and MAPK pathways by interaction with PI3K and Src kinase, respectively. Activation of downstream signaling kinases such as AKT, MAPK, PAK1, *etc.,* by growth factor signaling led to phosphorylation of ERα, which could further impact its nuclear activity. Phosphorylation of ERα by GSK3 also enhances ERα transcriptional activity. Methylation of ERα at arginine 260 by PRMT1 involves activation of FAK signaling in response to E2 rapid signaling. *Genomic signaling of E2/ERα:* Ligand binding to ERα ensures heat shock protein (HSP) dissociation and the receptor's nuclear entry. Upon nuclear translocation, ligand-bound receptor binds to its target genes to activate the transcription. If the HDAC complex is recruited to ERα chromatin, ERα-dependent transcription is repressed, whereas HAT complex recruitment activates ERα-dependent transcription. This model also illustrates that ERα can regulate the gene expression by extensive chromatin looping to bring genes together for coordinated transcriptional regulation.

1.1.8 ER coregulators, the orchestrators of E2 signaling: Since majority of the breast cancers are ERα positive and coregulators are proved to be crucial for ER transcriptional activity, a mounting interest in the field has led to the identification of a large number of coregulators (Lonard and O'Malley, 2006). The first discovered coregulator of ERα is coactivator SRC-3/AIB1 (Onate *et al*., 1995). So far about 200 coregulators have been identified for ERα, whereas very few coregulators are known for ERβ (Lonard and O'Malley, 2006). Though ERα and ERβ utilize E2 as their physiological ligand, they have both overlapping and distinct functions, partly due to the differential utilization of coregulators. The different classes of ER coactivators include members of steroid receptor coactivator (SRC)/p160 family, histone acetyltransferase, cAMP responsive element binding protein (CREB)-binding protein (CBP)/p300, ATP-dependent chromatin remodeling complexes like SWI/SNF, E3 ubiquitin-protein ligases, steroid RNA activator (SRA) etc. (Lonard and O'Malley, 2006). Large number of coactivators utilize specific motifs called NR boxes or

LXXLL motifs (X, any amino acid; L, Leucine) to facilitate their interaction with ligand-binding domains of ER (Heery *et al*., 1997). Conversely, corepressors inhibit ER-mediated gene transcription through a direct interaction with unliganded ER or utilizing CoRNR (Corepressor nuclear receptor) box present in it or competing with coactivators for ER binding (Hu and Lazar, 1999).

The interesting question to be addressed in the ER field is the presence of many coregulators for ER (over 200). The initial studies on various coregulators confirmed that different tissues express different amounts of the coregulators ie., a coregulator dosage, to account for cell-specific regulation of E2 target gene expression so that target cells express different levels of co-activators and co-repressors (Folkers *et al*., 1998; Misiti *et al*., 1998; Smith and O'Malley, 2004). This implies that the degree of coregulator expression is crucial to their ability to manipulate the transcriptional potential of the ERs that controls fine-tuning of target gene transcription in response to E2 (Brisken and O'Malley, 2010). Interestingly, the expression of several coregulators of ER is transcriptionally regulated by E2 and, in turn these coregulators regulate the expression of ERs, operating feedback mechanisms that are common in endocrine regulation (Mishra *et al*., 2004). This along with several posttranslational modifications (PTMs) such as phosphorylation, methylation, ubiquitination, SUMOylation and acetylation impact on several coregulators to further control their activity and eventually onto target gene expression (Lonard and O'Malley B, 2007; O'Malley *et al*., 2008; Han *et al*., 2009). Combined or individual modifications on a coregulator can confer distinct functions to the same coregulator. In this way, PTMs add additional complexity to a coregulator and provide a repertoire to a cell to utilize these regulatory molecules at appropriate time and conditions (Brisken and O'Malley, 2010).

1.1.9 Role of ER coregulators in mammary gland development: Although endocrine disruption procedures have elucidated the role of E2 in mammary gland development, the

precise role of its cognate receptor ER is established from the mouse knockout studies. *Esr1* (ER) gene-disruption in mouse lead to complete abrogation of the mammary gland development indicating ER is indispensible for mammary gland development (Mueller *et al*., 2002; Mallepell *et al*., 2006; Feng *et al*., 2007). Since an ER-mediated activity depends essentially on its coregulators, these are expected to play a crucial role in mammary gland development **(Figure 1.2)**. Knockout approaches have been used to address whether a coregulator is being employed in mammary development or not. Complete ablation of several ER coregulators resulted in distinct phenotypes that include embryonic lethality, metabolic diseases, impaired reproduction, mammary gland development etc. (Lonard *et al*., 2007; Dasgupta *et al*., 2014). Mammary gland-specific conditional knockouts are essential for those coregulators whose complete deletion in mouse resulted in embryonic lethality to understandtheir role in mammary gland development (Manavathi *et al*., 2014).

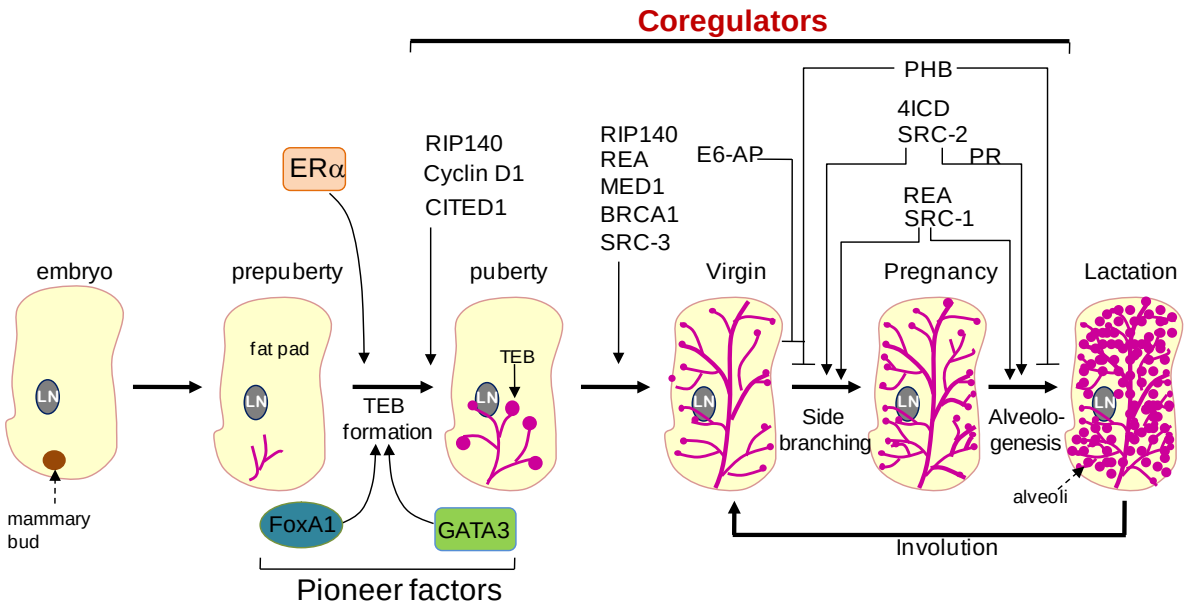


Figure 1.2: Schematic representation of the role of various coregulators and pioneer factors of ER in different stages of mammary gland development. The thin arrows points to the developmental defects at these stages of mammary gland development due to the loss of the expression of respective coregulator. Whereas the inhibitory lines (⊣) points to the suppressive effect of the respective coregulator at the indicated stages of mammary gland development. LN, lymph node; TEB, terminal end buds.

1.1.10 E74-like factor 3 (ELF3): The ETS family of transcription factors play very important role in the normal physiological and pathological processes that are critical for growth and development (Oikawa and Yamada, 2003). In addition to regulating key processes like cell proliferation, differentiation, migration, apoptosis, and epithelial to mesenchymal transition (EMT) during normal development, ETS proteins, upon deregulation of their expression levels and activity also contribute to the initiation and progression of human cancers (Sharrocks, 2001). In general, ETS transcription factors share a highly conserved 85 aminoacid long winged helix-turn-helix domain that binds to purine-rich DNA sequences with a central GGAA/T core consensus designate as EBS (Ets binding site) located on promoters of their target genes (Otero *et al*., 2012). The epithelial-specific ETS (ESE) family of proteins, which are members of the ETS family, include ELF3, ESE-2, ESE-3, and PDEF, has been implicated in epithelial cell differentiation (Feldman *et al*., 2003).

E74-like factor 3 (ELF3, also known as Ese-1, ESX, ERT and Jen) was initially reported as a regulator of epithelial cell differentiation as it regulates the expression of epithelial cell markers in keratinocytes, bronchial, and retina (Oettgen *et al*., 1997; Jobling *et al*., 2002). ELF3 protein consists of 371 amino acids with a molecular wt of ~42 kDa. The expression of ELF3 is epithelial-specific (Oettgen *et al*., 1997). ELF3 is composed of five perfectly defined domains: a N-terminal pointed domain, transactivation domain (TAD), serine and aspartic acid-rich (SAR) domain, an AT-hook domain, and an ETS domain (Agarkar *et al*., 2010). Pointed domain (PNT) is also known as SAM domain. The Ets-1 PNT domain forms a monomeric five-helix bundle. Proteins containing SAM domains include the Eph family of receptor, tyrosine kinases serine/threonine kinases, SH2 domain containing adaptor proteins, and many other proteins that allow the formation of homo and hetero-typic oligomers (Kim *et al*., 2001). This domain is evolutionally conserved and functions as a protein-protein interaction motif. Transcription activation (TAD) domain binds to DNA and activates

transcription. It has a highly conserved region of 8 amino acids (SWIIELLE) which forms a short alpha-helix which interacts with TAF (TBP-associated factors) and DRIP (these are ligand-dependent proteins which interact with the vitamin D receptor (VDR), together, these proteins constitute to form novel cofactor complex. DRIPs interact with several nuclear receptors both temporally and spatially and mediate ligand-dependent enhancement of transcription (Asada *et al*., 2002). Serine-aspartic acid rich (SAR) domain of 50 amino acids is rich in serine and aspartic acid. It is unique as it is found only in ELF3. AT hook domain binds preferentially to the minor groove of AT-rich regions in double-stranded DNA and is involved in the transcription regulation of genes. Erythroblast transformation specific (ETS) domain is rich in positively-charged and aromatic amino acid residues, and shows specificity to purine-rich segments of DNA. Many independent research groups have shown that ETS domain is also present in many other well known transcription factors such as human ELF-1, PU.1, human ERG, human ELK-1 and a number of others (Karim *et al*., 1990; Wasylyk *et al*., 1993). NMR analysis of the structure of the ETS domain revealed that it contains complex two dimensional structure comprising of four-stranded beta-sheets and three alpha helices arranged in the order alpha1-beta1-beta2-alpha2-alpha3-beta3-beta4 forming a winged helixturnhelix (wHTH) topology. It is shown that the third alpha-helix turn of ETS domain is responsive to make contact with the major groove of the DNA (Kodandapani *et al*., 1996).

ELF3 is expressed in organs that have secretory epithelial cells such as mammary gland, prostate, gastrointestinal tract, liver, lung, uterus, pancreas and kidney (Brown *et al*., 2004; Nishida *et al*., 2007). It either activates or represses gene transcription in cooperation with other members of transcription factors and co-factors to play crucial roles in embryonic development, mammary gland development, epithelial cell differentiation, proliferation, carcinogenesis and inflammation. ELF3 regulates transcription of variety of genes that are

involved in cellular transformation and inflammation (Choi *et al*., 1998; Scott *et al*., 2000; Rudders *et al*., 2001; Kwon *et al*., 2003; Brown *et al*., 2004; Grall *et al*., 2005; Otero *et al*., 2012). In support of this, deregulated activity and/or expression of ELF3 in human cancers has been reported (Prescott *et al*., 2004; Schedin *et al*., 2004; Neve *et al*., 2006; Longoni *et al*., 2013). Deregulated ETS protein activity and/or expression has been implicated in transformation of human mammary epithelial cells (hMEC) (Rudders *et al*., 2001; Neve *et al*., 2006). Recent reports suggest that ELF3 transforms human breast epithelium derived MCF12A cell line through the serine- and aspartic acid-rich (SAR) domain by an unknown cytoplasmic mechanism. Furthermore, Manavathi *et al*., 2007 reported that p21-acivated kinase-1 (Pak1) could phosphorylate ELF3 (ESE1) at serine 207 which is located in SAR domain. The phosphorylated ELF3 is more stable and activate MAPK to display increased tumorogenic activity (Manavathi *et al*., 2007).

In contrary to these reports, ELF3 is also shown to suppress the tumor growth (Shatnawi *et al*., 2014). A number of genes have been identified as transcriptional targets of ELF3, including transforming growth factor beta-type-II receptor (TGF-*β* RII), several differentiation-specific markers macrophage inflammatory protein 3 *alpha* (MIP3-α), nitric oxide synthase and tumor-associated genes (Park *et al*., 2001; Rudders *et al*., 2001). ELF3 is also a putative mediator of inflammatory shock and host defence (Rudders *et al*., 2001). In response to inflammatory stimuli, ELF3 expression is induced in non-epithelial cells (Oliver *et al*., 2012). In breast cancer, ELF3 expression prominently correlates with ErbB2 expression, and ELF3 is also a downstream transcriptional target of ErbB2 (Chang *et al*., 1997; Asada *et al*., 2002; Neve *et al*., 2002). ELF3 is a target of IL-1β-NF-κB pathway in prostate cancer cells (Longoni *et al*., 2013).

1.2 RATIONAL AND HYPOTHESIS: ERα plays a pivotal role in many physiological processes including mammary gland development. Its deregulation leads to breast cancer pathogenesis. Several reports suggest that ~70% of breast cancers are ER positive, indicating a strong association of ERα with disease progression. In this context, negative regulators of estrogen receptors are attracting significant clinical interest. Resistance to tamoxifen and various other SERMs (selective estrogen receptor modulators), ligand-independent activation, receptor hypersensitivity, cancer cell adaption to low concentration of estrogen and cancer cell invasion are mostly regulated by ER coregulators (Vadlamudi *et al*., 2001). Furthermore, the underlying mechanism involved in coregulator-mediated breast cancers is not fully understood. To address these problems, yeast two-hybrid (Y2H) screening has been performed with human mammary cDNA library using ERα as bait (Manavathi *et al*., 2006). From this screen, ELF3 was picked up as one of ERα interacting proteins.

ELF3, also a transcription factor, can activate or repress transcription genes involved in embryonic development; epithelial cell differentiation, proliferation, inflammation and carcinogenesis. Upon overexpression ELF3 could transform mammary epithelial cells. It is an oncogene and is found to be amplified in 40% of breast cancers. Since ELF3 and ERα are strongly expressed in breast tumors, it is possible that ELF3 could influence ERα functions in breast cancer cells. The aim of the study is to find therapeutic alternative to control breast cancer by developing a small peptide molecule that can block the association between ERα and ELF3.

1.3 MATERIALS AND METHODS:

1.3.1 Cell culture: Human breast cancer cell lines MCF7 and ZR-75-1 were obtained from National Centre for Cell Science (NCCS), Pune, India, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. When necessary, cells were grown in *improved minimum essential medium* (IMEM) supplemented with 2% dextran-charcoal stripped (DCC) serum for 48 hr and were treated with 10 nM of estrogen (E2) for indicated time points.

1.3.2 Plasmid constructs: pcDNA-*ELF3* and pcDNA-*ERα*, which expresses T7-tagged ELF3 and ERα respectively, were cloned into pcDNA3.1C vector. Generation of ELF3 constructs: ELF3 deletion mutants were amplified by PCR with specific primers and amplicons were digested with *EcoR*I and *Not*I, and ligated into pGEX4T1 vector. Generation of ERα constructs: ERα deletion mutants were amplified by PCR with specific primers and amplicons were digested with *EcoR*I and *Xho*I and ligated into pGEX4T1 vector. Full length GFP-*ELF3* and T7-*ELF3ΔETS* were generated using pcDNA-*ELF3* as template by PCR amplification using the appropriate primer pair as shown in **Table 1.1**, then ligated into pEGFP-C1 and pcDNA3.1 vectors, respectively. Similarly, HA tagged ERα was generated using pcDNA-*ERα* as template using appropriate primer pair and ligated into pCMV-HA vector (Clontech, USA).

*ELF3*shRNAs (ELF3 shRNA1 - 5' TCATTGAGCTGCTGGAGAAGGATGGCATG 3';

ELF3 shRNA2 - 5' TGCGAGACCTCACTTCCAGCTCTTCTGAT 3';

ELF3 shRNA3 - 5' GGAGAATCGGCATGAAGGCGTCTTCAAGT 3';

ELF3 shRNA4 - 5' GGATCAGCTACCAAGTGGAGAAGAACAAG 3') and control, shRNA plasmids were purchased from OriGene, USA.

1.3.3 Generation of stable cell lines: MCF-7 cells were transfected with 5 µg of either pEGFP-C1 or pEGFP-*ELF3* using Lipofectamine 2000 reagent (Life Technologies, USA) in 60 mm dishes. Twenty-four hours post transfection, culture medium was replaced with fresh RPMI medium and 500 µg/ml of G418 was supplemented to select for geneticin-resistant cells. After two weeks of antibiotic selection, pools of G418-resistant cells were replated and maintained in complete medium containing 250 µg/ml of G418 for experiments. Similarly, ELF3 stable knockdown was achieved in MCF7 cells by transfection of *ELF3*shRNA using Lipofectamine 2000 followed by puromycin selection (1 µg/ml).

1.3.4 Cell proliferation assay: For analyzing proliferation activity, MCF7 cells were seeded into triplicates at a starting density of 3000 cells per well in a 96-well plate. After 24 hr post seeding, cells were grown in IMEM (Improved MEM) supplemented with 2% dextran-charcoal stripped (DCC) serum for 48 hr and then cells were treated with E2 (10 nM) for different time points. Then 25 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/ml in PBS) was added to each well. After 4 hr of incubation at 37^{o}C followed by treatment with 100 µl of acidified isopropanol (4 mM HCl in isopropanal), colour intensities were determined by relative absorbance at 570 nm and 630 nm using a plate reader (Bio Rad, USA).

1.3.5 Electrophoretic mobility shift (EMSA) assay: Cells were resuspened in 5 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM kCl, 0.5 mM DTT (dithiothreitol), 1x protease inhibitor cocktail), and centrifuged at 800g for 10 min to isolate the nuclei. Nuclei were then resuspended in 2 packed nuclear volume of buffer B (20 mM HEPES, pH 7.9, 25 % Glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1X protease inhibitor cocktail). Protein concentrations were determined using the RC-DC protein assay (BIO-RAD, USA). 10 µl of equimolar amount of the 2 oligonucleotides ERE-1 and ERE-2 (diluted at 250 µg/ml) were mixed in 5 µl of 10X buffer and 25 µl of sterile water. Oligo

mixture was heated to 65°C for 5 min and slowly cooled to room temperature. The annealed oligonucleotides were then end-labeled with (γ ^{32}P) ATP using polynucleotide kinase (PNK) enzyme. Nuclear extracts were incubated with radiolabeled DNA probes. For oligomer competition, binding reactions were incubated with unlabelled double-stranded oligonucleotides for 20 min before adding radiolabeled oligonucleotide. Samples were run on 4% polyacrylamide gel and electrophoresed at 150 V in 0.5X TBE (22.5 mM Tris/borate and 0.5 mM EDTA) buffer for 5 hours at room temperature. Gels were dried and exposed to phosphoimager cassette for at least 16 hr and scanned by Typhon scanner.

1.3.6 Western blot analysis: Cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor mixture (Roche, USA) and lysate was centrifuged at 12000 rpm for 8 min to remove cell debris. The protein concentration was determined by the RC-DC protein assay by following manufacturer's protocol (Bio-Rad, USA). Approximately 120 µg of protein lysate was loaded and resolved on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Pall corporation, USA), and then probed with the following protein specific antibodies: anti-ERα (Cell Signaling Technology, USA or Abcam, USA); anti-ELF3 (Thermo Fisher Scientific, USA); anti-β-actin (Sigma, USA); anti-GAPDH (Cell Signaling Technology, USA); anti-T7 antibody (Novus Biologicals, USA) and anti-GFP (Invitrogen, USA). After incubation with HRP-conjugated secondary antibodies (GE Healthcare, USA), blots were developed with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, USA) using Chemidoc imaging system (Bio-Rad, USA).

1.3.7 Expression and purification of recombinant GST-tag proteins: *E. coli* BL21 (DE3) cells harbouring pGEX4T1-ELF3 domains and ERα domains were inoculated into Luria-Bertani broth (LB) medium supplemented with 100 µg/ml ampicillin and incubated at

37°C with shaking (200 rpm). Next day, 2% overnight culture was inoculated into 500 ml of LB medium supplemented with 100 µg/ml ampicillin. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM of a final concentration to induce protein expression when the OD_{600} reached 0.6. After 3 hrs of incubation at 30°C, the culture was then harvested by centrifugation at 8,000 rpm for 20 min and the cell pellet was stored at −80°C until further analysis. The harvested cell pellet was resuspended in bacterial lysis buffer (50 mM Tris–HCl pH 8.0, glycerol 10%, glucose 20% and protease inhibitor cocktail). After sonication, cell lysate was cleared by centrifugation at 11000 rpm for 10 min at 4°C and the soluble fraction was collected. Supernatant was incubated with Glutathione Sepharose beads (Clonetech, California, USA) for 90 min at 4°C, followed by four 30 min washes with NP 40 lysis buffer (50 mM Tris–HCl pH 8.0, glycerol 10%, NP 40 1% and 137 mM NaCl and protease inhibitor cocktail). To check the protein purity, 20-30 µl of beads were loaded onto SDS PAGE.

1.3.8 Glutathione S-Transferase (GST) pull-down assay: *In vitro* transcription and translation (TNT) of either full length [^{35}S]methionine ERα or ELF3 was performed using TNT kit (Promega Scientific, USA), where 1 µg template plasmid DNA was translated in presence of (^{35}S)-radiolabeled methionine in a reaction mixture of 50 µl. Equal aliquots were used for individual GST pull-down assays. *In vitro* translation and product size were confirmed by running 2 µl of the reaction mixture on SDS-PAGE followed by autoradiography. The GST pull-down assays were performed by incubating equal amounts of bacterial expressed GST-tagged proteins of either ELF3 or ERα immobilized onto glutathione-sepharose beads along with *in vitro* translated (^{35}S)-labeled protein to which the binding was tested. Purified GST alone was used as a control. Bound proteins were collected on glutathione-beads after washing with GST binding buffer to remove nonspecific binding of proteins. Later bound proteins were eluted with 2X SDS buffer and finally separated on

SDS-PAGE. After transferring the proteins onto nitrocellulose membrane, bound proteins ([^{35}S]-ELF3) were detected by autoradiography (Thyphoon scanner, USA).

1.3.9 Immunofluorescence studies: Cells were cultured on glass cover slips and fixed in 4% paraformaldehyde at room temperature for 20 min. The cells were permeabilized with ice cold acetone and methanol (1:3) followed by blocking with 3% BSA. Cells were then incubated with respective primary antibodies; anti-ERα (Abcam, USA), anti-ELF3 (Thermo Fisher Scientific, USA) for overnight at 4^{o}C. After incubation with secondary antibodies conjugated with either Alexa Flour 546 (red), Alexa Flour 488 (green) dye (Molecular Probes, Invitrogen, USA) for 1 hr, cover slips were mounted onto glass slide by adding one drop of prolonged gold antifade DAPI. Images were captured by using fluorescence microscope (Model IX81, Olympus, Singapore).

1.3.10 Chromatin Immunoprecipitation (ChIP) Assay: Approximately 1x10^{8} cells were fixed with formaldehyde at a final concentration of 1% for 20 min at room temperature followed by quenching of cross-links with glycine at a final concentration of 125 mM. Cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitors), followed by sonication on ice at 40 amplitude of 5 bursts each with 5 min gap. After lysate clarification, extracts were precleared using 50 µl of protein A/G-agarose beads (Santa Cruz Biotechnology, USA) (blocked with 400 µg/ml BSA and 20 µg/ml *salmon* sperm DNA) for 1 hr at 4°C on a rotator. Equal concentration of DNA was taken for all the samples and was diluted to 1 ml with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01% SDS, 165 mM NaCl, protease inhibitors). Protein-DNA complexes were immunoprecipitated using 1 µg of either ERα or IgG antibodies (CST, Santa Cruz Biotechnology USA respectively) at 4°C for overnight followed by incubation with a 40 µl of protein A/G beads on a rotator at 4°C for 1 hr. The beads were then washed once each with 1 ml of washing buffer I (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01%

SDS, 165 mM NaCl, protease inhibitors), wash buffer II (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01% SDS, 500 mM NaCl, protease inhibitors), LiCl/detergent solution (10 mM Tris-Cl, pH 8.1, 1% NP-40, 0.25 M LiCl, 1% sodium deoxycholate, 1mM EDTA), and finally with TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). Beads were then collected by centrifugation at 8000 rpm for 2 min at 4°C. Immune-complexes were eluted from the beads with 200 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) by rotating at room temperature for 30 min, and repeated once. Total 400 µl of elutes were reverse cross-linked by heating in 5 M NaCl (16 µl/ vial) and 1 µl DNA free RNase A (10 mg/ml) for 6 hr at 65°C. DNA was then purified by phenol:chloroform:isoamylalchohol mixture (1:1:0.04) and subjected to quantitative real time PCR analysis with suitable primers as shown in **table 1.1**.

1.3.11 Luciferase reporter assays: For reporter gene transient transfections, cells were cultured for 48 hours in IMEM with 2% DCC serum. pGL3-*ERE*-Luc plasmid along with internal Renilla-Luc plasmid were transfected using Lipofectamine 2000 reagent. Twenty four hour post transfection, cells were treated with or without E2 (10 nM) for 18 hr. Cells were then lysed in lysis buffer, and luciferase assay was carried out using dual luciferase reporter assay kit (Promega, USA).

1.3.12 RNA isolation by Trizol method: Total RNA was extracted from cells by TRIzol® Reagent (Life technologies, USA). TRIzol® Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and maintains the integrity of the RNA by effectively inhibiting RNase activity while disrupting cells. The growth medium was removed from culture dishes and the cell monolayer was rinsed once with ice cold PBS. Cells were lysed directly in the culture plates by adding 1ml of Trizol reagent and pipetting the cells up and down several times. Phase separation was carried out by adding 200 µl of chloroform and incubating the samples at room temperature for 5 minutes. The cell suspension was centrifuged at 12000

rpm for 15 minutes at 4^{o}C so that the mixture separates into lower red phenol- chloroform phase, an interphase and an upper aqueous layer containing RNA. The aqueous upper layer was carefully removed and transferred into fresh tubes, followed by RNA precipitation with 0.5 ml of isopropanol for 15 minutes at room temperature. Samples were centrifuged at 12000 rpm for 20 min at 4^{o}C and the supernatant was discarded. The pellet was washed with 70% ethanol and traces of ethanol were removed by air drying. The pellet was then dissolved in RNase free, DNase free ultra-pure water (Life technologies, USA). The quality of RNA extracted was verified by running 1-2 µl of sample on 1% agarose gel.

1.3.13 Quantitative Reverse transcriptase Polymerase Chain Reaction (RT-PCR): First strand cDNA synthesis was carried out using 1 µg of RNA by first strand cDNA Synthesis Kit from Takara Bio Inc, Japan which uses MMLV (Moloney Murine Leukemia virus)-derived reverse transcriptase. In the first step, RNA was incubated with 0.5 mM dNTP, 2.5 µM of oligo dT for 5 min at 65^{o}C. The second step involves incubation with 20 units of RNase inhibitor and 2 units of M-MuLV Reverse Transcriptase for 90 minutes at 42^{o}C followed by enzyme inactivation at 95^{o}C for 5 minutes. PCR reactions were setup using 1 µl of the synthesized cDNA using FastStart SYBR Green Master kit from Roche Life Sciences, USA in Roche LightCycler® 96 instrument. The primers used for amplification are listed in **Table 1.1**. The data was analysed by ΔΔCt method in which median cycle threshold values were used for analysis and threshold values were normalized to the expression of the housekeeping gene, *GAPDH*. The normalized mRNA level in control sample is arbitrarily given as 1 and the relative mRNA levels in test samples were compared to the levels in the control sample.

1.3.14 Statistical analysis: For reproducibility, all the experiments were performed 2-3 times. The results are expressed as means ± standard deviation, and differences between

groups were analyzed by one-way ANOVA using Prism software (Graph pad 5.0). P value <0.01 is considered as significant.

Name	Sequence 5'-3'	Target	Application
pS2 FP pS2 RP	CATCGACGTCCCTCCAGAAGAG CTCTGGGACTAATCACCGTGCTG	Human *pS2* mRNA	qPCR expression
Cathepsin D FP Cathepsin D RP	GTACATGATCCCCTGTGAGAAGGT GGGACAGCTTGTAGCCTTTGC	Human *Cathepsin D* mRNA	qPCR expression
GAPDH FP GAPDH RP	GTCCCCTCGAGGAGTTGTGT ATCTTCCATCATCTGAGGGC	Human *GAPDH* mRNA	qPCR expression
Cyclin D1 FP Cyclin D1 RP	TGGAGCCCCTGAAGAAGAG AAGTGCGTTGTGCGGTAGC	Human *Cylin D1* mRNA	RT-PCR expression
c-myc FP c-myc RP	TACCCT CTCAACGACAGCAG TCT TGACATTCTCCT CGGTG	Human *c-myc* mRNA	RT-PCR expression
Actin FP Actin RP	AGCCATGTACGTAGCCATCC CTCTCAGCTGTGGTGGTGAA	Human *actin* mRNA	RT-PCR expression
pS2 FP pS2 RP	GTGAGCCACTGTTGTCACG CGAGCCCCGGATTTTATAG	Human *pS2* promoter	ChIP-qPCR
Cathepsin D FP Cathepsin D RP	GGTTTCTCTGGAAGCCCTGTAG TCCTGCACCTGCTCCTCC	Human *Cathepsin D* promoter	ChIP-qPCR
ELF3 P.D FP ELF3 P.D RP	GATCGAATTCATGGCTGCAACCTGTGAG GATTGCGGCCGCGTCTCGCAGCTGGGCATG	Human ELF3 Pointed domain (1-128)	pGEX4T1-ELF3-P.D
ELF3 TAD FP ELF3 TAD RP	GATAGAATTCCTCACTTCCAGCTCTTC GATTGCGGCCGCAAAGGGCCCTGGGTCTAG	Human ELF3 TAD domain (128-159)	pGEX4T1-ELF3-TAD
ELF3 SAR FP ELF3 SAR RP	GATAGAATTCCCCTCCCCTGGCAGCTC GATTGCGGCCGCGCTGGGGAAGAGCTTGC	Human ELF3 SAR domain (189-229)	pGEX4T1-ELF3-SAR

ELF3 AT FP ELF3 AT RP	GATAGAATTCGGGGATCCCAAGCACGGG GATTGCGGCCGCGTCCCAGTACTCTTTGCT	Human ELF3 AT domain (238-259)	pGEX4T1-ELF3-AT
ELF3 ETS FP ELF3 ETS RP	GATAGAATTCCACCTGTGGGAGTTCATC GATTGCGGCCGCAAACTTGTAGACGAGTCG	Human ELF3 ETS domain (274-354)	pGEX4T1-ELF3- ETS
ERα A/B FP ERα A/B RP	GATCGAATTCATGACCATGACCCTCCAC GATCCTCGAGCTTGGCAGATTCCATAGCC	Human ERα A/B domain (1-180)	pGEX4T1-ERα-A/B
ERα C FP ERα C RP	GATCGAATTCATGGAATCTGCCAAGGAG GATCCTCGAGCATCATTCCCACTTCGTAG	Human ERα C domain (176-251)	pGEX4T1-ERα-C
ERα D FP ERα D RP	GGCCGAATTCATGAAAGGTGGGATACGA GGCCTCGAGGCTGTTCTTCTTAGAGCG	Human ERα D domain (251-305)	pGEX4T1-ERα-D
ERα E FP ERα E RP	CGGCGAATTCATGATCAAACGCTCTAAG GATCCTCGAGAGTGGGCGCATGTAGGC	Human ERα E domain (297-553)	pGEX4T1-ERα-E
ERα F FP ERα F RP	GATCGAATTCACTAGCCGTGGAGGGGC GATCCTCGAGGACCGTGGCAGGGAAAC	Human ERα F domain (553-595)	pGEX4T1-ERα-F
GFP ELF3 FP GFP ELF3 RP	GATCGAATTCTATGGCTGCAACCTGTGAG GATTGTCGACGTTCCGACTCTGGAGAAC	Human *ELF3*	pEGFPC1-ELF3
ELF3ΔETS FP *ELF3ΔETS* RP	GATCGAATTCATGGCTGCAACCTGTGAG GATTGCGGCCGCGTCCCAGTACTCTTTGCT	Human *ELF3-ΔETS*	pCDNA3.1-ELF3ΔETS

Table 1.1: Primer list and sequences. FP, forward primer and RP, reverse primer. Underlined are specific restriction enzyme sites for cloning, qRT PCR and ChIP-qPCR.

Name	Description	Insert size	Reference
BM 26	pcDNA3.1/His C-ELF3	1113 bp	(Manavathi *et al*., 2007)
BM180	pcDNA3.1/His C-ELF3-ΔETS	967 bp	in this study
BM 33	pcDNA3.1/His C-ERα	1788 bp	in this study
BM 91	pGL2-3X ERE TATA-luc	_	(Hall and McDonnell, 1999)
BM 230	pRL-SV40P-luc	_	(Chen and Prywes, 1999)
BM 81	pEGFP-C1-ELF3	1113 bp	in this study
BM 232	pGBKT7-ERα	1788 bp	in this study
BM 231	pGAD-C1-ELF3	1113 bp	in this study
BM 218	pCMV-ERα	1788 bp	in this study
BM 45	pGEX4T1-Pointed domain of ELF3	381 bp	in this study
BM 46	pGEX4T1-TAD domain of ELF3	93 bp	in this study
BM 47	pGEX4T1-SAR domain of ELF3	120 bp	in this study
BM 48	pGEX4T1-AT domain of ELF3	63 bp	in this study
BM 49	pGEX4T1-ETS domain of ELF3	240 bp	in this study
BM 44	pGEX4T1-ELF3	1113 bp	in this study
BM 61	pGEX4T1-A/B domain of ERα	540 bp	in this study
BM 62	pGEX4T1-C domain of ERα	225 bp	in this study
BM 63	pGEX4T1-D domain of ERα	162 bp	in this study

BM 64	pGEX4T1-E domain of ERα	768 bp	in this study
BM 65	pGEX4T1-F domain of ERα	126 bp	in this study
BM 145	pGFP-V-RS-ELF3 shRNA 1	-	From OrigGene, USA
BM 146	pGFP-V-RS-ELF3 shRNA 2	-	From OrigGene, USA
BM 147	pGFP-V-RS-ELF3 shRNA 3	-	From OrigGene, USA
BM 148	pGFP-V-RS-ELF3 shRNA 4	-	From OrigGene, USA
BM 150	pGFP-V-RS-ELF3 control shRNA	-	From OrigGene, USA

Table 1.2: Plasmids used in this study.

1. 4 RESULTS:

1.4.1 Identification of ELF3 as an ERα-interacting protein: To identify proteins that could be involved in regulation of ERα signaling, a genetic screen has been carried out using a human mammary cDNA library as prey and ERα as bait, respectively (Manavathi *et al*., 2006). ELF3 was one of the several ERα-interacting proteins identified in that screen. Although the link between ELF3 and breast cancer is established (Prescott *et al*., 2004; Schedin *et al*., 2004; Neve *et al*., 2006; Longoni *et al*., 2013), the functional interaction of ELF3 with ERα in breast cancer cells is largely unknown. Therefore, here we sought to determine the role of ELF3 on ERα functions in breast cancer cells. First, the specificity of the interaction between ERα and ELF3 was confirmed in one-to-one two-hybrid interaction assay **(Figure 1.3A)**. *In vitro* assays by GST pull-down experiments further demonstrated the interaction of GST-ELF3, but not GST, with [^{35}S]methionine-ERα **(Figure 1.3B)**. To ascertain the ELF3–ERα interaction in a more physiological context, we performed a coimmunoprecipitation assay using MCF7 cell lysate treated with E2. Endogenous ERα readily coimmunoprecipitated with ELF3, but E2 treatment reduced the interaction between ERα and ELF3 **(Figure 1.3C-D)**. Consistent with these results, immunofluorescence studies demonstrated a strong colocalization of ELF3 with ERα in the nucleus of MCF7 cells. But E2 treatment invoked a dissociation signal as witnessed by partial colocalisation of ERα with ELF3 confirming the E2-dependent nuclear interaction of ELF3 and ERα in breast cancer cells **(Figure 1.4E)**. Together these results indicated the nuclear interaction of ELF3 with ERα in breast cancer cells.

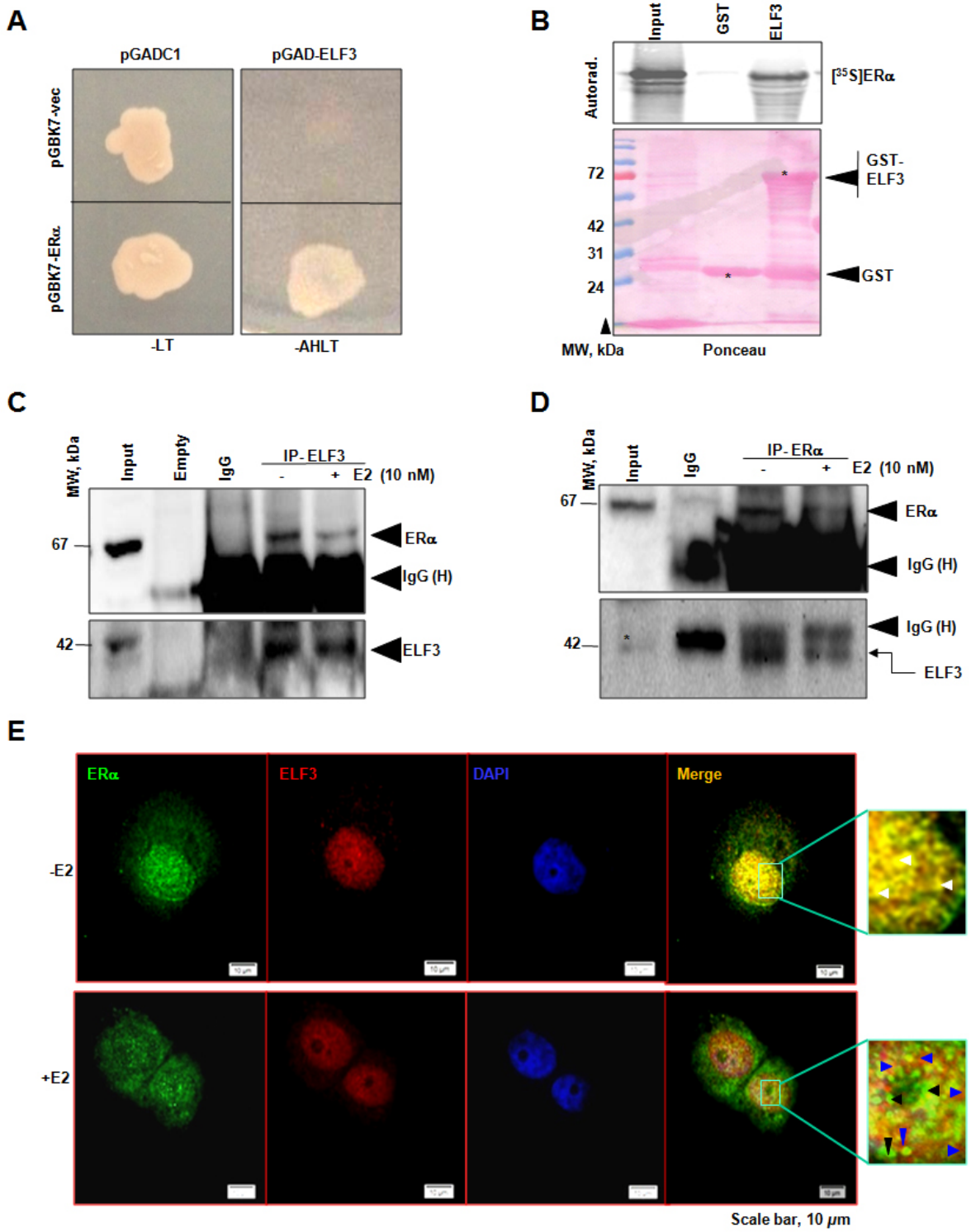


Figure 1.3: Interaction between ERα and ELF3 in breast cancer cells. A) Yeast one-to-one interaction assay demonstrates the interaction between ERα and ELF3 in yeast cells. Yeast strain AH109, cotransfected with either pGBK7-ERα or pGBKT7 vector and pBAD vector or pBAD-*ELF3* plasmids, were allowed to grow on either adenine (A) and histidine (H), leucine (L), tryptophan (T), dropout media or leucine (L) and tryptophan (T) dropout. The pGBKT7-ERα and pBAD-*ELF3* transformed yeast colonies grew in –AHLT medium, whereas the cells cotransformed with the control pGBKT7 vector and pBAD-ELF3 did not grow implying the interaction

between ERα and ELF3. B) *In vitro* GST-pull-down assay showing interaction of [S^{35}]ERα with GST-ELF3 but not with GST alone. C&D) Co-immunoprecipitation assay showing the effect of E2 on the interaction of ELF3 with ERα in MCF7 cells. E) Immunofluorescence analysis demonstrating the effect of E2 on colocalization of ERα and ELF3 in MCF7 cells in . ERα (red), ELF3 (green), nucleus (DAPI-blue). White arrows indicate colocalization of ERα with ELF3 (yellow). Black and arrows indicate ERα and ELF3 localization in MCF7 cells, respectively. Scale bar is 10 μm.

1.4.2 Mapping of ERα and ELF3 interacting domains: Next, to map the domains in the ERα that mediate the protein–protein interaction with ELF3, *in vitro* GST pull-down assays were performed using GST fusions of various ELF3 domains such as Pointed (1-128 aa) (aa-amino acid), trans-activation domain (TAD) (128-159 aa), serine aspartic acid-rich domain (SAR) (189-229 aa), AT hook domain (238-259 aa) and ETS domain (274-354 aa), and in vitro translated [^{35}S]methionine-ERα. As shown in **Figure 1.4A**, ERα bound to full-length ELF3 (1–371) and the ETS domain (274-354 aa). Conversely, *in vitro* translated [^{35}S]methionine-ELF3 interacted strongly with C domain (176-251 aa, DNA binding domain) and weakly with D domain (251-305 aa, hinge region) of ERα **(Figure 1.4B)**.

1.4.3 ELF3 acts as a transcriptional repressor of ERα: After establishing the interaction between ELF3 and ERα, we examined the effect of ELF3 on the transcriptional activity of ERα. ERα-positive breast cancer cell lines such as MCF7 and ZR75 cells were cotransfected with pERE-luc (an artificial estrogen-responsive element-containing reporter) **(Figure 1.5A)** and either pcDNA-ELF3 or vector control plasmids. ELF3 decreased E2-dependent transcriptional activity of ERα substantially in both cell lines **(Figure 1.5B-C)**. Similar results were observed in ER-negative cell line HeLa, which was transiently cotransfected with pcDNA-ERα, pERE-luc and either pcDNA-ELF3 or control plasmids **(Figure 1.5D)**. Next we verified whether ETS domain was responsible for ELF3's repressive activity on ERα-dependent transcription. In consistent with the interaction data, ETS deletion mutant, i.e., ELF3ΔETS, which although predominantly localizes into nucleus **(Figure 1.5E)**,

significantly lost its ability to repress E2-dependent ERα transactivation (P<0.001) **(Figure 1.5F)**. To further investigate the role of endogenous ELF3 on ERα transactivation, we knocked down ELF3 in MCF7 cells **(Figure 1.5G)** and ERE-luciferase activity was determined. ELF3 knockdown indeed significantly increased the transcriptional activity of ERα **(Figure 1.5H)**. To corroborate the results of the luciferase reporter assay, the effect of ELF3 on the expression of endogenous ERα target genes in MCF7 cells upon treatment with E2 was assessed by real-time quantitative PCR. ELF3 ectopic expression indeed decreased the expression of both *pS2* and *Cathepsin D* genes in MCF7 cells **(Figure 1.5I)**. Similar results were also obtained for *c-Myc* and Cyclin D1 (*CCND1*) genes and other ERα target genes **(Figure 1.5J)**. Together these results indicate that ELF3 represses ERα transcriptional activity in breast cancer cells.

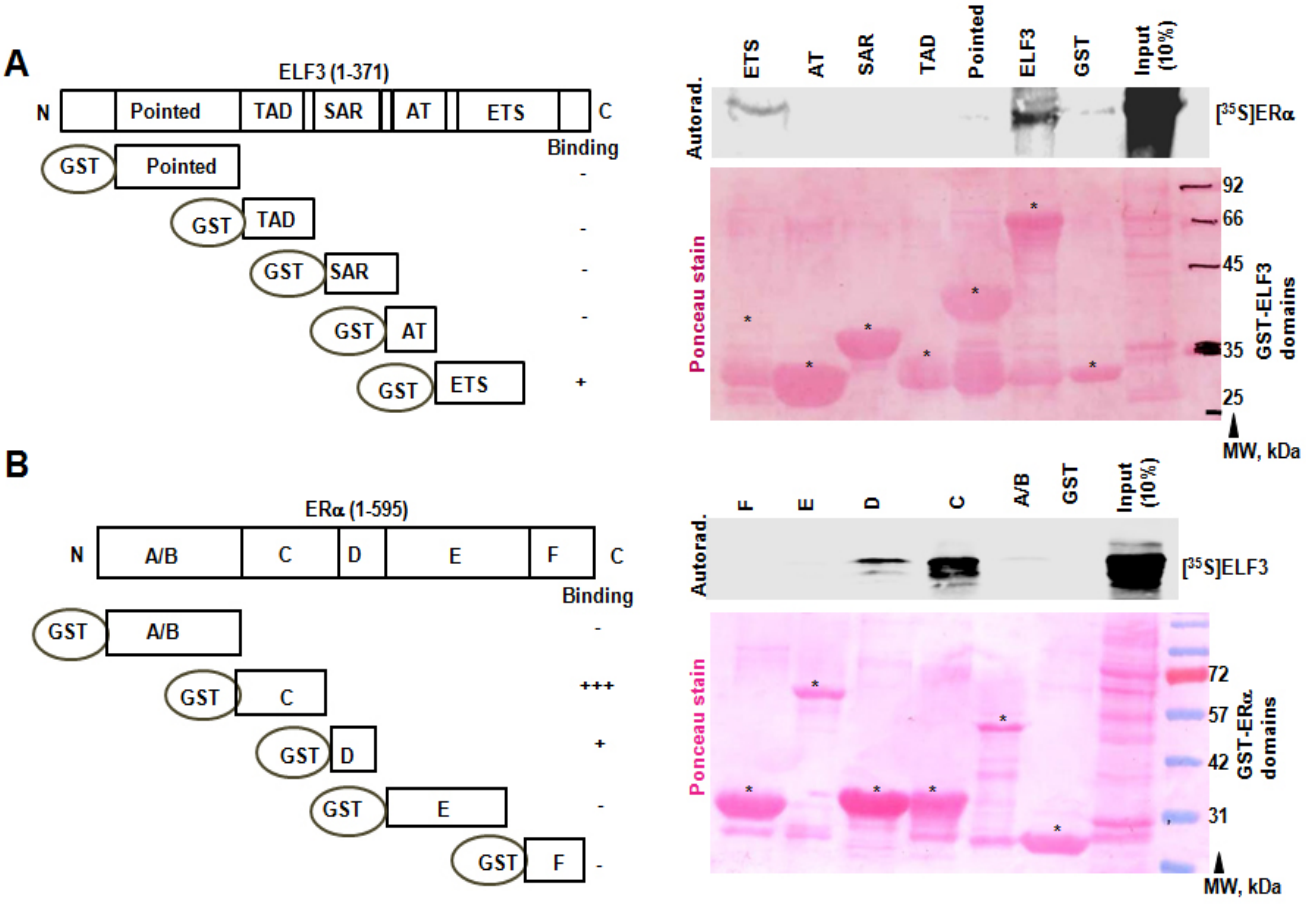
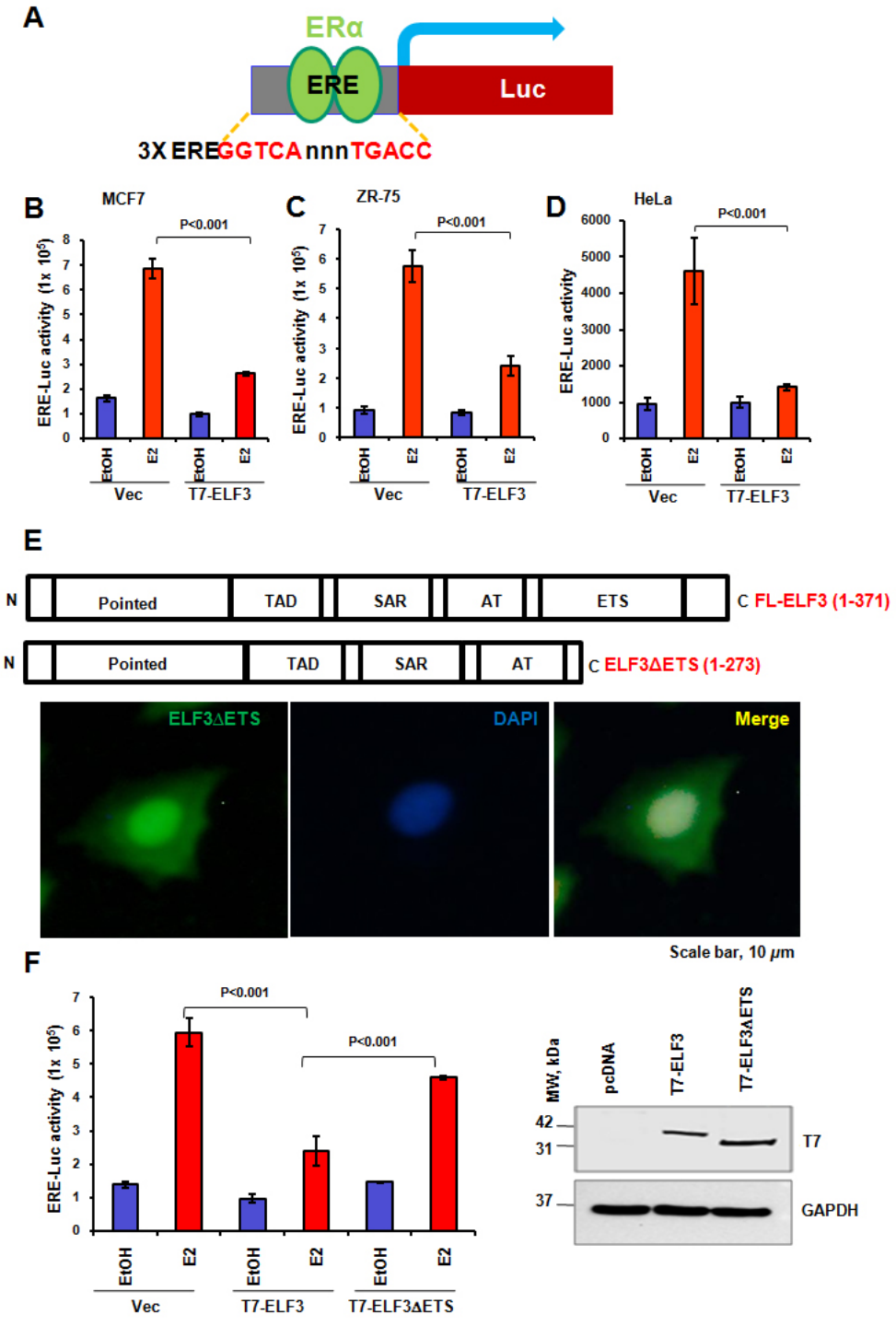


Figure 1.4: A) Physical map of ELF3 and various GST fusions of ELF3 domains, and summary of interaction

between various domains of ELF3 with ERα (left). Plus or minus indicates the strength of protein-protein interaction. +++, ++, + and – denotes strong, medium, low and no interaction, respectively. *In vitro* GST pull-down assay showing requirement of ETS domain of ELF3 for binding with ERα (right). Asterisk indicates corresponding ELF3 domains. B) Physical map of ERα and various GST fusions of ERα domains. Summary of interaction between various domains of ERα with ELF3 (left). *In vitro* GST pull-down assay showing requirement of D domain of ERα for binding with ELF3 (right). Asterisk indicates corresponding ERα domains.



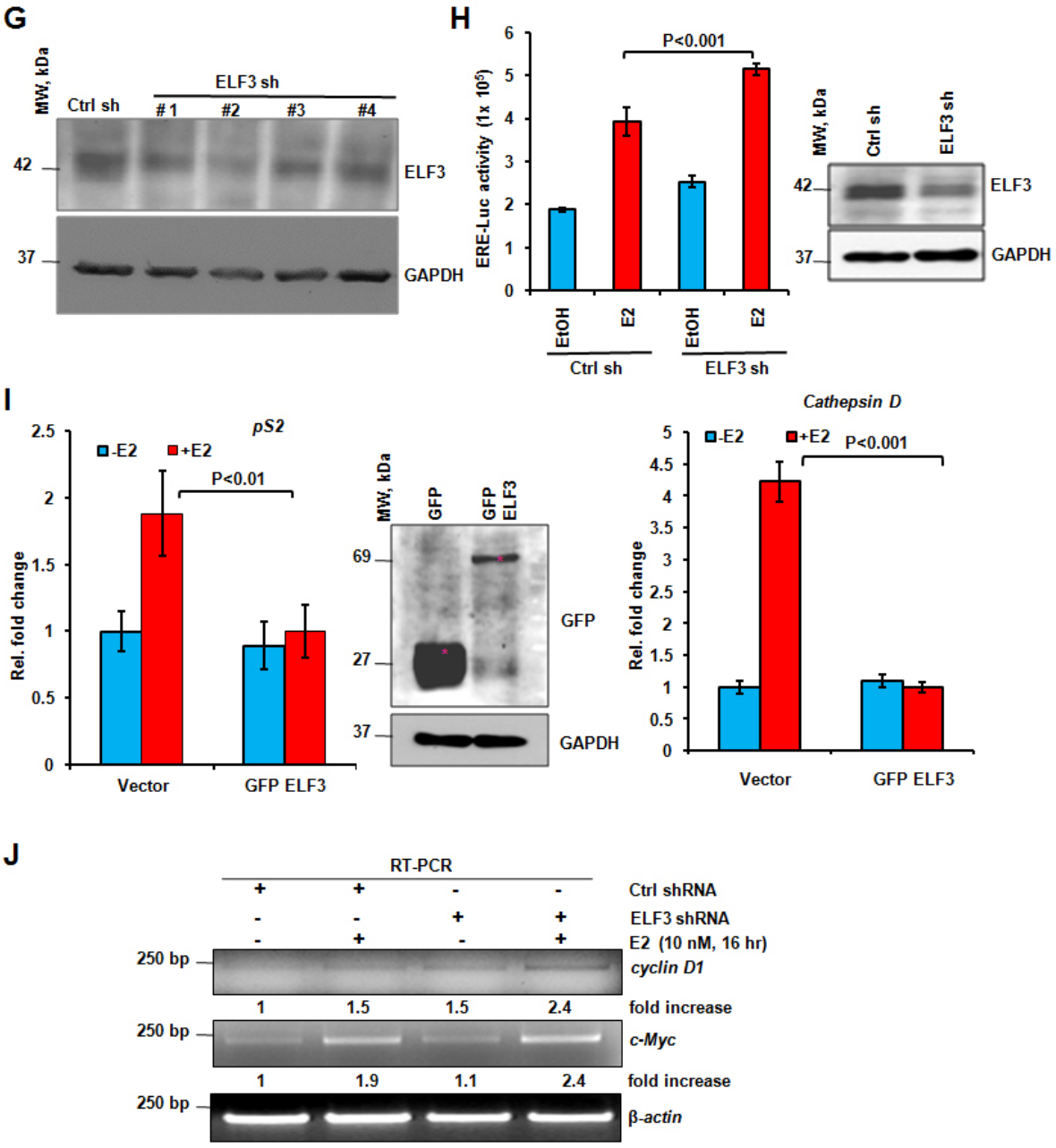


Figure 1.5: ELF3 represses ERα action in breast cancer cells. A) Schematic diagram of ERα binding site and an artificial estrogen-responsive element-containing luciferase reporter construct. Effect of ELF3 on ERE-luciferase activity in MCF7 (B), ZR75 (C) and HeLa (D) cells. Cells were transfected with either pcDNA vector or pcDNA-ELF3 along with ERE-luc plasmid and after treatment with either ethanol (EtOH) or E2 (10 nM) for 18 hr, luciferase activity was determined. E) Physical maps of full length ELF3 and ETS deletion mutant of ELF3 i.e., ELF3ΔETS and immunofluorescence analysis showing the localization of T7-tagged ELF3ΔETS (green) in MCF7 cells. DAPI is used for nuclear staining. F) Effect of wt-ELF3 or ELF3ΔETS on ERE-luciferase activity in MCF7 cells. Western blot analysis showing the expression of wt-ELF3 and

ELF3ΔETS in MCF7 cells (inset). GAPDH serves as internal control. G) Western blot analysis showing the knockdown of ELF3 by various ELF3shRNA clones in MCF7 cells. GAPDH is used as an internal control. H) Effect of ELF3 knockdown on ERE-luciferase activity in MCF7 cells. Western blot analysis showing the knockdown of ELF3 in MCF7 cells (inset). I) Quantitative RT-PCR (qRT-PCR) showing the effect of ELF3 knockdown on expression of ERα target genes, *pS2* and *Cathepsin D* in MCF7 cells. J) Semi-quantitative RT-PCR showing the effect of ELF3 knockdown on expression of ERα target genes, *c-Myc* and *cyclin D1* in MCF7 cells.

1.4.4 ELF3 inhibits ERα recruitment to its target gene promoter by blocking its dimerization: As our domain mapping studies revealed the involvement of ETS domain in interaction with DNA binding domain (C domain) and hinge region (D domain) of ERα, we first tested whether ELF3 inhibits ERα binding to oligos containing ERE elements by gel shift (EMSA) assay. E2–ERα complex from control lysate (vec-treated) readily bound to ERE containing oligos **(Figure 1.6A**, lane 7). However, supplementing wt-ELF3 (lane 8), but not mutant ELF3ΔETS, decreased ERα binding to ERE oligos (lane 9). Furthermore, we observed a super shift with vector- as well as T7-ELF3ΔETS-tranfected MCF7 lysates incubated with ERα antibody (lanes, 11 and 13) but not with wt-ELF3 lysate (lane 12). Next, promoter occupancy of ERα upon ELF3 knockdown in response to E2 treatment at the ERE elements within the endogenous *pS2* and *Cathepsin D* gene promoters was analyzed by chromatin immunoprecipitation (ChIP) assay. ELF3 knockdown significantly increased ERα recruitment to *pS2* and *Cathepsin D* gene promoters as compared to control shRNA cells ($p<0.001$) **(Figure 1.6B)**.

ERα is a ligand-dependent transcription factor. Upon ligand binding it undergoes conformational dependent dimerization to be recruited and to associate with target gene chromatin (Kumar and Chambon, 1988; Winkler *et al*., 2006). To test whether the inhibitory activity of ELF3 on ERα recruitment to target gene chromatin was due to its ability to affect the ERα dimerization, we cotransfected pcDNA-ERα (T7-ERα) and pCMV-ERα (HA-ERα)

along with either GFP or GFP-ELF3 expressing HEK293T cells, which do not express neither ERα nor ELF3 endogenously, and CoIP assay was performed after treating the cells with E2 (10 nM) for 18 hr. HA-ERα readily coimmunoprecipitated with T7-ERα in GFP-transfected cells upon E2 treatment (lane 4) but not in GFP-ELF3-transfected cells (lane 6) **(Figure 1.6C)**. Thus, ELF3-mediated repression of ERα transcriptional activity that was observed **(Figure 1.6A-B)** could be due to inhibitory activity of ELF3 on ERα recruitment to its target gene promoters by blocking ERα dimerization *in vivo* **(Figure 1.6C)**

1.4.5 ELF3 inhibits estrogen-dependent breast cancer cell proliferation: To further investigate the functional relationship between ERα and ELF3 with respect to breast cancer cell growth, cell proliferation assay was performed under conditions of ELF3 ectopic expression as well as knockdown in MCF7 cells. Preliminary cell cycle analysis using flow cytometry (FACS) indicated that ELF3 suppresses cell proliferation (reduced S phase cells; vec vs. ELF3: 20% vs. 16%) **(Figure 1.7)**. After confirming the ectopic expression of either T7-ELF3 or T7-ELF3ΔETS (**Figure 1.8A**, inset) in MCF7 cells, cells were treated with either ethanol or E2 (10 nM) for 1–5 days and cell proliferation was determined. ELF3 ectopic expression indeed decreased cell proliferation compared to either control Vec-transfected or T7-ELF3ΔETS-transfected cells. Conversely, ELF3 knockdown increased E2-dependent cell proliferation in MCF7 cells **(Figure 1.8B)**. Because ELF3 displayed inhibitory activity towards ERα functionality, we tested if it confers tamoxifen, an ERα antagonist, resistant to MCF7 cells. In consistent with this notion, ELF3 ectopic expression increased tamoxifen resistance in MCF7 cells **(Figure 1.8C)**. Together, these results indicate that ELF3 suppresses ERα-mediated cell proliferation and confers tamoxifen resistance to breast cancer cells.

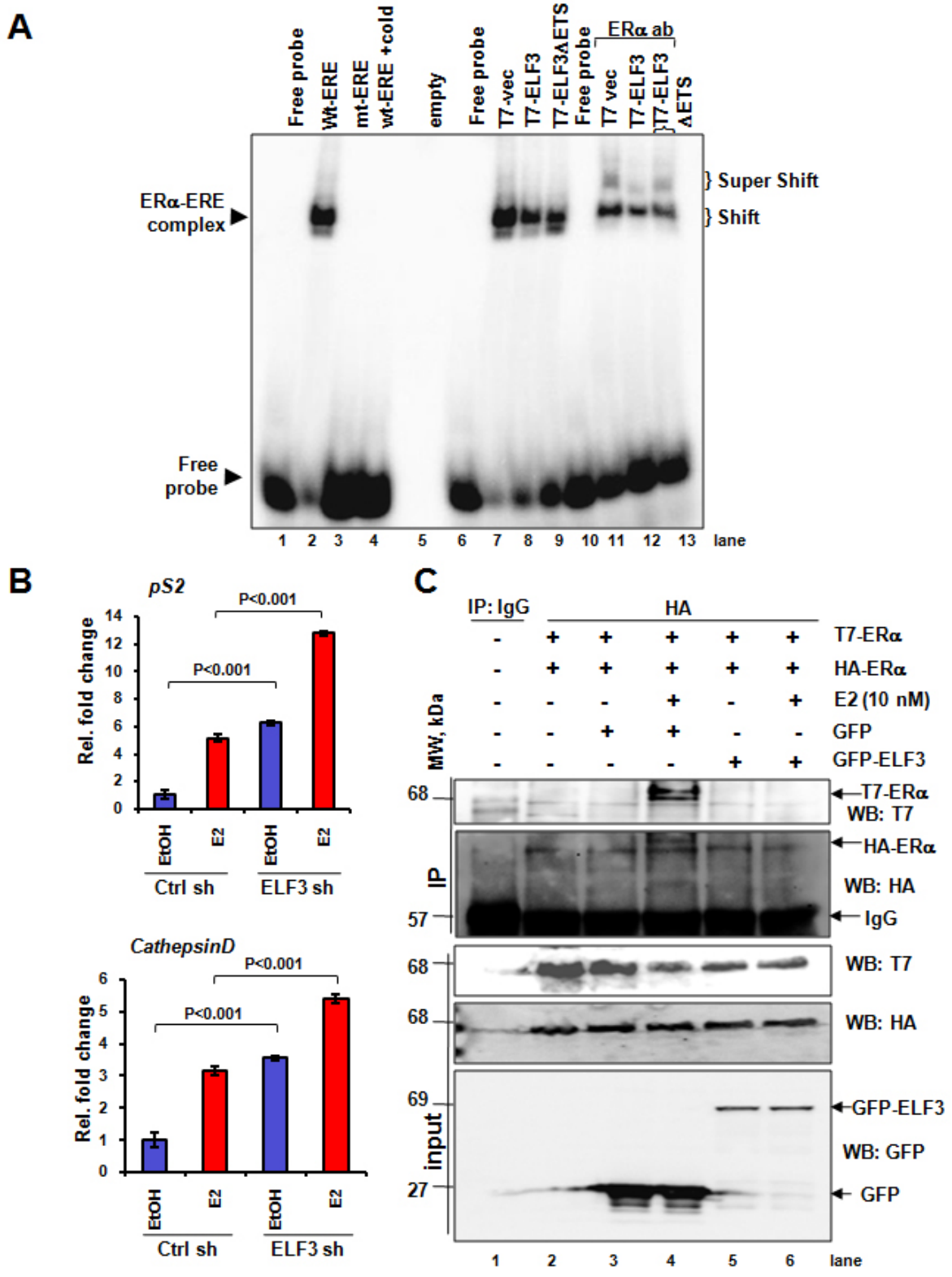


Figure 1.6: ELF3 inhibits ERα DNA binding capacity and recruitment to target gene promoters by affecting its dimerization. A) Electrophoretic mobility shift assay (EMSA) demonstrating inhibition of ERE binding capacity of ERα by ELF3. Increasing concentrations (2.5, 5 and 10 µg) of either GFP or GFP-ELF3 transfected MCF7 +++cell (treated with E2 at 10 nM concentration for 1 hr) nuclear lysates (NL) were incubated with ERE oligos

and subjected to EMSA. B) Chromatin immunoprecipitation assay demonstrating the increased recruitment of ERα to *pS2* and *Cathepsin D* gene promoters upon ELF3 knockdown in MCF7 cells. C) Effect of ELF3 overexpression on ERα dimerization in HEK293T cells. Cells were transfected with either control GFP vector or GFP-ELF3 plasmid along with HA-ERα and T7-ERα encoding plasmids. After treatment with E2 for 18 hr, cell lysates were subjected to co-immunoprecipitated followed by Western blotting with indicated antibodies.

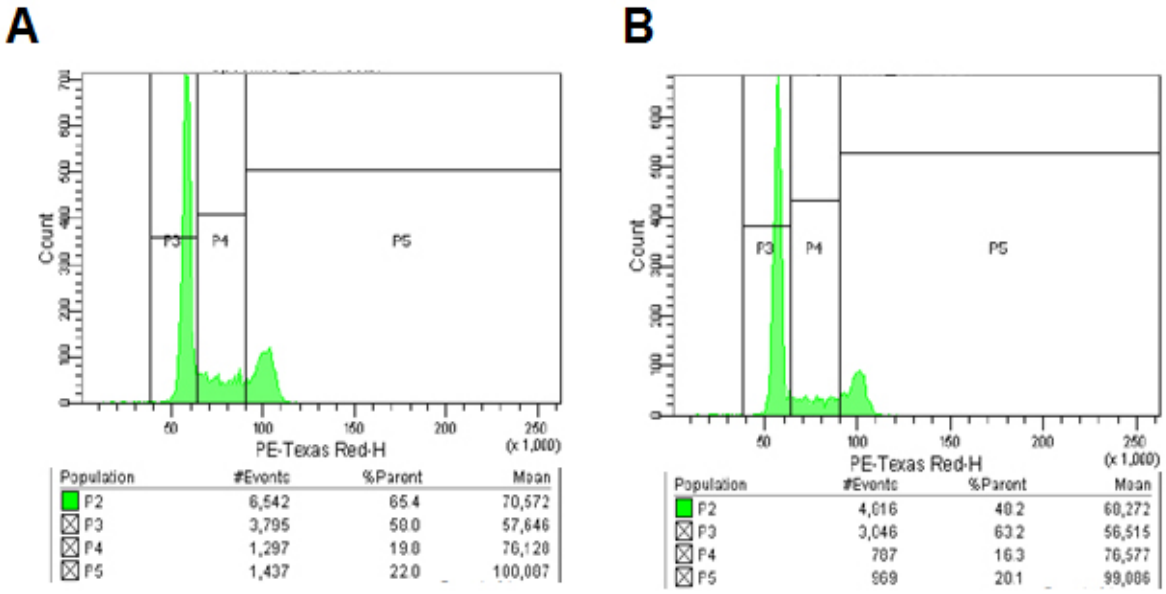


Figure 1.7: FACS analysis showing the effect of ELF3 ectopic expression on MCF7 cell cycle. pcDNA-vec transfected MCF7 cells were used as internal control. Table describes the statistics of cell cycle in respective samples. P3-G1 phase; P4-S phase; P5-G2/M phase.

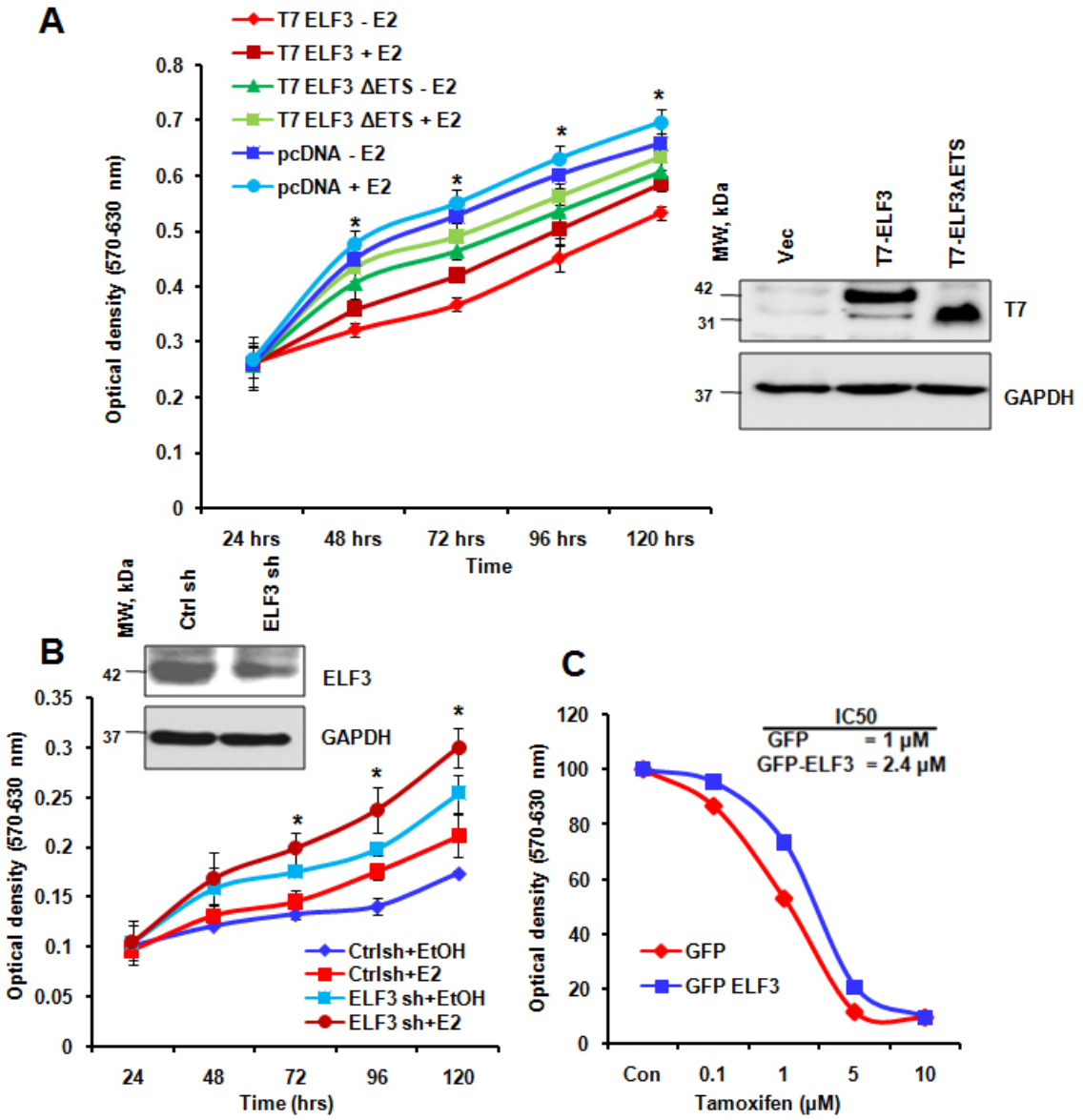


Figure 1.8: ELF3 suppresses ERα-mediated breast cancer cell proliferation. A) Effect of ELF3 overexpression on E2-mediated MCF7 cell proliferation demonstrated by MTT assay. Vec, T7 ELF3 or T7 ELF3 ΔETS transfected cells were treated with E2 (10 nM) for indicated time points. Western blot analysis showing the expression of T7 ELF3 or T7 ELF3 ΔETS in MCF7 cells (inset). B) Effect of ELF3 knockdown on E2-mediated MCF7 cell proliferation demonstrated by MTT assay. Ctrl *shRNA* or *ELF3shRNA* transfected cells were treated with E2 (10 nM) for indicated time points. Western blot analysis showing the knockdown of ELF3 in MCF7 cells (inset). C) Half maximal inhibitory concentration ($\mathbf{IC_{50}}$) values of Tamoxifen in GFP or GFP ELF3 ectopic expressed MCF7 cells.

1.5 DISCUSSION: Because majority of breast cancers are ERα-positive and existing therapies to treat breast cancer are not effective, understanding the molecular mechanisms that regulate ERα functions in breast cancer cells is of continued interest. Furthermore, as coregulators are proven to be critical factors in regulating ERα functions both in normal development as well as in cellular transformation, considerable amount of interest led to identifying several ERα coregulators (Manavathi *et al*., 2014). With this background, here we identified ELF3 as an ERα interacting protein that represses ERα transactivation functions in breast cancer cells. Mechanistic studies revealed that ELF3 represses ERα transactivation functions by blocking ERα dimerization thereby, its DNA binding activity towards ERα target genes.

The role of ETS family factors in mammary gland development and breast cancer has been well documented (Myers *et al*., 2005). For instance, ETS-1, which has been shown to promote breast tumor metastasis, is an ERα coactivator in breast cancer cells (Kalet *et al*., 2013). ERG, another ETS factor, is reported to inhibit ERα transcriptional activity implying an inherent association of ETS activity with ERα (Vlaeminck-Guillem *et al*., 2003). In this study, we determined the functional interaction between epithelial-specific ETS factor, ELF3 and ERα in breast cancer cells. As the functionality of ERα is dependent on its ability to bind and transcriptionally upregulate the genes that are involved in cell proliferation to cause cellular transformation (Nilsson *et al*., 2001; Brisken and O'Malley, 2010), we report that ELF3 inhibits breast cancer cell proliferation by repressing ERα transcriptional activity. We found a strong interaction between ERα and ELF3 in MCF7 cells. But E2 treatment alleviated such interaction implying ligand-induced conformational change in the receptor might rendered ELF3 dissociation from ERα. The other possibility could be the recruitment of coactivators to the receptor upon treatment with E2. Furthermore, decreased interaction of ELF3 with ERα upon E2 treatment is attributed to the reduced repressive activity of ELF3

towards ERα transcriptional functions in response of E2 **(Figure 5-7)**. Remarkable in this respect is the involvement of ETS domain in interaction with DNA binding domain of ERα suggests ELF3-mediated transcriptional repression at ERα target genes occur through inhibition of ERα DNA binding activity. This is not the case for ERG inhibiting ERα functions, where ERG does not physically interact with ERα (Vlaeminck-Guillem *et al*., 2003). Rather, N-terminal and C-terminal transactivation domains of ERG are involved in repressive functions (Kalet *et al*., 2013). This argues that the transcriptional repressive activity displayed by ELF3 on ERα is different from ERG and offers a repertoire of inhibitory mechanisms to control ERα activity in breast cancer cells.

Ligand binding triggers conformational change in the ERα that promotes its dimerization (Kumar and Chambon, 1988; Tamrazi *et al*., 2002). Dimerization is an important step for ERα binding to DNA and subsequently for its transcriptional activity (Li *et al*., 2004; Ahlbory-Dieker *et al*., 2009). Therefore, blocking of ERα dimer formation has serious implications on its transcriptional activity and breast cancer cell growth. Both ligand binding domain (LBD) as well as hinge region is shown to participate in ERα dimerization (Schwabe *et al*., 1990; Ruff *et al*., 2000). Proteins like Calmodulin binds at hinge region and enhance ERα dimerization (Li *et al*., 2003; Zhang *et al*., 2012). In this study, employing HEK293 cells, which do express neither ERα nor ELF3, we demonstrate that ectopically expressed GFP-ELF3 inhibits interaction of T7-ERα with HA-ERα i.e., ERα dimerization. Furthermore, residues present in 'D box' (D for dimerization), which is located in DNA binding domain, have been shown to involve in receptor dimerization (Schwabe *et al*., 1990; Ruff *et al*., 2000). As our *in vitro* studies shown weak interaction of ELF3 with hinge region of ERα, which is a flexible region that connects ligand binding domain (LBD) and DBD, we can't rule out the possibility of hinge region-mediated ERα dimerization aided by Calmodulin protein. With this data, our model represents inhibition of DNA binding capacity of ERα

onto ERE elements by ELF3 involves direct interaction of ELF3 with DBD of ERα that alleviates receptor dimerization, DNA binding and subsequently its transcription activity **(Figure 1.9)**.

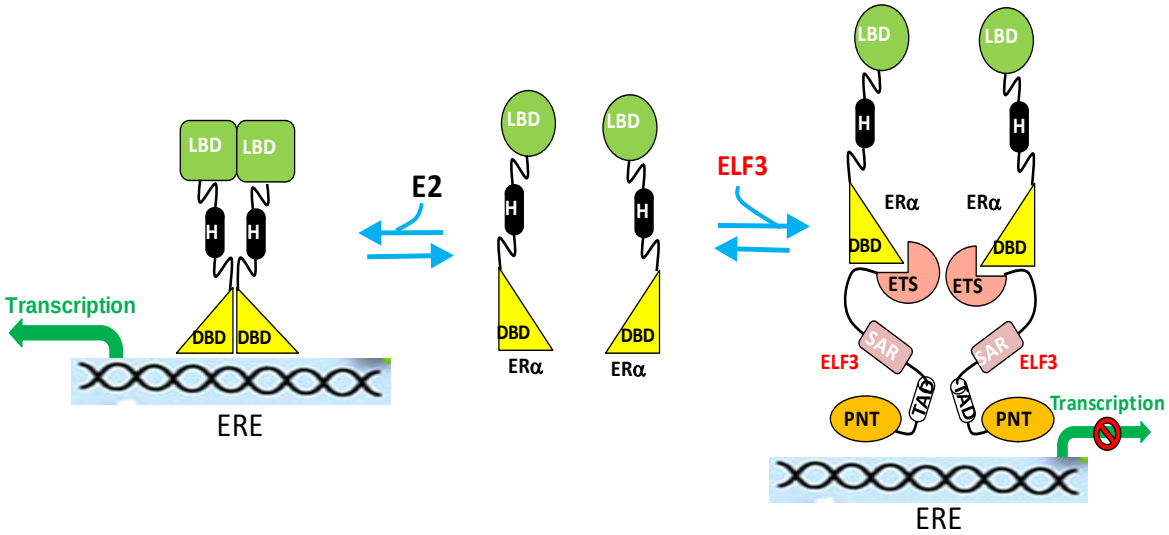
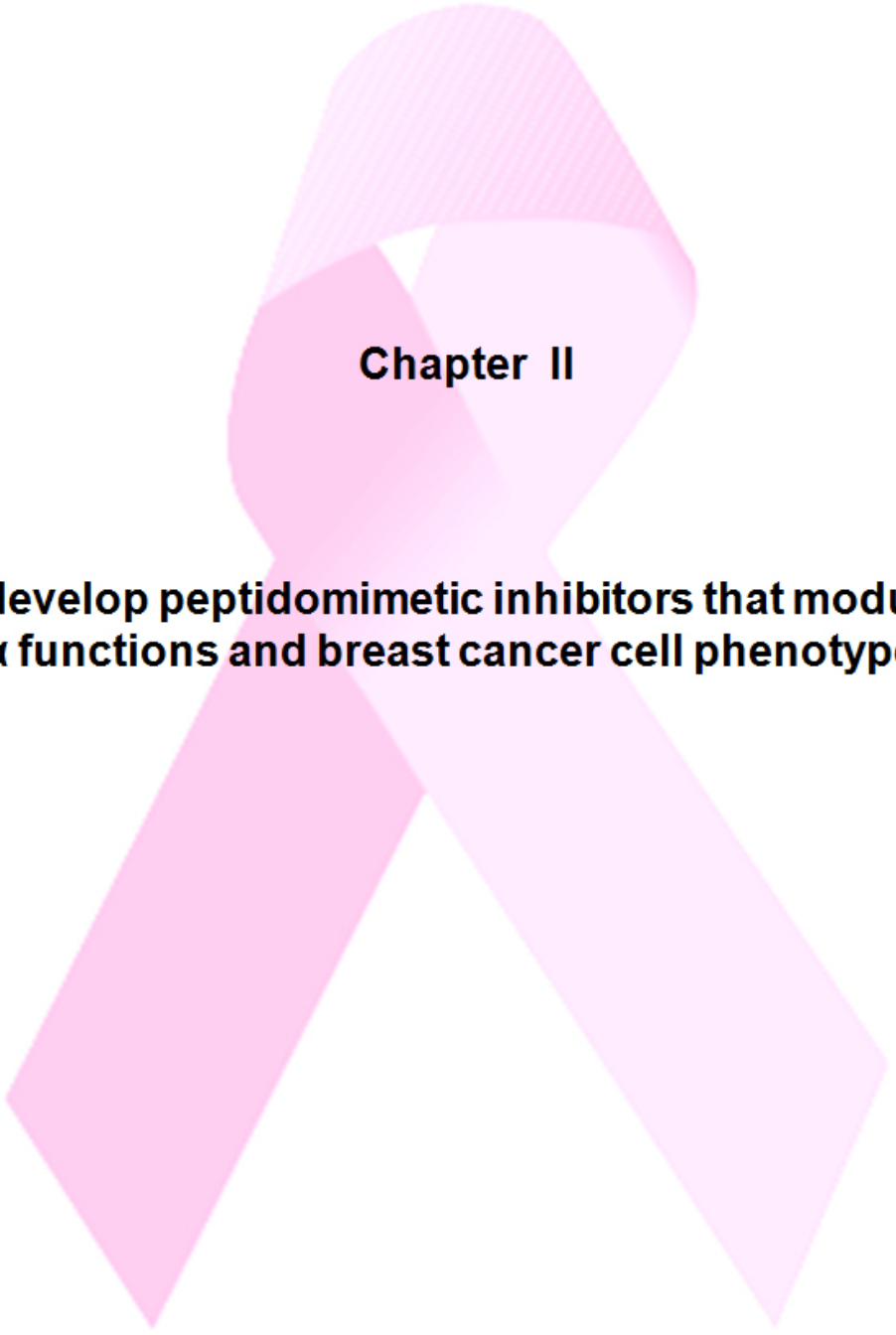


Figure 1.7: Model illustrating the repressive activity of ELF3 on ERα functionality. ELF3 prevents ERα dimerization subsequently its DNA (ERE) binding capacity.

1.6 CONCLUSION: Our study on interaction between ERα and ELF3 has provided key insights into the progression of ERα-dependent breast cancers. Our data highlights the previously unidentified interaction between ELF3 and ERα in both *in vitro* and cell line models and also we were able to pin down on the key functional domains in these proteins that facilitate this interaction. The evidences for this cross talk were gained from the Y2H studies where ERα was used as bait. Later on the interaction was confirmed between the proteins by various standard molecular techniques like colocalization, GST pull-down and immunoprecipitaion assays. To further evaluate this interaction, different domains of these two proteins were cloned, expressed, purified and used for *in vitro* binding studies. Interestingly, we found that the ETS domain of ELF3 and C domain of ERα interact with each other. ERα-C region is very critical for ERE binding and dimerization of the receptor. ETS deletion mutant of ELF3 lost its ability to suppress ERα dependent transcriptional activity and proliferation as compared to wild type ELF3.

Many researchers claimed that ERα-C domain is crucial for the dimerization of ERα. Any molecule or compound that stereo-chemically clouds this C domain of ERα that is sufficient to prevent the dimerization of the receptor and, acts as an inhibitor. Interestingly in our investigation, the same was found to be true in case of ELF3, where transient overexpression of ELF3 hindered ERα dimerization and knockdown of ELF3 has the converse effect. Also, we found that overexpression of ELF3 reduced the transcriptional activity. The transcriptional activity of ERα is key driver in the development and progression of ER positive breast cancers subtype and is also critical for their survival. The presence of ELF3 obliterated the ERα transcriptional activity as is evident from the reduced levels of its downstream targets in ELF3 over expressed cells and also the same has inhibitory effect on the breast cancer cell proliferation and survival. The binding of these proteins and conformational changes thus occurred are such significant that this interaction leads to tamoxifen resistance in MCF7 cells over expressing ELF3 possibly due to conformational changes arising in ERα after binding to ELF3, giving little opportunity for tamoxifen to bind to its direct target ERα. Taken together these results implicate the interaction of two diverse classes of proteins and how detrimental the conformational changes resulting out of this interaction would be off to breast cancer cell proliferation.



Chapter II

To develop peptidomimetic inhibitors that modulate ERα functions and breast cancer cell phenotype

2.1 INTRODUCTION

2.1.1 Breast cancer: Breast cancer is a malignant cell growth in the breast. Breast cancer begins in the breast tissue that is made up of glands for milk production, called lobules, and the ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissue. Breast cancer is the most common cancer among women, although it exists in men in rare cases. If left untreated, cancer spreads to other areas of the body (Saunders *et al*., 2009). Breast cancer remains one of the common malignancies affecting millions of women all over the world. It is said that breast cancer is the fifth most common cause of cancer death (WHO, 2006). In 2012, 1.7 million women were diagnosed with breast cancer worldwide; men have a 100-fold lower risk of developing breast cancer than women. Across the world, over 522,000 women died as a direct result of their breast cancer (GLOBOCAN 2012 IACR). In India, every year 1,00,000-1,25,000 new breast cancer cases are registered and estimated that this number will be doubled by 2025.

2.1.2 Breast cancer risk factors: Extensive research over the last 30 years has shown different risk factors associated with higher incidence of women breast cancer. The two strongest risk factors for breast cancer are sex (gender) and age (Roses DF, 2009). Some of the common risk factors include: age at diagnosis, genetic mutations, previous breast cancer, race, previous premalignant tumor biopsy, prior radiation treatment in the chest area, age of mensus onset, age of menopause (earlier onset of menstruation and late menopause increases the breast cancer risk), use of oral contraceptives, pregnancy, hormone replacement therapy, obesity, poor diet, failing to exercise, fail to breast feed and excessive alcohol intake (more than one alcoholic drink a day) (Veronesi U *et al*., 2005).

2.1.3 Breast cancer types: Approximately 95% of the breast cancers originate from epithelial cells and hence are termed carcinomas (Richie and Swanson, 2003). Breast cancer

can originate from different areas of the breast that include the ducts, the lobules, or in some cases, between the tissues. Breast cancers are classified into two major types, based on the invasive nature of the cancer cells.

A. Non-invasive breast cancers: In this category cancerous cells remain in a particular location of the breast, without spreading to surrounding tissue, lobules or ducts. These are further classified based on the location. The ductal carcinoma in situ (DCIS) is defined by a mass of proliferating cancer cells confined to ducts, and doesn't invade through the basement membrane (Richie and Swanson, 2003; Burstein *et al*., 2004). The second type of non-invasive carcinoma is lobular carcinoma in situ (LCIS) that arises and is confined to the milk producing glands or lobules (Visvanathan, 2011).

B. Invasive breast cancers: This is the type of cancer that spreads outside the membrane of the lobule or duct into the breast tissue. Consequently, the cancer cells will metastasize to other parts of the body hence giving rise to metastatic breast cancer (MBC). Approximately 80% of all breast cancers are invasive ductal carcinomas (IDC) spreading through the cells of the ducts. On the other hand, invasive lobular carcinoma (ILC) starts spreading through lobules (Weigelt *et al*., 2005).

2.1.4 Molecular classification of breast cancer: Breast cancer is a heterogeneous disease comprising of numerous distinct cell types having different biological features and clinical behaviour. Therefore, classification of breast cancer cannot be limited to the one based on localization and the extent of the tumor. On molecular basis (gene expression profile) breast cancers are further classified into five major subtypes of breast cancers:

1) Basal-like breast cancers: Basal-like breast tumors are aggressive in nature and are associated with high rate of proliferation and metastasis events. Chemotherapy is the only treatment option available currently; however resistance often occurs due to deprivation of anti-apoptotic signals resulting in recurrence and patient death. This is also called as triple

negative breast cancer (TNBC). It is a heterogeneous group of tumors that accounts for up to 15% of all breast cancers. Basal-like breast cancers have been defined based on immunehistochemical markers as follows: (1) lack of ER- (estrogen receptor), PR- (progesterone receptor) and HER2- (human epidermal growth factor receptor 2) expression ('triple-negative' immunophenotype); (2) expression of one or more high-molecular-weight/basal cytokeratins (CK5/6, CK14, and CK17); (3) lack of expression of ER and HER2 in conjunction with expression of CK5/6 and/or epidermal growth factor receptor (EGFR); and (4) lack of expression of ER, PR, and HER2 in conjunction with expression of CK5/6 and/or EGFR (Badve *et al*., 2011). *BRCA1* and *BRCA2* mutations are associated with this type of tumors (Sorlie *et al*., 2003)**.** For this group, chemotherapy with personalized medicine is only the option (Cancer Genome Atlas, 2012).

2) Luminal A: Luminal A breast cancers are estrogen-receptor-positive, HER2- negative and low-grade. These tumors grow very slowly and have the best prognosis than luminal B type (Kim *et al*., 2012).

3) Luminal B: Similar to Luminal A type, Luminal B type is also estrogen-receptor-positive and HER2-negative, but often high grade. Proliferation signature (expression of MKI67 (encoding Ki-67) and Cyclin B1) is high in Luminal B tumors than in luminal A tumors. Tumors of this subgroup A and B are associated with a good prognosis and can be treated with targeted therapies, e.g. selective oestrogen receptor modulators (SERMS), such as tamoxifen or, in post-menopausal women, aromatase inhibitors such as anastrozole (Prat and Perou, 2011).

4) HER2 overexpressing: *HER2* gene encodes a transmembrane tyrosine kinase receptor of the epidermal growth factor (EGFR) family. HER2 positive breast tumors are characterised by lack of expression of luminal/ER-related genes and overexpression of *HER2*-related genes. This subtype comprises approximately 10% of all breast tumors (Carey *et al*., 2006).

They are frequently high-grade and 50% of them exhibit p53 mutations, and associate with poor prognosis (Carey *et al*., 2006; Parker *et al*., 2009).

5) Normal breast like: These are characterised by expression of genes found in benign breast tissue, e.g. basal epithelial cells and adipose cells. The stem cell marker, aldehyde dehydrogenase 1 (ALDH1) is found to be highly expressed in the normal breast-like group (Prat and Perou, 2011).

2.1.5 ER-positive-breast cancers: These are defined by the expression of ERα in breast tissues. ERα is over expressed in approximately 75% of breast cancers at the time of diagnosis and ER status serves as a major prognostic marker. Patients with ER-positive breast tumors generally have a better prognosis than those with tumors that lack ER and these cancer patients positive to ER usually undergo treatment with endocrine therapeutics such as the selective ER modulator (SERM) tamoxifen or aromatase inhibitors like anastrozole or letrozole. It is unfortunate that, many patients with ER-positive breast tumors fail to respond to endocrine therapy with tamoxifen, and most tumors that are initially responsive acquire resistance due to various reasons (Clarke *et al*., 2001). In recent years, accumulating studies, using high throughput gene expression profiling have attempted to unravel specific gene expression signatures that predict response to endocrine therapy and to channel breast cancer patients into more appropriate therapeutic avenues. For example studying gene expression profiling using tamoxifen in ER-positive tumors has led insights into a number of specific signature codes that are predictive of clinical outcome of breast cancer patients relying upon tamoxifen theraphy (Loi *et al*., 2007; Huang *et al*., 2011). To further validate the outcome of patients to breast cancer therapies gene profiling studies of both ER-positive and ER-negative breast tumors were conducted. Interestingly ER-positive tumors were classified into two intrinsic subtypes, Luminal A type and Luminal B type that differ prominently in relapse-free and overall survivability. ER positive breast tumors of the Luminal A subtype are found to be

associated with greater overall patient survivability, whereas the Luminal B subtype patients are associated with significantly worse patient outcome in response to breast cancer therapeutics. These gene expression profiling investigations by many groups led to the development of breast cancer intrinsic classifier assay termed as PAM50, that analyses the expression levels of 50 different genes in order to classify the intrinsic tumor sub type, and provide key additional prognostic information beyond current conventional pathological tumor characterisation that is crucial in determining the therapeutic approach (Sorlie *et al*., 2003; Parker *et al*., 2009; Nielsen *et al*., 2010). Although gene expression profiling has the potential to provide greater insights into predictive ability for responsiveness of some ER-positive tumors to endocrine therapeutic drugs like tamoxifen, the underlying biology causing tumor heterogeneity is yet to be fully understood. What is known so far of these studies is that ER-positive breast tumors with poor response to endocrine therapy tend to have lower ER expression and high levels of proliferation-associated genes (Bardou *et al*., 2003; Loi *et al*., 2007; Musgrove and Sutherland, 2009; Creighton *et al*., 2010). A number of studies suggest that growth factors like IGF-I and cell signaling pathways, such as phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT), IGF-I, and protein kinase C alpha (PKCα), contribute to poor outcome to treatments for women with ER-positive tumors causing preying on agony (Creighton *et al*., 2010; Fox *et al*., 2011; Miller *et al*., 2011).

2.1.6 ER-positive-breast cancer therapy: It is also called as endocrine therapy, in which ERα is targeted by blocking receptor binding with an antagonist or by depriving the tumor of estrogen. It is the most effective treatment for ER-positive metastatic breast cancers. Various endocrine therapies work by different mechanisms to antagonize the growth promoting activity of estrogen. Selective estrogen receptor modulators (SERMs) such as tamoxifen, toremifene, raloxifene and ICI 182,780 (ICI) or fulvestrant primarily act by competing with estrogens for binding to the estrogen receptor (ER) and are the most widely administered

endocrine agents for the management of ER-expressing breast cancers. These drugs interact with the ligand binding domain of the receptor and induce different conformations of the receptor and antagonize the effects of estrogen on ER target genes (Frasor *et al*., 2004; Jordan, 2006; Osborne and Schiff, 2011). Endocrine therapy prevents ovarian synthesis of estrogens either with the luteinising hormone-releasing hormone (LHRH) analog goserelin in premenopausal women or postmenopausal patients, who have hormone-dependent breast cancer, or can be treated with aromatase inhibitors, which blocks estrogen production. Aromatase inhibitors include anastrozole, letrozole, or exemestane (Geisler *et al*., 2001; Geisler *et al*., 2008).

2.1.7 ER-positive-breast cancers and drug resistance: Cancer drug resistance is the decreased in effectiveness of a drug or failure of a cancer patients to respond to a specific therapy. The main reasons for drug resistance in cancer patients, is due to alterations in the drug target, ineffective induction of cell death or it could be due to activation of prosurvival pathways.

Several signaling cascades have been implicated in the development of endocrine resistance, including MAP kinase, HER2, PI3K-mTOR, FGFR and IGF-IR pathways (Roop and Ma, 2012). Furthermore, coregulators disregulated expression has been linked to tamoxifen resistance. For instance, PELP1 which serves as a coactivator of ERα is involved in ERα cross talk with the cell cycle machinery and mediates E2-induced breast cancer cell proliferation, and its overexpression confers tamoxifen resistance (Nair *et al*., 2010). Recently it has been reported that the BET (bromodomain and extraterminal domain) protein BRD3/4 plays a key role in tamoxifen resistance by recruiting WHSC1 (Wolf-Hirschhorn syndrome candidate 1), a histone H3K36 methytransferase, to the *ESR1* gene and positively regulating its expression (Feng *et al*., 2014). AKT1 is a serine/threonine kinase and it phosphorylates ERα at serine 167. Clinical studies on tamoxifen treated breast cancer patients

revealed tamoxifen resistance was due to phosphorylation of ERα at serine 167 (Kirkegaard *et al*., 2005).

ERα coactivators such as BCAS3 and AIB1/SRC3, known to be overexpressed and amplified in breast cancers, are associated with tamoxifen resistance in breast cancers (Osborne *et al*., 2003; Gururaj *et al*., 2006). Proline, glutamic acid, and leucine-rich protein (*PELP1*) is as a coactivator of ERα and its overexpression confers tamoxifen resistance (Nair *et al*., 2010). PELP1 can act as an adaptor protein between ERα and Src, thereby activating of E2-dependent Src and the ERK/MAPK downstream signaling cascade. Interestingly, this pathway confers tamoxifen resistance in breast cancer cells (Vadlamudi *et al*., 2005). FOXM1 is a ERα target gene and its deregulation is shown to be involved antiestrogen resistance (Millour *et al*., 2010). Nuclear receptor corepressor 1 (NCOR1) is well-defined corepressor of ERα. Decreased NCOR1 protein levels correlate with tamoxifen resistance in mouse models of breast cancer (Lavinsky *et al*., 1998). *BRCA1* is a E2-responsive gene and downregulation of BRCA1 expression leads to tamoxifen resistance due to increased coactivator and decreased corepressor recruitment onto ERα-targeted gene promoters (Wen *et al*., 2009).

Increasing evidence suggest that there are number of ERα splice variants involved in breast cancer development. The ERα variant such as ERα36 initiates its transcription from exon 2 of the ERα gene and lacks both transcriptional activation domains (AF1 and AF2) but retains the DNA binding domain and the ligand binding domain of ERα. ERα36 was shown to be involved in tamoxifen resistance in breast cancer cells (Wang *et al*., 2006). ERα mRNA contains several putative binding sites for miRNA. These miRNA act by decreasing ERα mRNA translation or stability. miRNA-221/222 negatively regulates ERα and confers fulvestrant and tamoxifen resistance in breast cancers (Zhao *et al*., 2008; Rao *et al*., 2011). Posttranslational modifications such as sumoylation, ubiquitination, phosphorylation and

methylation regulate ERα activity and are also shown to have potential implications in drug resistance and breast cancer development (Le Romancer *et al*., 2011).

2.1.8 Peptide drugs: The conventional chemotherapy to target cancers exclusively relies upon either inducing cell death by apoptosis, necrosis or keeps the proliferation of tumor in check. But very few of these drugs come without side effects. Majority of the compounds used in chemotherapy lacks specific targets and therefore lead to cytotoxicity in noncancerous cells. This non-specific targeting of normal cells is called systemic toxicity which causes severe undesirable side effects. Peptide drugs have been evolved recently as future therapeutic agents in the treatment of cancer.

"Molecularly targeted cancer therapies" using peptides, proteins and related biomolecules are gaining momentum due to the possibility of enhanced drug efficacy with minimal side effects. Discovery of several protein/peptide receptors, tumor-related peptide molecules and proteins is expected to induce a new wave of more efficient and selective anti-malignant drugs in the future, capturing the prospects of large share in the cancer therapeutic market. The “defensive” treatment option against cancer include the use of proteins, peptides and monoclonal antibodies (Thundimadathil, 2012). The advantage of peptide drugs over chemical compounds are many, such as small size, ease of modification and synthesis, tumor penetrating ability, and good biocompatibility (Borghouts *et al*., 2005).

Through combined efforts of biochemists, biophysicists and with the development of robust bioinformatics tools, many peptides with anti tumor activity have been designed and validated. The number of peptide drugs being engineered and reaching the clinical trial phase has been increasing steadily over the years from 1.2 peptide drugs per year in 1970’s to 16.8 per year in 2000’s (Borghouts *et al*., 2005). Out of the four peptide drugs that have proven successful in different clinical trial phases three peptides are used to directly target cancerous

cells or to treat different episodes of cancer. These three peptide drugs hold a major share of cancer therapeutics with sales recorded over and above 1$ billion per year (Borghouts *et al*., 2005). Unlike the conventional drugs that are mainly used to induce apoptotic pathways in cancer cells, peptide drugs can be strategically used in different modes such as carriers of cytotoxic molecules, radioactive nucleides, mimetics of hormones, vaccines and also using them directly as drugs with high specificity to target cells (Borghouts *et al*., 2005; Thundimadathil, 2012).

The strategy employed by Schally *et al* in treatment of prostate cancer using peptide drugs could be referred to as one classical example of versatile ways these drugs could be used to treat cancer. Schally and his associates developed oligopeptides that when administered caused the down regulation of LHRH receptors in the anterior pituitary, consequential to which the release of LH and FSH from this endocrine gland was inhibited. This inhibition resulted in decreased production of testosterone from Leydig cells owing to loss of stimulatory impulses by LH and FSH and finally resulting in circumventing the growth of prostate cancer. Studies implicated that administration of these peptides lowers the activity of LHRH receptors in the pituitary gland leading to inhibition of LH and FSH release, and with a concomitant decrease in the production of testosterone from Leydig cells (Schally *et al*., 2000). Goserelin, Histrelin, Leuprolide, Triptorelin and Cetrorelix are LHRH agonists and antagonists currently available in the market for breast cancer and prostate cancer treatment (Thundimadathil, 2012).

Blocking ERα functioning is the major treatment modality in luminal breast cancer. The main drawback with the conventional chemotherapy is the lack of proper delivery methods and also to deliver the correct amount of drug directly to malignant cells without affecting surrounding normal cells. Drug resistance, altered biodistribution of drugs, biotransformation, pharmacokinetics and drug clearance are also common problems. Many coactivators interact

with ERα and other nuclear receptors in a hormone-dependent manner via small hydrophobic and amphiphatic a helical peptide sequences with the common signature motif, LXXLL (L = leucine, and X = any amino acid) (Akram *et al*., 2014). Coactivators contain nuclear receptor (NR) boxes within their central nuclear receptor interaction domain. These characteristic features have raised the idea that the ERα coactivator interaction is a specific amendable interface for LXXLL motif like inhibitors, preventing transactivation by ERα. Inhibitors of coactivator binding (ICBs) may only bind to hormone occupied ERα, thus featuring a different mechanism than the classic anti-estrogen drugs. These ICBs might thus be useful in anti-estrogen therapy for patients with tamoxifen resistant breast cancers or could provide a possibility to target orphan nuclear receptors for which antagonists are difficult to identify (Thundimadathil, 2012)**.**

Recently Carraz *et al* shown the physical inhibition of the ERα coactivator interaction by the nona-arginine tag LXXLL-motif is shown to inhibition of ERα mediated gene transcription. In their experiments, peptides that featured both a nona-arginine tag and an LXXLL-motif showed higher toxicity to the cells than either the nona-arginine tag alone or the peptide featuring an arginine tag but with the LXXLL motif mutated to an AXXAA motif. The probe feature a oligopeptide bearing charge such as nona-arginine tag that facilitates cellular entry owing to its positive charge and induces probe localization in nucleoli. The nucleolar localization of the probe provides an excellent tool for evaluating the interaction of LXXLL motif with ERα. These probes compete with coactivators for specific binding sites on ERα, and recruit it into the nucleoli (Carraz *et al*., 2009). Similarly, singh *et al* 2006; also developed a LXXLL based peptide to which could bind to ERα and inhibit ER-dependent proliferation and cell growth.

2.2 RATIONAL AND HYPOTHESIS: After conforming the interaction between ELF3 and ERα, and the significance of this interaction in inhibiting cell proliferation and ERα transactivation functions, we planned to design and engineer a biopeptide bearing resemblance to ETS domain of ELF3 that can interact with ERα and inhibit its activity similar to its full length counterpart. Unlike to normal cells, cancer cells rely upon signaling pathways that are either unique to them or pathways that are aberrantly regulated in them for their survival. Conventional and economically feasible treatment methods like radiotherapy and chemotherapy exploit this particular property of cancer cells but with limited success because of lack of specificity in radiotherapy. But alternatively using chemotherapeutic drugs like tamoxifen, acquired drug resistance during the course of treatment will dampen the success of the chemotherapy. However versatile strategies employed using biopeptides over rides these hindrances and can prove to be a fascinating tool in the hands of biochemists to explore the new avenues in the treatment of cancer.

Careful examination of amino acid sequence and secondary structure of ETS domain of ELF3 revealed one such potential site that has capability to form favourable secondary structures necessary to interact with ERα inside the cells and the same was cross verified using entropy data gathered using HEX docking software. With this information, we made an attempt to chemically synthesize a biologically active peptide that can easily penetrate the cell membrane and specifically interfere in ERα functions.

2.3 MATERIALS AND METHODS:

2.3.1 Liposomal encapsulation of the peptide: Liposomal formulation of wild and mutant type peptides was prepared as follows. 10 ml of t-butanol was added to 10 mg wild or mutant type peptide, dimyristoyl phosphatidyl choline (Sigma, USA) was added to t-butanol (125 mg of dimyristoylphosphatidylcholine:25 ml of t-butanol). After lipid was dissolved, Tween-20 (187 µl) was added to the lipid solution. The peptide and lipid solutions combined and mixed.

After freezing in liquid nitrogen the solutions were lyophilized for 24 hr. The resulting white powder was dissolved by addition of PBS. This process was repeated in a separate sample with addition of peptide and was considered as control liposome.

2.3.2 GTS pull-down assay for peptide competitive binding studies: Equal aliquots of *in vitro* translated [^{35}S]methionine-labeled full-length ELF3 was incubated with GST or GST-ERα-C fusion proteins in presence of either wild type (wERIPE) or mutant (mERIPE) peptides at different concentrations ranging from 10 nM to 1 µM. The reaction mixture was diluted to 0.5 ml with GST binding buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% Nonidet P-40, 10% glycerol) and incubated for 2 hr 4°C on a rotator. Bound proteins were collected on glutathione-beads after washing with GST binding buffer to remove nonspecific binding of proteins. Later bound proteins were eluted with 2X SDS buffer and finally separated on SDS-PAGE. After transferring the proteins onto nitrocellulose membrane, bound proteins ([^{35}S]-ELF3) were detected by autoradiography (Thyphoon scanner, USA).

2.3.3 Soft agar colony-formation assay: *In vitro* tumor formation of MCF7 cells in presence of ELF3 peptide was determined by anchorage-independent growth assay. Cells were treated with control, wild type or mutant peptides with 2 µg/ml concentration for 48 hr. Cells were harvested by trypsinization, and 10000 cells were mixed in 0.3% (0.3% agarose in DMEM with 10% FBS) in top agarose and plated onto 0.5 % base agarose (0.5% agarose in DMEM with 10% FBS) in 35 mm plates as a duplicates. Cells in the agarose were fed twice weekly with DMEM with 10% FBS and plates were incubated at 37°C in a 5% CO_2 atmosphere for 21 days. Colonies visible in the top agar were counted and images were captured by bright field microscope (Model IX81, Olympus, Singapore).

2.3.4 Flow cytometry (FACS) analysis: Approximately $2x10^5$ cells were treated with control, wild or mutant peptides (2 µg/ml) for 18 hours. After incubation, cells were

harvested and washed with PBS. Cells were then fixed in 1 ml of ice cold 70% ethanol for overnight at -20^{o}C. Following ethanol fixation, cells were washed with 1XPBS and treated with DNAse-free RNAse A (1 mg/ml) and propidium iodide (PI) (50 µg/ml), incubated at room temperature for 30 min, and then analysed on a FACS Aria (BD Biosciences, USA).

2.3.5 Cytotoxicity assay: To determine the cytotoxic effect of ELF3-derived peptides (wild type and mutant peptides), cells were seeded into triplicates at a starting density of 5000 cells per well in a 96-well plate. After treating the cells with control, wild type or mutant peptides with different concentrations from the range of 0.25 µg/ml to 2 µg/ml for 48 hours at 37^{o}C, MTT assay was performed as described previously and then, IC50 values were calculated using sigma plot software.11.

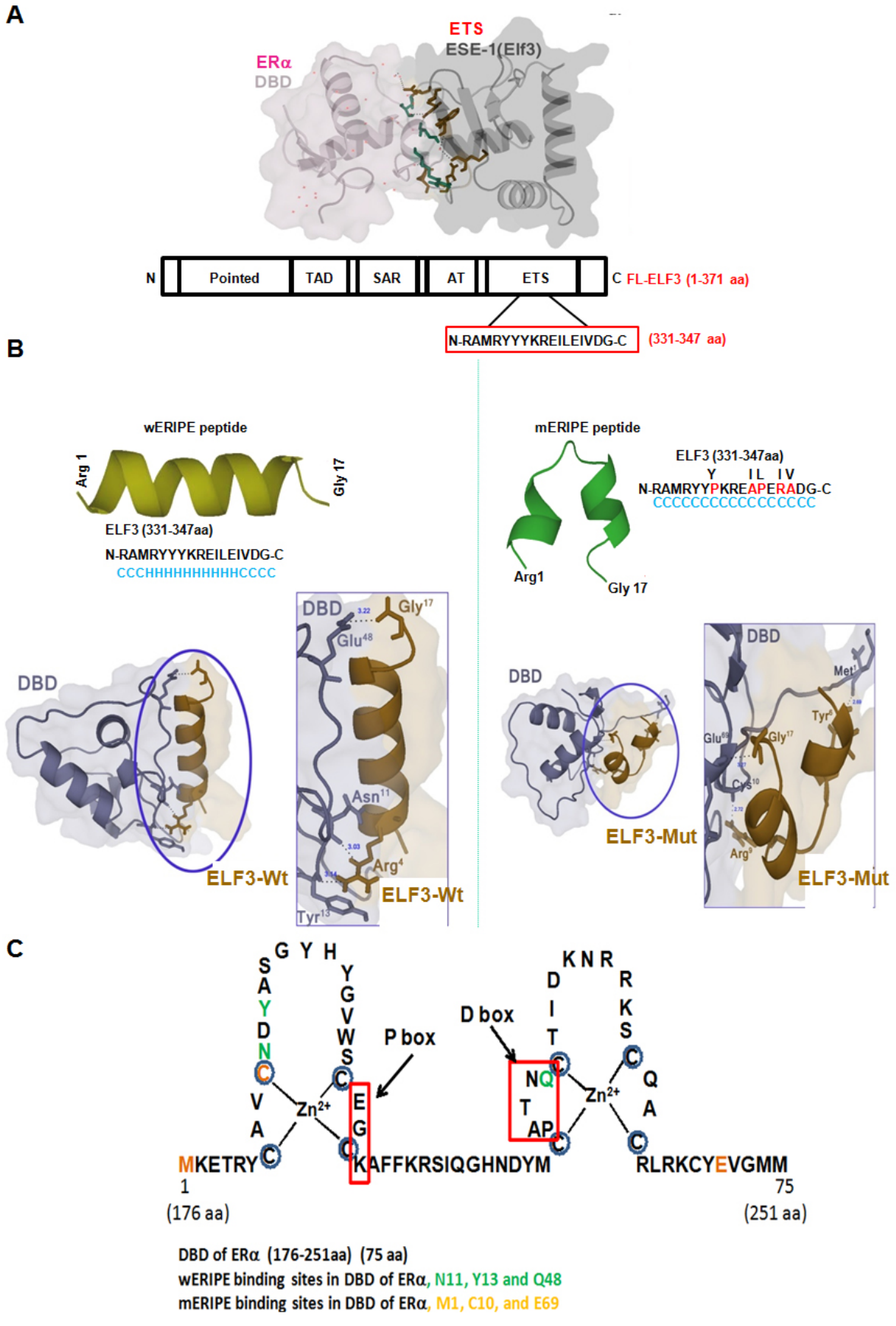
2.3.6 *In silico* analysis of protein domain interactions: A *de novo* approach to modelling from small number of amino acids was used for modelling of ELF3 peptides. Modelling is based on structural alphabet (SA) letters to describe the conformations of four consecutive residues to a greedy algorithm and a coarse-grained force field in PEP-FOLD. Best 3D models with an averaged RMSD of 2.1A starting from a single amino acid sequence were obtained. PEP-FOLD runs up to 200 simulations and the best conformation of the 5 models are developed. The crystal structure of ERα (DBD) (PDB ID 1hcq) and ETS domain of ELF3 (PDB ID 3jtg) were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) PDB for *in silico* analyses. To find out the amino acid residues involved in the binding between DBD of ERα with ETS domain of ELF3, we developed 3D structures of wERIPE (wild type ELF3 peptide) and mERIPE (mutant ELF3 peptide), and performed the protein-protein docking in HEX (http://hexserver.loria.fr/).

2.3.7 Statistical analysis: For reproducibility, all the experiments were performed 2-3 times. The results are expressed as means ± standard deviation, and differences between

groups were analyzed by one-way ANOVA using Prism software (Graph pad 5.0). P value <0.01 is considered as significant.

2.4 RESULTS:

2.4.1 ELF3 peptides inhibits DNA binding activity of ERα: Having established the functional interaction between ELF3 and ERα, we next explored to map the minimal region of ELF3 protein that participates in interaction with DNA binding domain (DBD) of ERα and to further develop it as therapeutic agent to treat ER-positive breast cancers. Our initial analysis indicated that a highly conserved region between 331 and 347aa (aa, amino acid) in ELF3 is involved in interaction with DBD of ERα (**Figure 2.1A**). Protein secondary structure analysis predicted a helical structure for this 17aa peptide named 'wERIPE' (wild type ERα-interacting peptide of ELF3). Molecular docking studies further indicated that Arg 334 and Gly 347 (corresponds to Arg4 and Gly 17 of wERIPE) of ETS domain of ELF3 were involved in hydrogen bonding with Asn 217 and Tyr 219 (corresponds to Asn 11 and Tyr 13 of DBD domain of ERα as mentioned in **Table 2.1**); and Gln 226 was located in 'D box' region (corresponds to Gln 48 of DBD domain of ERα) in the DBD of ERα, respectively (Figure 2.1B, left panel). Residues present in 'D box' have been shown to involve in receptor dimerization (Schwabe *et al*., 1990; Ruff *et al*., 2000). Replacement of the amino acids, Tyr 7, Ile 11, Leu 12, Ile 14 and Val 15 with Pro, Ala, Pro, Arg and Ala, respectively, in this 17 aa peptide of ETS domain, resulted in loss of peptide helical structure and also its interaction with critical residues of D box domain in the DBD of ERα (**Figure 2.1B**, right panel) (**Figure 2.1C**) and considered it a mutant peptide named mERIPE (mutant ERα-interacting peptide of ELF3).


A
ETS
ESE-1(Elf3)
ERα
DBD
N
Pointed
TAD
SAR
AT
ETS
C FL-ELF3 (1-371 aa)
N-RAMRYYYKREILEIVDG-C
(331-347 aa)
B
wERIPE peptide
Arg 1
Gly 17
ELF3 (331-347aa)
N-RAMRYYYKREILEIVDG-C
CCCHHHHHHHHHHCCCC
mERIPE peptide
ELF3 (331-347aa)
Y I L I V
N-RAMRYYPKREAPERADG-C
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Arg1
Gly 17
DBD
ELF3-Wt
Gly17
Glu48
Asn11
Arg4
Tyr13
ELF3-Wt
DBD
ELF3-Mut
DBD
Met1
Tyr2
Glu69
Gly17
Cys10
Arg3
ELF3-Mut
C
G Y H
S A Y D N C V A
Y G V W S C
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Zn2+
E G
C
K A F F K R S I Q G H N D Y M
MKETRY
1
(176 aa)
D box
K N R
D I T C
N Q T A P C
R K S C
Q A C
Zn2+
RLRKCYEVGMM
75
(251 aa)
DBD of ERα (176-251aa) (75 aa)
wERIPE binding sites in DBD of ERα, N11, Y13 and Q48
mERIPE binding sites in DBD of ERα, M1, C10, and E69

Figure 2.1: *In silico* analysis of interaction between ERα and ELF3-derived peptides. A) Protein sequence spanning 331-348 region of ETS domain in ELF3. B) Binding mode of DBD of ERα with either ESE-wt (wild type ELF3 peptide) (top panel) or ELF3-mt (mutant ELF3 peptide) (lower panel). 3D structures of wt-peptide (mercury color) and mt-peptides (green) are shown. Zoom view showing amino acid interactions between DBD (light blue sticks) and ESE-wt (brown) or ELF3-mt (brown). C, coil; H, helix. C) Physical map of DNA binding domain (DBD) of ERα displaying the zinc finger along with P and D boxes. wERIPE or mERIPE binding sites (amino acids) in DBD of ERα are indicated. wERIPE, but not mERIPE, binds to D box region of ERα-DBD.

S. No	Name of the protein domain	Name of the ELF3 peptide	Binding energy in K.jouls/Mole	No. of Hydrogen bonds	Amino acids involved from the respective domains	
					ERα-DBD	ERIPE
1	ERα- DBD	wERIPE	-4.085726e+02	3	Asn11,Tyr13,Gln48	Arg4,Gly17
2	ERα- DBD	mERIPE	-4.033301e+02	3	Met1,Cys10,Glu69	Tyr6,Arg9,Gly17

Table 2.1: Docking and binding energies of ERα-DBD with either wERIPE or mERIPE.

Because ELF3 is interacting with ERα through the ETS domain, we assumed that 17aa peptide could compete with ELF3 to bind to ERα. To test this hypothesis, GST pull-down assay was performed by incubating [^{35}S]ELF3, GST-ERα-C and increasing concentrations of either wt- or mt-ELF3 peptides. As shown in **Figure 2.2A**, wt-peptide (wERIPE) could inhibit the interaction between ELF3 and ERα effectively at 1 μM concentration, whereas the mutant peptide (mERIPE) failed to do so. Next we examined the effects of wERIPE and mERIPE peptides on the DNA binding activity of ERα by electrophoretic mobility assay (EMSA). wERIPE indeed displayed more inhibitory activity towards ERα's DNA binding than mERIPE (**Figure 2.2B**). Together, these results indicate that wERIPE could inhibit the DNA binding activity of ERα and potential to suppress ERα functions.

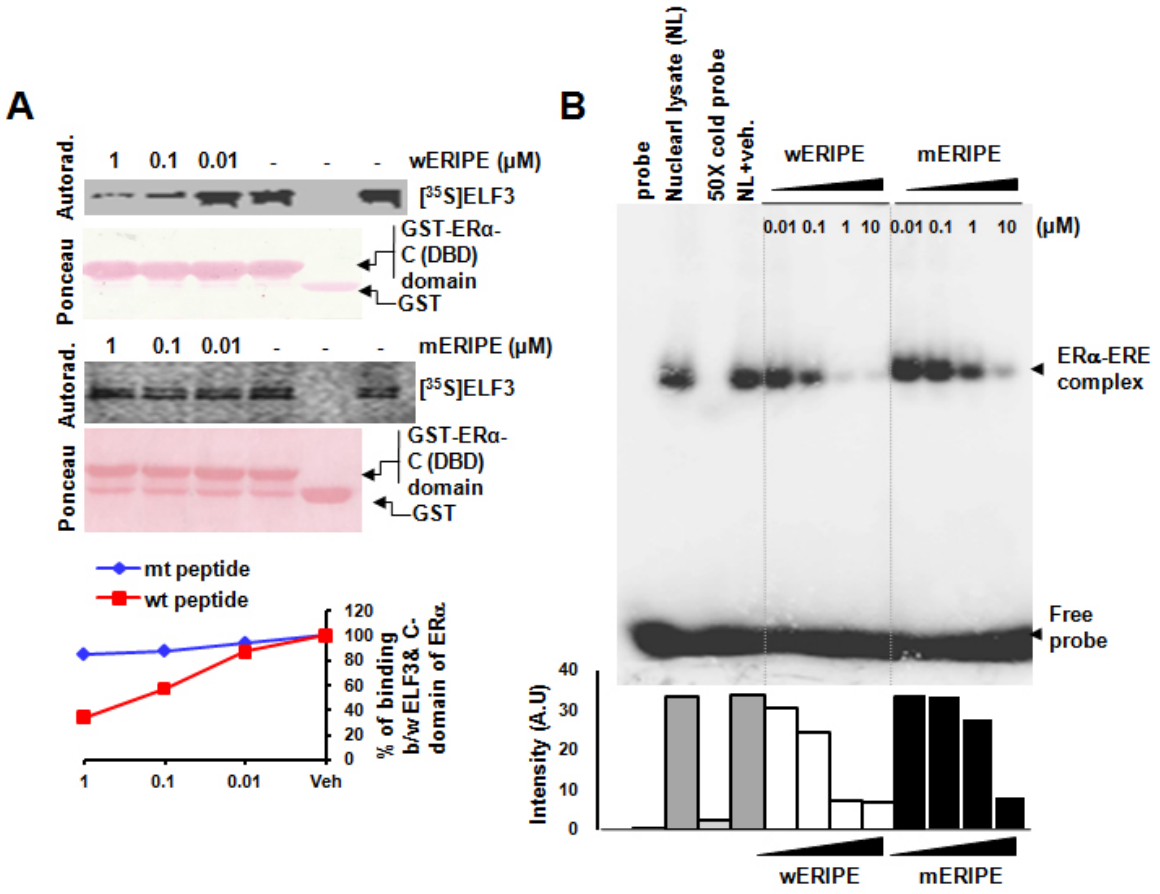


Figure 2.2: *In vitro* analysis of interaction between ERα and ELF3-derived peptides. A) GST pull-down assay showing the effect of wERIPE(top) or mERIPE(lower panel) on the interaction between DBD-ERα and [S^{35}]ELF3. B) Electrophoretic mobility shift assay (EMSA) demonstrating the inhibition of ERE binding capacity of ERα by wERIPE or mERIPE. Increasing concentrations of wERIPE or mERIPE were incubated with ERE oligos along with nuclear lysate of MCF7 and DNA-protein complexes were run on 4% polyacrylamide gel followed by autoradiography.

2.4.2 Effect of ERα interacting peptides of ELF3 (ERIPEs) on breast cancer cell growth: As *in vitro* studies indicated the inhibitory effect of ERIPEs towards ERα DNA binding activity, we next explored the effect of ELF3 peptides on breast cancer cell growth. First we examined the cellular uptake of wERIPE and mERIPE (biotin-labeled) in MCF7 cells by immmunofluorescence studies. As shown in **Figure 2.3A**, both wERIPE and mERIPE were localized to both cytoplasm and nuclear compartments. We next examined the

cytotoxic effect of these peptides on ER-positive (MCF7 and ZR-75) and ER-negative (MDA-MB231) breast cancer cells. wERIPE showed ~5 fold more cytotoxic effect than mERIPE (IC_{50} values: 0.15 µg/ml, wERIPE vs. 0.75 µg/ml, mERIPE) in MCF7 cells **(Table 2.2)**. Similar observations were made in ZR-75-1 cells. However, no change in the IC_{50} values for wERIPE and mERIPE in MDA-MB231 cells were observed. To verify whether the cytotoxic effect of ELF3-derived peptides was due to arrest of cells at sub-G1 stage (apoptotic), we next treated the MCF7 cells with either wERIPE or mERIPE for 24 hrs, and the cell cycle stages were analyzed by FACS assay. As shown in **Figure 2.3B**, wERIPE-treated cells showed more apoptosis (~40%, sub-G1 cells) than in either control (5%) or mERIPE-treated cells (15%). Furthermore, wERIPE treated MCF7 cells showed increased PARP proteolysis (**Figure 2.3C**, lane 3) as compared to either vehicle or mERIPE (lanes 1-2) treated cells **(Figure 2.3C)**. In support of these findings, we next analyzed the effect of ELF3-derived peptides on anchorage independent growth ability of MCF7 cells. wERIPE could significantly reduced the colony forming ability of MCF7 cells than either control or mERIPE **(Figure 2.3D)**. Together these results indicate that wERIPE could inhibit breast cancer cell growth partly by inducing cellular apoptosis.

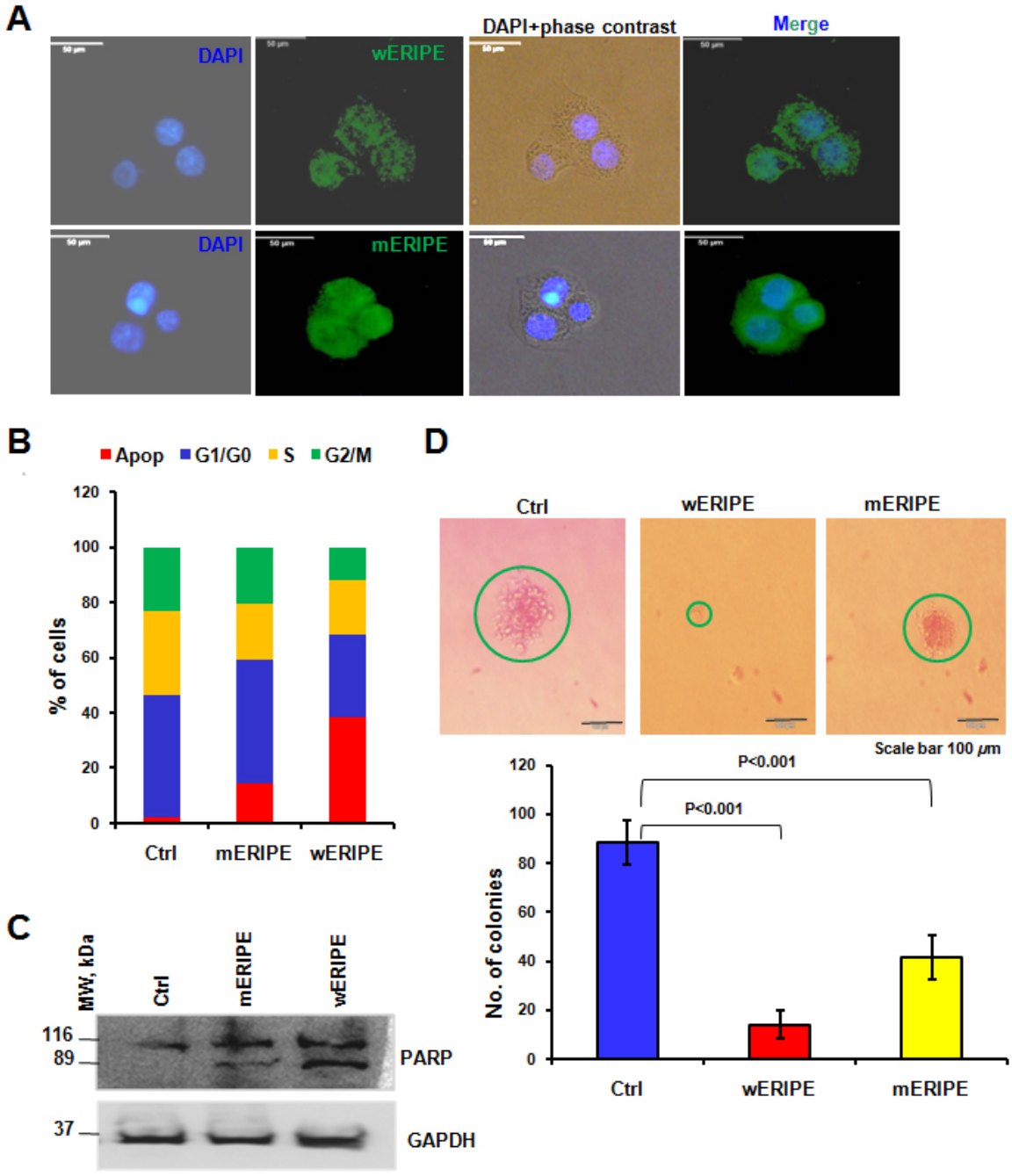


Figure 2.3: Effect of ERα interacting peptides of ELF3 on breast cancer cell growth. A) Immunofluorescence analysis showing cellular distribution of wERIPE and mERIPE in MCF7 cells. B) FACS analysis showing the effect of wERIPE or mERIPE on different stages of cell cycle in MCF7 cells. subG1 or Apop-apoptotic cells. C) Westrenblot analysis showing the effect of wERIPE or mERIPE on levels of cleaved PARP in MCF7 cells. D) *In vitro* anchorage independent cell growth assay demonstrating the effect of wERIPE or mERIPE on the anchorage independent growth ability of MCF7 cells. Representative images of MCF7 colonies formed after

treatment with either control or wt or mt peptides (top panel). Colonies formed after 21 days of treatment in presence or absence of control or wt- or mt-peptides were plotted in a bar graph (lower panel).

S.No	Peptide	Cells		IC_{50} µg/ml	Fold reduction of IC_{50}
1	Control	MCF7	ER (+)	1.09	
2	wERIPE-peptide	MCF7	ER (+)	0.15	7.3
3	mERIPE-peptide	MCF7	ER (+)	0.72	1.5
4	Control	ZR75	ER (+)	0.77	
5	wERIPE-peptide	ZR75	ER (+)	0.35	2.2
6	wERIPE-peptide	ZR75	ER (+)	0.57	1.35
7	Control	MDA-MB231	ER (-)	0.99	
8	wERIPE-peptide	MDA-MB231	ER (-)	1.00	1
9	wERIPE-peptide	MDA-MB231	ER (-)	0.99	1

Table 2.2: Half maximal inhibitory concentration (**IC_{50}**) values of MCF7, ZR75 and MDA-MB-231 cells treated with wERIPE or mERIPE.

2.5 DISCUSSION: Approximately 70% of the breast cancers are ER-positive (Perou *et al*., 2000; Creighton *et al*., 2006; Sotiriou and Pusztai, 2009). Therefore, selective estrogen receptor modulators (SERMs) have been one of the important therapeutic options for treating estrogen receptor-dependent breast cancers (Viedma-Rodriguez *et al*., 2014). One such drug is tamoxifen, which inhibits ERα functions by blocking ligand binding and suppresses ER-dependent transcription and, therefore it is being used widely to treat breast cancers. However, longer treatments with tamoxifen resulted in the development of drug resistance and posed increasing risk of endometrial cancers (Osborne, 1998; Sengupta and Jordan, 2008). In view of these clinical associated problems, developing alternative approaches to treat breast cancer has become a challenging task. Exploring the domain mapping studies, we identified a 17 aa region of ELF3 called ERIPE that could inhibit ERα DNA binding activity. Further, liposome encapsulated ERIPE could be transported into breast cancer cells tested and exhibited cytotoxic effect in MCF7 cells, an ER-positive breast cancer cells, but not in MDA-MB231 cells, an ER-negative cell line. Consistent with the breast cancer cell growth, wt-peptide could decrease the anchorage independent growth capacity of breast cancer cells

as compare to mt-peptide. It is possible that wt-peptide may inhibit ER-recruitment to its target genes, so the target gene transcription required for breast cancer cell growth. In this context, we discovered a minimal region of ELF3 protein that could inhibit ERα-dependent breast cancer cell growth and may be useful in breast cancer therapy.

Previously, the interaction between Her-2 and ELF3 was explored to design a peptide drug to treat Her-2 positive breast cancers. Asada *et al* showed that the expression of the *Her2* gene can be decreased by inhibiting the interaction of the two cancer-linked proteins, DRIP130/CRSP130/Sur-2 (a Ras linked subunit of human mediator complexes) and ELF3. Disruption of the interaction between these proteins by a short cell-permeable TAT-ELF3$_{129–145}$ peptide reduced the expression of the *Her2* gene and specifically impaired the growth and viability of Her2 overexpressing breast cancer cells. Although TAT-ELF3$_{129–145}$ specifically killed Her2-positive cells by inhibiting Her2 expression, the peptide is not practical to use clinically because of its rapid degradation and poor cellular uptake (Asada *et al*., 2002).

Similarly, the interaction between metastasis tumor-associated 1 short form (MTA1s) and ERα was explored to target ER-positive breast cancers. Singh *et al* used a C-terminal 33-amino acid region containing a nuclear receptor (NR)-box motif (-LRILL-), which mediates binding of MTA1s with ERα, to target this interaction. This 33 amino acid MTA1s peptide has ability to compete with the coactivator recruitment to ERα and effectively repressed ERα transactivation functions. In addition, the MTA1s peptide also blocked the progression of tumors formed by MCF-7 cells in a xenograft-based assay (Singh *et al*., 2006). However the cell type specificity was not determined in this study.

Beltran *et al* identified that Engrailed 1 (EN1), a neural-specific transcription factor, is exclusively overexpressed in Basal-type breast tumors. Overexpression of EN1 transcription factor in these cells mediates prosurvival pathways. Beltran *et al* engineered synthetic interference peptides (iPeps) to block EN1 comprising of EN1-specific sequences that are

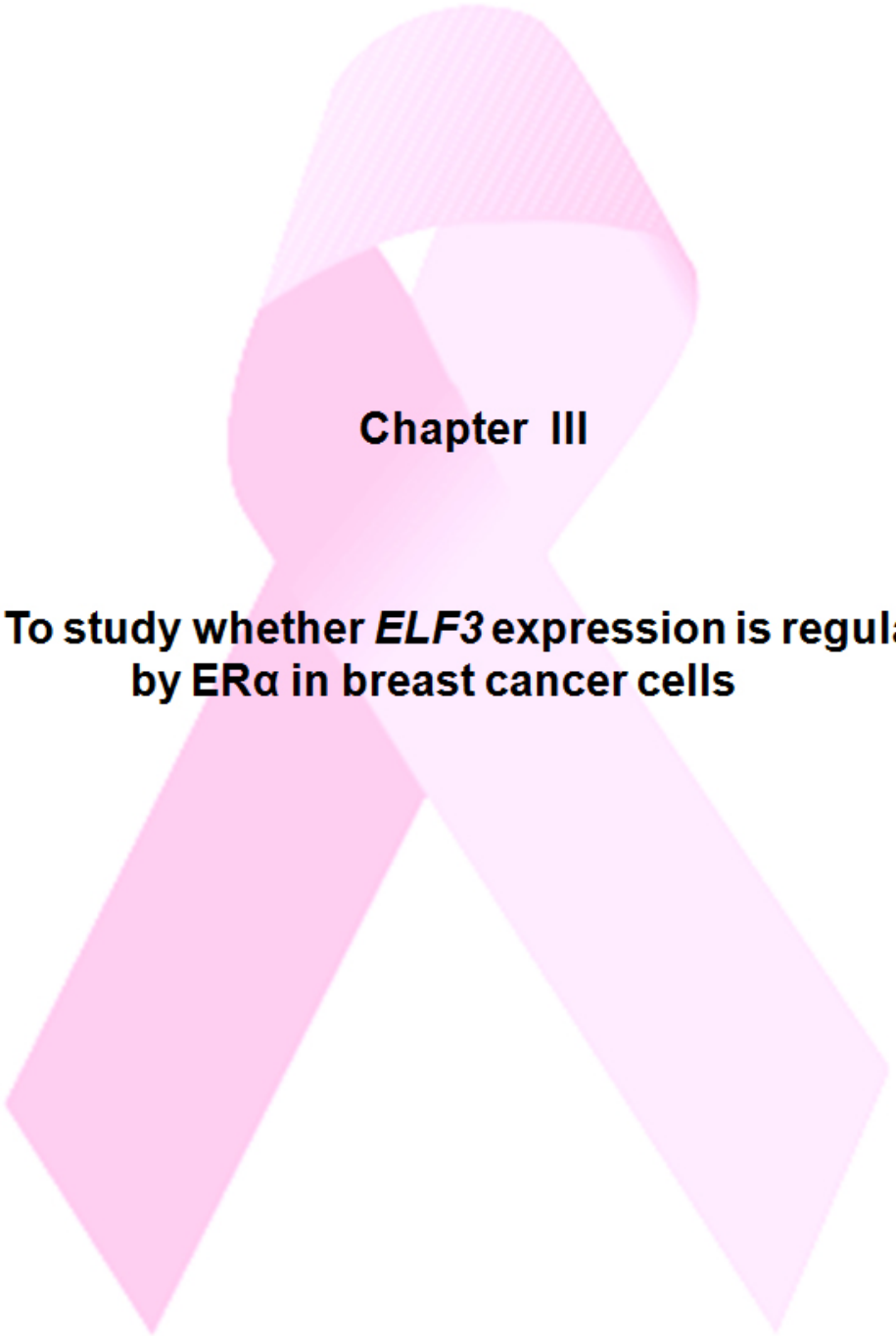
remarkable in mediating essential protein-protein interactions necessary for functioning of EN1 protein. This group also designed an N-terminal cell-penetrating peptide/nuclear localization sequence. The EN1-iPeps thus developed rapidly mediated a strong apoptotic response in tumor cells overexpressing EN1, with negligible cytotoxicity to normal or non EN1-expressing cells, when it administered.

Unlike to the TAT-ELF3$_{129-145}$ peptide and MTA1s peptide, but similar to EN1-iPeps, ELF3-derived peptide i.e., wERIPE displayed good cytotoxic effect and cellular uptake. Furthermore, wERIPE exhibited cytotoxic specificity towards ER-positive breast cancer cells but not ER negative cells.

2.6 CONCLUSION: We designed and functionally characterized a peptide called ERIPE (ER interacting peptide of ELF3) bearing similarity to ETS domain of ELF3 as described. The design of ERIPE was based upon on well-established HEX molecular docking platforms and secondary structure analysis modules. Different properties like selective affinity towards C-domain of ERα, favoured secondary structure, biological stability were analysed using bioinformatics tools. Also, a mutant ERIPE lacking necessary amino acid sequence required to interact with ERα was used as control in all our studies.

Specifically, wERIPE was shown to readily interact with ERα when compared to mERIPE where no interaction was observed. Also, liposomes loaded with ERIPE showed decrease in ERα DNA binding activity accompanied by decreased cell proliferation, reduced tumor forming ability and increased apoptosis. Interestingly all these effects of ERIPE were very specific and observed only in ERα positive breast cancer cells and not in ERα negative breast cancer cells indicating high affinity and specificity of ERIPE to its target.

These results together establish that ERIPE may prove to be an effective alternative therapeutic in treatment of ER positive breast cancers, as ERIPE has negligible nonspecific targets and cytotoxicity.



Chapter III

To study whether *ELF3* expression is regulated by ERα in breast cancer cells

3.1 INTRODUCTION

3.1.1 Estrogen signaling: Estrogen actions are mediated mainly through the classical estrogen receptors (ERα and ERβ), which are members of the nuclear receptor superfamily. They share similar domain organization and have overlapping but nonidentical tissue distributions. These estrogen receptors are encoded by different genes and have distinct expression patterns that depend on tissue type. ERα is predominately expressed in the uterus, kidney, liver and heart whereas ERβ is primarily found in the ovary, lung, bladder, prostate, gastrointestinal tract, and nervous system. However, ERα and ERβ are co-expressed in the mammary gland, thyroid gland, bone, and adrenal glands (Marino *et al*., 2005). Moreover, estrogen-regulated genes, such as *cyclin D1(CCND1), PR, cathepsin D (CTSD), GATA-3*, and *c-myc* are important for cell survival and proliferation (Nagai and Brentani, 2008).

ERα genomic signaling: In the absence of estrogen, ERα is sequestered in complex with an inhibitory heat shock protein. Upon ligand binding, the receptor dissociates from the heat shock protein complex and undergoes dimerization (Klein-Hitpass *et al*., 1986). The interaction of ERα with target gene promoters can occur either directly, through specific estrogen response elements (ERE), or indirectly through contacts with other DNA-bound transcription factors such as specificity protein 1 (SP1), activation protein1 (AP-1), or nuclear factor κ-light-chain-enhancer of activated B cells (NF-kB). Upon ligand binding to LBD of ERα, receptor dimers and translocate into nucleus, where it recruits co-activators or co-repressors, as well as chromatin-remodelling factors, to estrogen response elements (EREs), which is the 15-bp (AGGTCAnnnTGACCT) (n=any nucleotide) palindromic inverted repeat (IR) separated by any three nucleotides sequence on target gene promoters (Mason *et al*., 2010). Once this complex tethered to DNA, the receptor can either positively or negatively regulate target gene transcription (O'Lone *et al*., 2004). ERα regulates many genes that are involved in mammary gland development, and their deregulated expression is associated with

breast cancer progression. In general genomic actions induce biological responses more slowly than the nongenomic actions (Welboren *et al*., 2009).

3.1.2 Estrogen genomic signaling targets: With successful completion of the human genome project and introduction of novel technologies, a plethora of novel targets of ERα have been identified. Using chromosomal walking, Brown and colleagues (Carroll *et al*., 2005) revealed that only a minor fraction of ERα binding sites are located in promoter regions, whereas a vast majority is located at long distances from target genes. Similarly, using the circular chromosome conformation capture method, it has been shown that multiple ERα binding sites interact at classical ERα target genes of *pS2/TFF1*, *GREB1*, *carbonic anhydrase 12 (CA12)*, and *B-cell lymphoma 2* via looping to regulate transcription (Deschenes *et al*., 2007; Pan *et al*., 2008). Fullwood *et al.* mapped the chromatin interaction network bound to ERα in the human genome by utilizing chromatin interaction analysis by paired end tag through long-range chromatin interactions like looping (Fullwood *et al*., 2009). Similar three-dimensional chromatin interaction studies in cancer patient samples revealed that the clinical outcome of the breast cancers is decided at the level of chromatin interaction by ERα (Ross-Innes *et al*., 2012). Using various techniques to isolate differentially expressed genes, a number of previously unidentified estradiol-inducible genes have been reported in ER-positive human breast cancer cell lines. Few important genes identified to be induced by estrogen-ERα include *progesterone receptor* (*PR*), *pS2*, *cathepsin D*, *heat shock protein 27* (*HSP27*), *aldolase A*, *dehydrogenase*, *α-tubulin*, *gene regulated by estrogen in breast cancer 1 (GREB1)*, *glyceraldehyde-3-phosphate* (*GAPDH*), *cyclin D1* (*CCND1*), *breast cancer amplified 1 (BRCA1)*, *breast cancer amplified 2 (BRCA2)*, etc (Ghosh *et al*., 2000).

E2 regulates cell proliferation, migration, and differentiation etc, primarily by controlling the expression of its target genes. *Trefoil factor 1* or *pS2* is an ERα regulated gene. Trefoil factors

are a group of small molecular weight polypeptides associated with mucin secretion in mucous secreting epithelial cells. In mammals, there are three kinds of trefoil peptides: breast cancer-associated peptide (pS2 or TFF1), spasmolytic polypeptide (sp or TFF2) and intestinal trefoil factor (ITF or TFF3). The peptides are so named because they are folded into three highly stable loops that are held by pairs of disulphide bridges which make the peptides extremely stable against proteolytic digestion, acid and heat degradation. They are expressed in different gastrointestinal lesions (gastritis, peptic ulcer, inflammatory bowel disease) since they act on mucosal surface protection, epithelial restitution and repair after injury (Abdou *et al*., 2008).

Cathepsin D, also an E2-responsive gene, is a lysosomal aspartyl protease. It is a member of the peptidase A1 family and exhibits some similarity with that of pepsin A. Its catalytic activity depends critically on protonation of its active site Asp residue and gets activated at pH 5 in endosome of hepatocytes where it degrades insulin (Barrett, 1992; Leto *et al*., 1992; Rochefort *et al*., 2000). Cathepsin D is also frequently used as a breast tumor marker.

Gene regulated by estrogen in breast cancer 1 (*GREB1*) is another ERα target gene that mediates E2-induced proliferation in breast cancer cells (Sun *et al*., 2007). It was first identified as an estrogen-regulated gene expressed in breast cancer in a subtractive hybridization screen in MCF-7 cells. The three cDNAs of GREB1 (GREB1a, GREB1b, and GREB1c) have distinct 5′-untranslated regions but share extensive coding sequences, indicating different promoter usage (Ghosh *et al*., 2000).

Among different cyclins, *cyclin D1* (*CCDN1*) is a target of E2-ERα signaling (Sabbah *et al*., 1999). Although c*yclin D1* promoter lacks either ERE or ERE-like elements, E2-ERα regulates cyclin D1 expression by recruiting various transcription factors involving ATF-2 and c-Jun (Herber *et al*., 1994; Sabbah *et al*., 1999). Because cyclin D1 regulates cyclin-dependent kinase (CDK) 4 activity and retinoblastoma protein functionality, which decides

the transcriptional activity of E2F transcription and S phase progression, it is expected that up-regulation of cyclin D1 gene expression in response to E2 promotes G_1 to S transition by activating CDK4 through cyclin D1 induction (Dyson, 1998).

Both *BRCA1* and *BRCA2* are tumor suppressor genes, and loss-of-function mutations in these two proteins are predisposed to breast cancer development because they are key components of the genome maintenance network (Huen *et al*., 2010). Both *BRCA1* and *BRCA2* are E2-responsive genes, and BRCA1 in turn regulates ERα activity through posttranslational mechanisms (Spillman and Bowcock, 1996; Ma *et al*., 2010). For instance, BRCA1/BARD1 complex monoubiquitinate ERα in MCF7 cells, and thus, ubiquitinated ERα becomes transcriptionally inactive (Ma *et al*., 2010).

3.2 RATIONAL AND HYPOTHESIS: Progress made by the recent advancements in gene expression profiling has shown that ERα directly regulates the expression of few of its coregulators by directly binding to ERE elements on their promoters. In our preliminary experiments we identified that ELF3 is found to be expressed only in ERα positive but not in ERα negative cell lines. This led us to examine the ELF3 gene for the possible presence of ERE elements on its promoter. To our surprise, we found eight putative half ERE (1/2 ERE) elements that have the potential to regulate its expression by one of the ERα-dependent mechanisms. In addition, mining the openly available gene expression databases like gene expression omnibus (GEO) revealed that *ELF3* is a response gene. This preliminary evidence postulated us to study the effect of E2 on *ELF3* expression in breast cancer cells and the underlying molecular mechanism.

3.3 MATERIALS AND METHODS:

3.3.1 Cloning of *ELF3* promoter: *ELF3* promoter (3 kb) was amplified by PCR using specific primers as shown in **Table 3.2.** Genomic DNA isolated from MCF-7 cells by

DNAzol method, MCF7 cells ($1X10^7$) were lysed in 1 ml of DNAzol reagent (Life technologies, USA) following manufacturer protocol. The cell lysate was subjected to centrifugation at 1000 rpm for 10 min to clear the cell debris. Genomic DNA was then pelleted using 100% ethanol by centrifugation. After 70% ethanol wash, DNA was dissolved in 8 mM NaOH by slowly passing the pellet through a pipette tip. PCR was carried out using 50 ng of genomic DNA as template and *ELF3* promoter-specific primers to amplify 3 kb *ELF3* promoter. The amplicon was digested with *Xho*I and *Hind*III enzymes (NEB, USA), ligated into pGL3 vector (promoter probe vector) (Promega, USA) and sequence verified.

3.3.2 Chromatin Immunoprecipitation (ChIP) Assay: Approximately $1x10^8$ cells were fixed with formaldehyde at a final concentration of 1% for 20 min at room temperature followed by quenching of cross-links with glycine at a final concentration of 125 mM. Cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitors), then sonication on ice at 40 amplitude of 5 bursts each with 5 min gap. After lysate clarification, extracts were precleared using 50 µl of protein A/G-agarose beads (Santa Cruz Biotechnology, USA) (blocked with 400 µg/ml BSA and 20 µg/ml salmon sperm DNA) for 1 hr at 4°C on a rotator. Equal concentration of DNA was taken for all the samples and diluted to 1 ml with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01% SDS, 165 mM NaCl, protease inhibitors). Protein-DNA complexes were immunoprecipitated using 1 µg of either ERα (Cell Signaling Technology, USA) or IgG (Santa Cruz Biotechnology USA) at 4°C for overnight followed by incubation with 40 µl of protein A/G beads on a rotator at 4°C for 1 hr. The beads were then washed once each with 1 ml of washing buffer I (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01% SDS, 165 mM NaCl, protease inhibitors), wash buffer II (16.7 mM Tris-HCl, pH 8.1, 1% Triton X-100, 1.5 mM EDTA, 0.01% SDS, 500 mM NaCl, protease inhibitors), LiCl/detergent solution (10 mM Tris-Cl, pH 8.1, 1% NP-40, 0.25 M LiCl, 1% sodium

deoxycholate, 1mM EDTA), and finally with TE (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). Beads were then collected by centrifugation at 8000 rpm for 2 min at 4°C. Immunocomplexes were eluted from the beads in 200 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) by rotating at room temperature for 30 min, and repeated once. Total 400 µl of elutes were reverse cross-linked by heating in 5 M NaCl (16 µl/ vail) and 1 µl DNA free RNase A (10 mg/ml) for 6 hr at 65°C. DNA was then purified by phenol:chloroform:isoamylalchohol mixture and subjected to real time quantitative PCR amplification using suitable *ELF3* promoter-specific primers as shown in **Table 3.2.**

3.3.3 Reverse transcriptase Polymerase Chain Reaction (RT-PCR): First strand cDNA synthesis was carried out using 1 µg of RNA by first strand cDNA Synthesis Kit from Takara Bio Inc, Japan which uses MMLV (Moloney Murine Leukemia virus)-derived reverse transcriptase. In the first step, RNA was incubated with 0.5 mM dNTP, 2.5 µM of oligo dT for 5 min at 65^{o}C. The second step involves incubation with 20 units of RNase inhibitor and 2 units of M-MuLV Reverse Transcriptase for 90 minutes at 42^{o}C followed by enzyme inactivation at 95^{o}C for 5 minutes. PCR reactions were setup using 1 µl of the synthesized cDNA using FastStart SYBR Green Master kit from Roche Life Sciences, USA in Roche LightCycler® 96 instrument. The primers list used for amplification is showed in **Table 3. 2**. The expression of *ELF3*, *pS2*, *Cathepsin D* was compared with housekeeping gene, *GAPDH.*

3.3.4 Luciferase reporter assays: For reporter gene transient transfections, MCF7 cells were cultured for 48 hours in IMEM with 2% DCC serum. *ELF3*-promoter cloned into pGL3 basic vector along with internal Renilla-Luc plasmids were transfected using Lipofectamine 2000 reagent. Twenty four hour post transfection, cells were treated with or without E2 (10 nM) for 18 hr. Cells were then lysed in lysis buffer, and the luciferase assay was carried out using dual luciferase reporter assay kit (Promega, USA)

3.3.5 Bioinformatic analysis: *ELF3* 5'-UTR of 3 kb in length upstream to transcriptional start site (TSS) of gene region from 202007562 to 202011137 bp located on chromosome 1q32.1 retrieved from National Center for Biotechnology Information (NCBI) in FASTA format. This 3 kb promoter sequence was submitted to online web based software called TFBIND which provided us with all the binding sites for transcription factors on the promoter. ERα binding sites ½ ERE located on juxtra position to Sp1 and AP-1 sites on *ELF3* promoter were annotated.

3.3.6 Statistical analysis: For reproducibility, all the experiments were performed 2-3 times. The results are expressed as means ± standard deviation, and differences between groups were analyzed by one-way ANOVA using Prism software (Graph pad 5.0). P value <0.01 is considered as significant.

Name	Description	Insert size	Reference
BM 102	pGL3-ELF3 pro-luc	3000 bp	in this study
BM 230	pRL-SV40P-luc	_	(Chen and Prywes, 1999)

Table 3.1: Plasmids used in this study.

Name	Sequence 5'-3'	Target	Application
ELF3 R-1 FP ELF3 R-1 RP	TTGCTGAATCTCTGGAATTTAG TCTGGGGTTGGGCACGGGG	Human *ELF3* promoter (-410 to -196)	ChIP-qPCR
ELF3 R-2 FP ELF3 R-2 RP	ATACTATGCATTCCCTCTTCTTT CTGGACTCCATCCAAGCATCAGG	Human *ELF3* promoter (-903 to -640)	ChIP-qPCR
ELF3 R-3 FP ELF3 R-3 RP	GGGGGTCACACAGATCCTACAT GACTCCTGGGTTCAAGCGATTCTCC	Human *ELF3* promoter (-1864 to -1540)	ChIP-qPCR
ELF3 R-4 FP ELF3 R-4 RP	AAGGGTCAGTGCAAGGAAAGGC AGGTCAGTGCCTTGGAGGCATGG	Human *ELF3* promoter (-2817 to -2453)	ChIP-qPCR
GAPDH FP GAPDH RP	GCCTCTGCGCCCTTGAGCTA GATGCGGCCGTCTCTGGAAC	Human *GAPDH* promoter	ChIP-qPCR
ELF3 FP ELF3 RP	AGAACAGCAACTGACCTACGA CCGATCCTTAATTCCGACTC	Human *ELF3* mRNA	RT-PCR expression
Cathepsin D FP Cathepsin D RP	GTACATGATCCCCTGTGAGAAGGT GGGACAGCTTGTAGCCTTTGC	Human *Cathepsin D* mRNA	RT-PCR expression
pS2 FP pS2 RP	CATCGACGTCCCTCCAGAAGAG CTCTGGGACTAATCACCGTGCTG	Human *pS2* mRNA	RT-PCR expression
GAPDH FP GAPDH RP	GTCCCCTCGAGGAGTTGTGT ATCTTCCATCATCTGAGGGC	Human *GAPDH* mRNA	RT-PCR expression
Promoter *ELF3* FP Promoter *ELF3* RP	ATGACTCGAGTTTGACAGCAAACTG CTTCC AGTCAAGCTTGGCTTTATAGTGTGT CCCCTG	Human *ELF3* promoter (-1 to -3000 bp)	pGL3-ELF3-promoter

Table 3.2: Primer list and sequences. FP, forward primer; RP, reverse primer. Underlined are specific restriction enzyme sites used for cloning of respective genes/gene products.

3.4 RESULTS:

3.4.1 ELF3 is an estrogen-inducible gene: Because ELF3 displayed repressive activity towards ERα-dependent transcription and functions, increasing the expression of endogenous ELF3 may provide an option to treat ER-positive breast cancers. In view of this idea, we analyzed human *ELF3* promoter. Surprisingly, we found eight ½ ERE sites spanning 3 kb promoter region (202007562-202011137 bp) of *ELF3* gene which is located on chromosome 1q32.1 (**Figure 3.1&Table 3.3**).

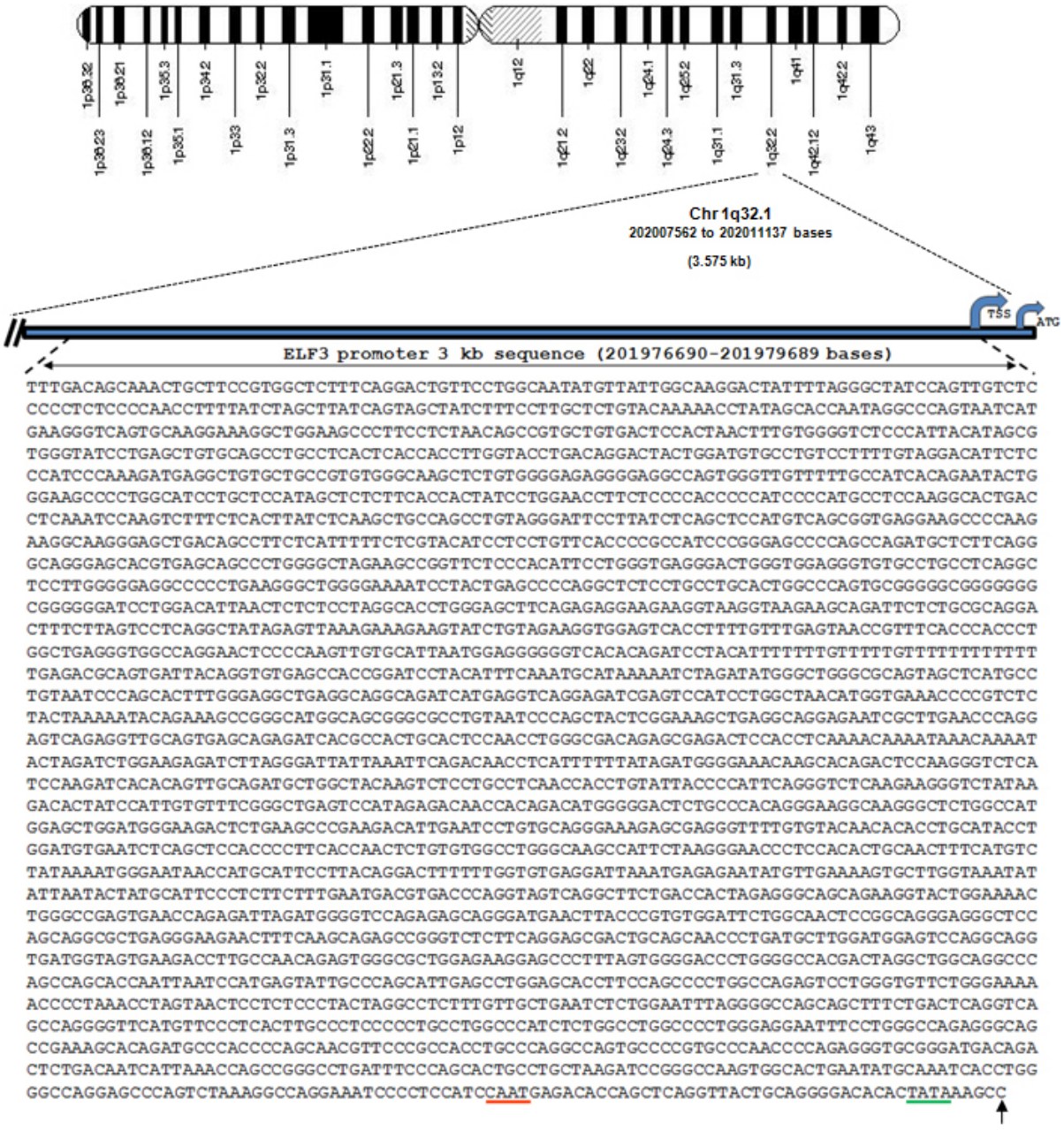


Figure 3.1: Sequence analysis of *ELF3* gene promoter located on chromosome 1q32.1. Arrow denotes TSS, transcription start site; TATA (green) and CAAT (red) are underlined.ATG is start codon.

Upstream to transcription start site	Sequence	Strand
-2729 to -2711 bp	CGTGGGTATCCTGAGCTGT	+
-2277 to -2259 bp	TCAGGGCAGGGAGCACGTG	-
-1864 to -1846 bp	GGGGGTCACACAGATCCTA	-
-1540 to -1522 bp	AGAGGTTGCAGTGAGCAGA	-
-1061 to -1041 bp	CCAACTCTGTGTGGCCTGG	+
-883 to -865 bp	TTTGAATGACGTGACCCAG	+
-369 to -351 bp	TCAGGTCAGCCAGGGGTTC	-
-221 to -203 bp	CCAGTGCCCCGTGCCCAAC	+

Table 3.3: Identification of annotated ER binding sites in *ELF3* promoter region using TFBIND search tool. Predicted ER binding sequences and their position upstream to transcription site on *ELF3* promoter are shown. +, sense and -, denotes anti-sense strand.

It was previously reported that ERα binds to ½ ERE sites (AGGTC or TGACCT) when they are juxtaposed to Sp1, a transcription factor, sites and activates target gene transcription (Klinge, 2001). This observation led us to test whether *ELF3* is an estrogen target gene. Further gene expression Omnibus (GSO) data sets (GDS1326; GDS4061) indicated the induction of *ELF3* in response to E2 in ERα- positive cell line but not in ERα-negative cells (**Figure 3.2A&B**).

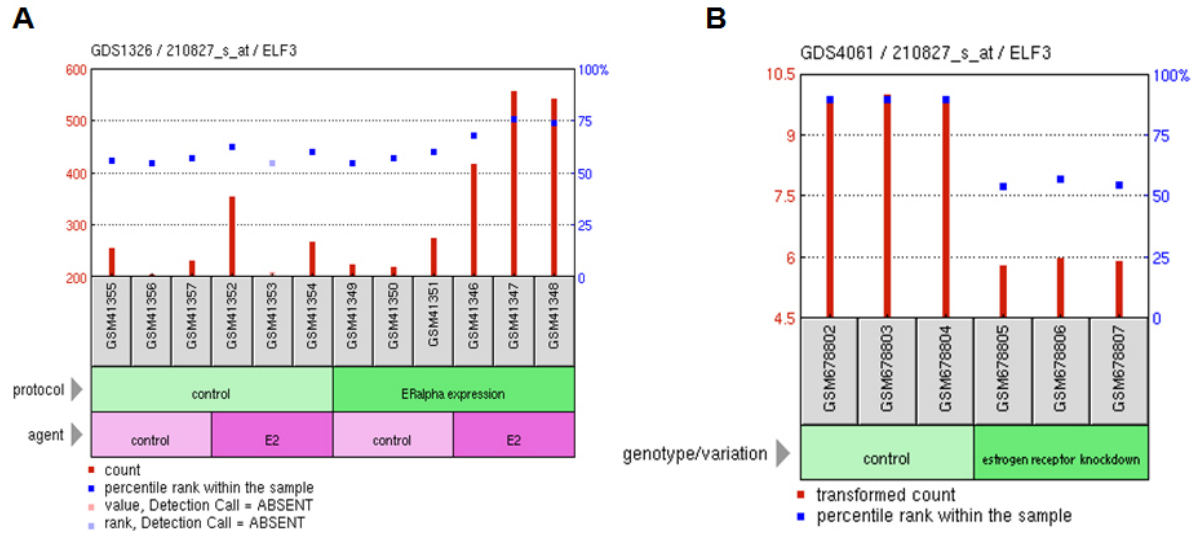


Figure 3.2: A) GEO profile data set (GDS1326). showing the effect of E2 and requirement of ERα on *ELF3*/*ELF3* gene transcription B) GEO profile data set (GDS4061) showing the effect of ERα knock down on ELF3 expression in MCF7 cells.

To confirm whether *ELF3* is an E2 responsive gene, we cloned 5' UTR of 3 kb in length upstream to transcriptional start site of *ELF3* gene into a pGL3-Luciferase reporter vector and luciferase assay was performed in MCF7 cells. As shown in **Figure 3.3A**, *ELF3* promoter-luciferase activity was significantly increased upon E2 treatment as compared to the untreated cells. Further we noticed a significant increase in *ELF3* transcript levels upon E2 treatment in MCF7 cells **(Figure 3.3B)**. Consistent with the qRT-PCR data, Western blot analysis showed increased expression of ELF3 protein levels upon E2 treatment **(Figure 3.3C)**. To further confirm the direct involvement of ERα in *ELF3* transcription, MCF7 cells were treated with E2 in presence or absence of ICI-182,780, an ERα antagonist, and *ELF3* transcripts levels were analyzed by RT-PCR. As shown in **Figure 3.3D**, ICI treatment decreased the *ELF3* levels in E2 treated cells. Together these results suggest that ELF3 is an E2 target gene in breast cancer cells.

To further validate ERα occupancy at ½ ERE sites on ELF3 promoter in response to E2 was further analyzed by ChIP assay using ERα antibody. MCF7 cells were treated with either ethanol or E2 (10 nM) for 45 minute before fixing with formaldehyde. The eluted DNA samples after ChIP were subjected to real-time quantitative PCR for *ELF3* gene with primers designed across various ½ ERE sites on ELF3 promoter. ERα readily recruited to all ½ ERE sites on ELF3 gene promoter, albeit with different affinities **(Figure 3.3E-F)**. Thus, these results indicate that ELF3 is an E2 responsive gene in breast cancer cells.

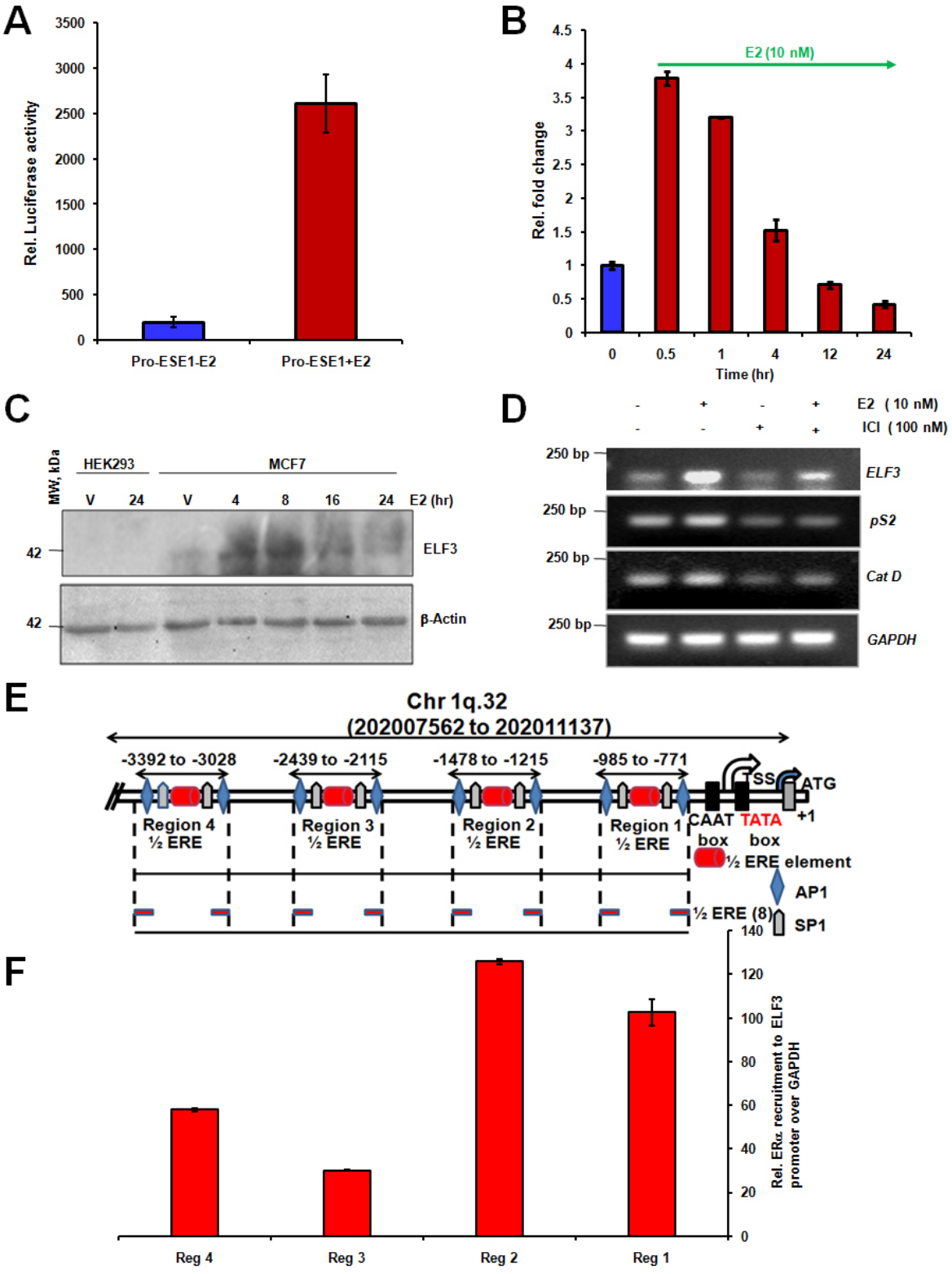


Figure 3.3: *ELF3* is an E2 inducible gene in breast cancer cells. A) Luciferase assay showing the effect of E2 (10 nM, 18 hr) on ELF3 promoter activity in MCF7 cells. EtOH, ethanol. B) Real-time qRT-PCR analysis showing the effect of E2 on *ELF3* transcription MCF7 cells. C) Western blotting analysis showing the effect of

E2 on ELF3 protein synthesis in either HEK293 or MCF7 cells. D) RT-PCR analysis showing the effect of E2 and/or ICI on *ELF3*, *pS2* and *Cathepsin D* transcription in MCF7 cells. GAPDH used as loading control. E) Physical map of *ELF3* promoter located on chromosome 1q32.1. Eight ½ ERE elements located at four different regions (Region 1-4) spanning in 3 kb *ELF3* promoter were chosen for ChIP assay (top panel). F) ChIP analysis showing the recruitment of ERα onto *ELF3* promoter over *GAPDH* promoter as compared to IgG control.

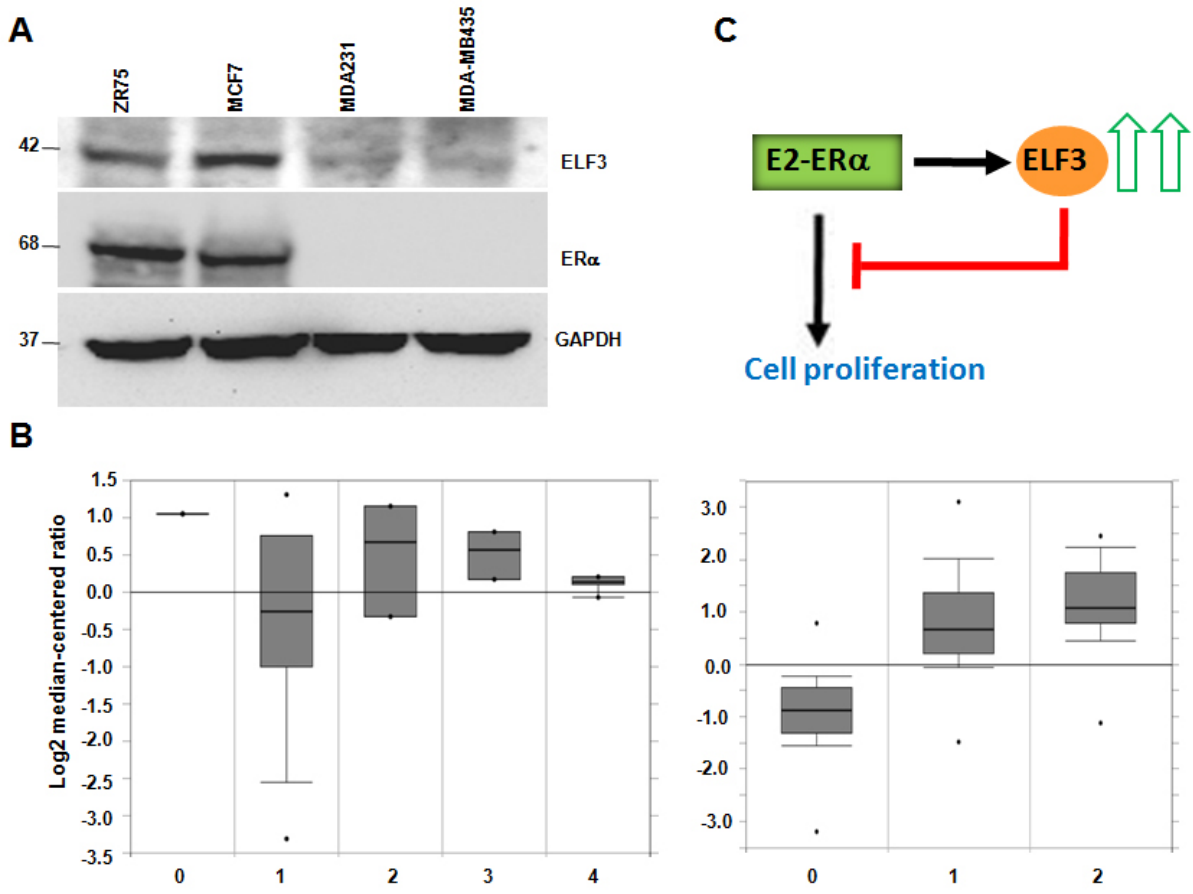


Figure 3.4: ELF3 expression correlates with ER status in breast cancers. A) Western blotting analysis showing the correlative expression of ERα and ELF3 in MCF7 cells. B) Oncomine microarray data was used to analyse *ELF3* expression (mRNA) in ER negative vs. ER positive breast cancers. *ELF3* expression in Hedenfalk breast tumor dataset (left). 0. No value (n=1), 1. Estrogen Receptor Negative (n=11), 2. Estrogen Receptor Weakly Positive (n=3), 3. Estrogen Receptor Strongly Positive (n=3), 4. Estrogen Receptor Very Strongly Positive (n=4). E. *ELF3* expression in Minn breast tumor dataset (right). 0. No value (n=22), 1. Estrogen Receptor Negative (n=42), 2. Estrogen Receptor Positive (n=57). $P<10^{-4}$. Coexpression (R) =0.758. C) We propose a model wherein E2-ERα-mediates *ELF3* expression in breast cancer cells. ELF3 thus expressed in turn

antagonizes E2-ERα-dependent cell proliferative functions, establishing a negative feedback loop between ELF3 and ERα in breast cancer cells.

3.4.2 Correlation of ELF3 expression in ER positive vs. ER negative breast cancers:

E2-dependent expression of ELF3 in breast cancer cells predicted a potential positive correlation between ELF3 and ERα expression in breast cancer cell lines or subclasses of breast tumors where this pathway played a significant role. To test this hypothesis, we first analysed the expression of ELF3 in ER positive and ER negative breast cancer cell lines. Western blot analysis indicated the co-expression of ELF3 and ERα in ER-positive breast cancer cells (MCF7 and ZR-75) but not in ER negative breast cancer cells (MDA-MB231 and MBA-MD435) **(Figure 3.4A)**. Next we focused on microarray datasets that classified tumor samples based on ER subtypes. Two datasets obtained from Oncomine data base showed moderately high expression of ELF3 in ER positive breast cancer subtype over ER negative, indicating that ELF3 expression in response to E2-ERα observed is functionally relevant to ER positive breast cancer **(Figure 3.4B)**(Hedenfalk et al., 2001; Minn et al., 2005). These results together indicate that ELF3 is an E2 responsive gene. ELF3 thus expressed feeds back to suppress ERα functions in breast cancer cells **(Figure 3.4C)**.

3.5 DISCUSSION: Estrogens play crucial role in various cellular processes in diverse organs in human body primarily by controlling expression of its target genes through genomic signaling mechanism. In genomic signaling E2 exerts its function through either upregulation or downregulation of its targeted genes. In this study, we found that *ELF3* is a genomic target of E2-ERα in breast cancer cells. ERα regulates *ELF3* expression by binding to ½ ERE sites located on its promoter. Lin and his colleagues mapped whole genome ERα binding sites in MCF7 cells using chromatin immunoprecipitation-paired end diTag cloning and sequencing strategy. They reported 1,234 high confidence binding clusters for E2-ERα on the MCF7 genome. Among them, 71% are putative full EREs, 25% are half ERE sites,

and only 4% had no recognizable ERE sequences (Lin *et al*., 2007). It implies that one quarter of E2-ERα targets contains ½ EREs and ELF3 will fall into this category.

ERα regulates the transcription of its target genes directly binding to either complete EREs or ½ EREs (Gruber *et al*., 2004). ERα binds to ½ ERE sites (AGGTC or TGACCT) when they are juxtaposed to Sp1 or AP-1, transcription factors, sites and activates target gene transcription (Klinge, 2001). Using promoter-luciferase assays, we found that *ELF3* is an E2 response gene. Further RT-PCR analysis followed by Western Blot analysis indicated that E2 upregulates ELF3 in breast cancer cell line, MCF7. Interestingly, ELF3 mRNA levels were peaked at 30 minutes of E2 treatment and then gradually declined. Decrease in ELF3 mRNA levels after 30 minutes of E2 treatment could be due to instability of *ELF3* transcript as it contains AUUUA sequence in its 3'UTR. Gene transcripts with AUUUA motifs are known to be degraded by HU complex (Winstall *et al*., 1995; Sully *et al*., 2004). The other possibility could be miRNA-mediated gene silencing on *ELF3* gene expression. Because E2 is known to upregulate miRNAs to control various cellular processes through genomic signaling (Klinge, 2012), ELF3 targeting miRNAs, which are E2 responsive, might have silenced *ELF3* expression. Inhibitor studies using ICI-182,780, an ERα antagonist, we demonstrate that ELF3 expression is E2-ERα dependent. Chromatin immunoprecipitation assays further supported this finding that E2-ERα directly recruits to *ELF3* promoter, albeit with different affinities to regulate *ELF3* expression in ER-positive breast cancer cells. Similar to ELF3, few known E2 target genes such as *LRP16*, *HSP27*, progesterone receptor (*PR*), prolactin (*PRL*), also contained ½ EREs (Porter *et al*., 1996; Anderson and Gorski, 2000; Han *et al*., 2003). Proximal region of the *LRP16* contains a ½ ERE half element juxta position with Sp1 site (Han *et al*., 2003). Similarly Porter *et al* reported that *HSP27*, an E2 responsive gene, contain one ½ ERE element with Sp1 site (Porter *et al*., 1996). For *PR* gene, Petz *et al* reported that an ERE half site and a Sp1 sites are sufficient for its upregulation in MCF7 cells

(Petz *et al*., 2004). Together, our studies clearly demonstrate that E2-ERα regulates ELF3 expression in a ½ ERE-dependent mechanism.

Another intriguing finding from this study is that correlation of ELF3 expression with ER positive breast cancer phenotype. E2-dependent expression of ELF3 in breast cancer cells led us to test the correlative expression between ELF3 and ERα expression in breast cancer cell lines. In consistent with this notion, we found a positive correlation of ELF3 expression with ER positivity in breast cancer cell lines tested. Our Western blot analysis using two ER positive breast cancer cells such as MCF7 and ZR75, and ER negative breast cancer cells such as MDA-MD231 and MD-MBA435, indicated ELF3 expression confined to ER positive cell line. In support these results, analysis of ELF3 expression in publicly available cancer datasets, a positive correlation of ELF3 expression with ER positive breast cancers was observed. The marked phenotypic and physiological difference between the ER-positive and -negative breast tumors is due to differences in gene expression between these two tumor types. Although significant number of genes found to be differentially expressed in ER-positive cancers were not E2 responsive, it is possible that E2 elicits the expression of a repertoire of genes involved in cell proliferation and growth (Lin *et al*., 2004). In case of ELF3, being an E2 response gene its expression was positively correlated with ER positivity in breast cancers.

Literature suggests that E2 regulates a plethora of genes in various organs in humans (Klinge, 2001; Carroll *et al*., 2006). Many of these gene products are directly or indirectly associated with mammary gland development and, their expression has been correlated with breast cancer when they are dysregulated. In this report, we identified *ELF3* as an E2 target gene in breast cancer cells. The finding that *ELF3* is an E2-ERα-responsive gene suggests the existence of a negative regulatory loop between the two proteins in which E2-dependent

upregulation of ELF3 feeds back to inhibit ERα function and down regulate the estrogenic response in breast cancer cells (**Figure 3.5**).

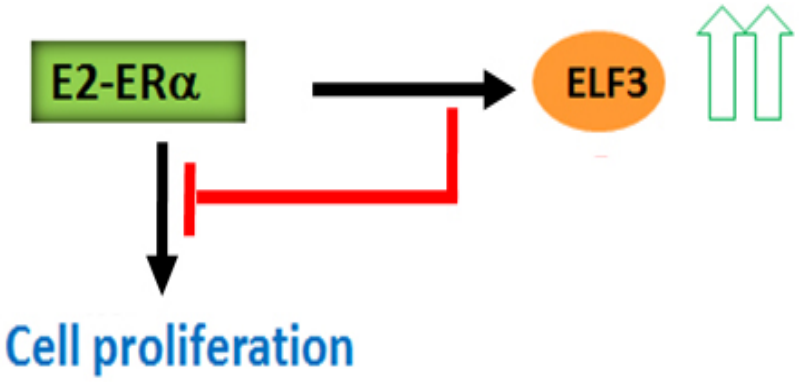


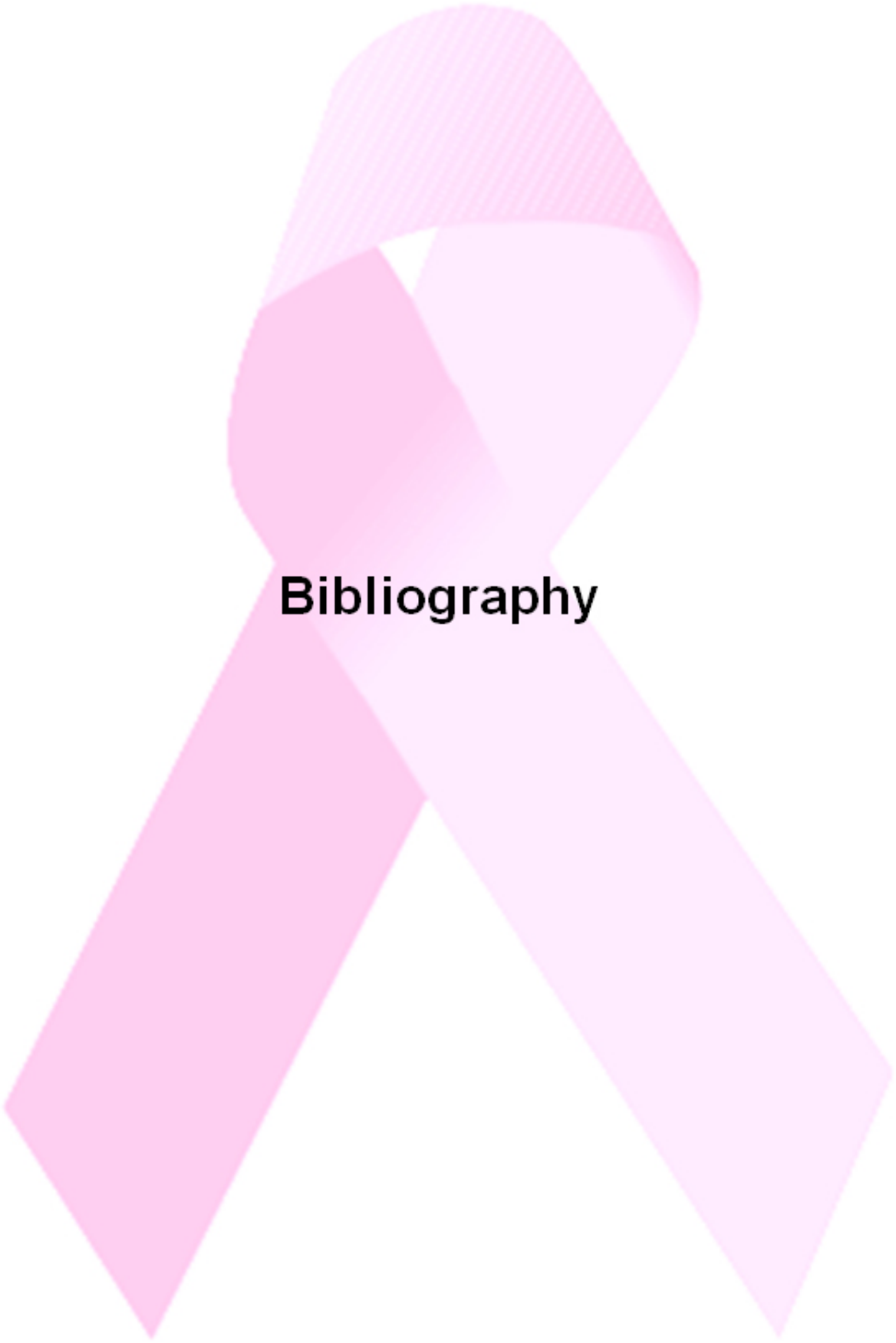
Figure 3.5: Model illustrating the repressive activity of ELF3 on ERα functionality. E2 induces ELF3 expression which in turn antagonizes ERα cell proliferative functions implying a negative feedback loop existing between ELF3 and ERα in breast cancer cells.

3.6 CONCLUSION: An attempt was made to trace out the transcriptional regulation of ELF3 by E2 in this study. The Western blot analysis of ELF3 revealed the presence of ELF3 in ER positive cells only, leaving a strong clue that ELF3 expression could be regulated by ERα. At first instance, we analysed the upstream promoter region of ELF3 using a couple of bioinformatic tools (TFBIND, TFSEARCH and ALGGEN-PROMO) and found out that eight ½ ERE elements were juxtaposition within the 3 kb upstream of *ELF3* gene which could act as putative binding sites for ERα. To further analyse the functional significance of these half ERE sequences, 3 kb region to the upstream of transcription start site of *ELF3* was cloned into luciferase reporter vector. As expected, the luciferase activity increased by several folds in the cells treated with E2 indicating that the promoter was directly under the control of ERα genomic signaling. Besides this, treatment of cells with E2 showed increase in mRNA and protein levels of ELF3 and treatment of cells with ICI-182,780 (Fulvestrant), an ERα antagonist, was enough to abrogate this transcriptional activation. To dissect the molecular mechanism of this transcriptional regulation chromatin immunoprecipitation with ERα

antibody was performed and, as anticipated ERα bound directly with these ERE elements indicating that ELF3 is under direct transcriptional control of ERα.

All these results provided evidence of previously unidentified feedback regulation of ERα by ELF3 in breast cancer cells. It appears that ERα may keeps ELF3 levels under check at times of robust cell proliferation as the expression of ELF3 has negative impact on the ER transcriptional activity and proliferation of cells. It may seem to be very naive and amateur idea here to propose that disruption of this feedback regulation in ERα negative breast cancer cells gives these cancers the benefit of aggressive cell division and malignancy due to lack of ELF3 which shown anti-proliferative activity in MCF7 cells.

Hence, the peptidomimetic ERIPE engineered by us might prove to be an effective drug for targeting ER positive breast cancer cells as wERIPE has the potential to specifically target cells expressing ERα and has negligible activity in ER-negative breast cancer cells. Also, there is every possibility to modify the amino acid residues of ERIPE without affecting its affinity to ER and crosslink them to cytotoxic agents, and engineer hybrid therapeutics that can deliver the drugs at desired place in desired time.



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Publications

Derailed Estrogen Signaling and Breast Cancer: An Authentic Couple

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Estrogen or 17β-estradiol, a steroid hormone, plays a critical role in the development of mammary gland via acting through specific receptors. In particular, estrogen receptor-α (ERα) acts as a transcription factor and/or a signal transducer while participating in the development of mammary gland and breast cancer. Accumulating evidence suggests that the transcriptional activity of ERα is altered by the action of nuclear receptor coregulators and might be responsible, at least in part, for the development of breast cancer. In addition, this process is driven by various posttranslational modifications of ERα, implicating active participation of the upstream receptor modifying enzymes in breast cancer progression. Emerging studies suggest that the biological outcome of breast cancer cells is also influenced by the cross talk between microRNA and ERα signaling, as well as by breast cancer stem cells. Thus, multiple regulatory controls of ERα render mammary epithelium at risk for transformation upon deregulation of normal homeostasis. Given the importance that ERα signaling has in breast cancer development, here we will highlight how the activity of ERα is controlled by various regulators in a spatial and temporal manner, impacting the progression of the disease. We will also discuss the possible therapeutic value of ERα modulators as alternative drug targets to retard the progression of breast cancer. (***Endocrine Reviews*** **34: 1–32, 2013)**

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I. Introduction

Breast cancer is heterogeneous in nature that originates from the mammary epithelial cells. Despite advances made in the understanding of the molecular and cellular events that underlie the disease, it remains the leading cause of cancer deaths among females worldwide (1). A woman's risk of breast cancer is influenced by her reproductive history, *i.e.*, lifetime exposure to reproduc-

Abbreviations: AIB1, Amplified in breast cancer-1; AKT, serine/threonine protein kinase; ALDH, aldehyde dehydrogenase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and rad3-related protein; BCAS3, breast carcinoma amplified sequence 3; BRCA1, breast cancer 1; BrCSC, breast cancer stem cell; BT-IC, breast tumor-initiating cell; CDK4, cyclin-dependent kinase; Ciz1, CDKN1A-interacting zinc finger protein 1; DACH1, dachshund homolog 1; DBC1, deleted in breast cancer 1; DNAPK, DNA-dependent protein kinase; E2, estrogen or 17β-estradiol; Efp, estrogen-responsive finger protein; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; ERE, estrogen response element; GPR30, G protein-coupled receptor 30; GREB1, growth regulation by estrogen in breast cancer 1; GSK3β, glycogen synthase kinase 3β; HAT, histone acetyl transferase; HDAC, histone deacetylase; HPIP, hematopoietic PBX-interacting protein 1; MaSC, mammary stem cell; miRNA, microRNA; MTA, metastasis-associated protein; MTA1s, MTA1 short form; NCOR1, nuclear receptor corepressor 1; NuRD, nucleosome remodeling and histone deacetylation complex; PAK1, serine/threonine p21-activated kinase; PELP1, proline, glutamic acid and leucine-rich protein; PHB, prohibitin; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PR, progesterone receptor; PRMT1, protein arginine N-methyltransferase 1; REA, repressor of ER activity; SAFB, scaffold attachment factor B; SCID, severe combined immunodeficiency; SERM, selective ER modulator; SIRT1, sirtuin 1; S6K1, S6 kinase 1; SP, specificity protein; TFF1, trefoil factor 1; UTR, untranslated region.

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Estrogen receptor coregulators and pioneer factors: the orchestrators of mammary gland cell fate and development

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The steroid hormone, 17β-estradiol (E2), plays critical role in various cellular processes such as cell proliferation, differentiation, migration and apoptosis, and is essential for reproduction and mammary gland development. E2 actions are mediated by two classical nuclear hormone receptors, estrogen receptor α and β (ERs). The activity of ERs depends on the coordinated activity of ligand binding, post-translational modifications (PTMs), and importantly the interaction with their partner proteins called "coregulators." Because coregulators are proved to be crucial for ER transcriptional activity, and majority of breast cancers are ERα positive, an increased interest in the field has led to the identification of a large number of coregulators. In the last decade, gene knockout studies using mouse models provided impetus to our further understanding of the role of these coregulators in mammary gland development. Several coregulators appear to be critical for terminal end bud (TEB) formation, ductal branching and alveologenesis during mammary gland development. The emerging studies support that, coregulators along with the other ER partner proteins called "pioneer factors" together contribute significantly to E2 signaling and mammary cell fate. This review discusses emerging themes in coregulator and pioneer factor mediated action on ER functions, in particular their role in mammary gland cell fate and development.

Keywords: estrogen, estrogen receptor, coregulators, pioneer factors, mammary gland development

INTRODUCTION

Mammary gland development occurs postnatally unlike other human organs (Russo and Russo, 2004). The ovarian hormones, 17β-estradiol (hereafter referred to as E2) and progesterone play a pivotal role in mammary gland development. Although prenatal development of mammary gland is relatively independent of these steroid hormones, pronounced growth occurs during puberty which requires E2. Hence, the hormone-dependent mammary gland development occurs only after puberty. The ovarian hormones impact profound morphogenetic changes in the development of gland by inducing ductal elongation, side branching, terminal end bud (TEB) formation and alveologenesis (Brisken and O'malley, 2010).

E2 exerts its biological functions through specific ligand-inducible nuclear receptors, namely estrogen receptors (ERs) ERα and ERβ. These receptors are encoded by genes located on two different chromosomes and share considerable sequence homology (Nilsson et al., 2001). These proteins regulate the transcription of a diverse array of target genes during development and, in response to specific physiological and pathological signals (Klinge, 2000). Knockout mouse studies have clearly demonstrated that ERα is indispensible for the postnatal development of mammary gland while ERβ is not (Mueller et al., 2002; Mallepell et al., 2006; Feng et al., 2007). The canonical action of the ER involves binding to its ligand with a concomitant dissociation from HSP chaperone proteins, receptor dimerization, nuclear entry and binding to E2 response elements (EREs) located within the promoter/enhancer regions of the target genes to regulate transcription (Klein-Hitpass et al., 1988; Echeverria and Picard, 2010). Accumulating evidence suggests that ERα is preferentially recruited at enhancer regions of target genes upon E2 stimulation (Carroll et al., 2005; Welboren et al., 2009; Gertz et al., 2012; Ross-Innes et al., 2012; Xiao et al., 2012). These enhancer elements modulate target gene expression by forming chromatin loops (Lieberman-Aiden et al., 2009; Sanyal et al., 2012). This is strengthened by the fact that ERα depletion leads to transcriptional repression and loss of chromatin loops, thereby supporting the notion that ERα indeed participates in chromatin loop formation in the breast cancer cells (Fullwood et al., 2009).

In contrast, a recent study suggests that unliganded ERα also binds to large number of chromatin sites in breast cancer cells and, this binding is specifically linked to genes with developmental functions (Caizzi et al., 2014). ERα can also affect gene transcription indirectly through its physical interaction with other transcription factors, such as activator protein 1 (AP1), SP1, nuclear factor-κB (NF-κB) and E2F1 (Safe, 2001). Functionality of the ERα is further regulated by the post-translational modifications (PTMs) and its cooperative interaction with a special category of proteins called "coregulators," which exhibit with a coactivator or corepressor activities (Lonard and O'malley,

Abbreviations: TEBs, terminal end buds; KO, knockout; Tg mice, transgenic mice; ERα, estrogen receptor alpha; Esr1, estrogen receptor alpha gene 1; SHR, steroid hormone receptor; PTMs, post-translational modifications; PgR, progesterone receptor; MaSC, mammary stem cells.

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