Tamoxifen inhibits ERα positive breast cancer progression by disrupting the ERα-JMJD2B signaling axis

by

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A thesis submitted in conformity with the requirements for the degree of Master’s
Graduate Department of Medical Biophysics,
University of Toronto

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1. Abstract

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Rashi Gupta, 2015

For the Degree of Master’s in Science

Department of Medical Biophysics, University of Toronto

JMJD2B is a histone demethylase for H3K9me3. Our lab has shown that JMJD2B is a co-factor of estrogen receptor in breast cancer proliferation: JMJD2B knockdown severely impairs estrogen-induced cell proliferation and tumor formation of breast cancer cells. Resistance to tamoxifen poses major problems in ERα + bc management. I hypothesize that tamoxifen inhibits breast cancer cell proliferation by targeting the ERα-JMJD2B signaling axis. My results indicate that tamoxifen leads to inhibition of estrogen-induced enrichment of ERα, JMJD2B and RNAPII at the enhancer of an ERα target gene, c-MYB. Tamoxifen interfered with demethylation of H3K9me3 at the c-MYB- enhancer. These effects reduced expression of ERα target genes and diminished ERα positive breast cancer cell proliferation. This indicates a critical role for JMJD2B that may be amenable to therapeutic targeting in tamoxifen resistant ERα positive tumor cells. I discuss alternative roles for JMJD2B and outline potential experimental directions that will help elucidate its role in tumor response to tamoxifen.
Acknowledgements

No amount of this project would have been possible without the support of some essential people. Since this is the only time I have to formally thank them, this is going to be lengthy and I apologize for that.

Dr. Hitoshi Okada gave me a chance to become a graduate student in the Univ. of Toronto, when all other doors seemed closed to me by virtue of my being an expensive international student. Without his faith in me, I could not have achieved even a fraction of this research.

Dr. Kayoko Saso, or simply, Kayoko, was my research mentor from day one. Dr. Okada might not have been available always, but Kayoko was a shout away for most laboratory emergency questions, despite having a very busy roles as lab manager and mother.

Jun Ikura, my friend and colleague, who was my life-support system during good days, bad days, failed gels and spilled buffers. I miss occupying the desk next to yours.

Rania Chehade from the Gauthier Lab, who was everything to me during some extremely difficult times – best friend, sister, mentor, motivator, shoulder to cry on, colleague to brainstorm ideas with, and companion to gossip with during long incubation periods.

My supervisory committee members – Dr. Mitsu Ikura, Dr. Eldad Zacksenhauss and Dr. Jack Greenblatt. They were not always pleased with the progress of work, but they never let that lead me to believe that I could not do better. The wealth of their knowledge and wisdom saved me from wasting countless months on fruitless experiments and unwise guidance. Their scientific intuition and concern for my future and time might have prevented me from making some very expensive mistakes.

There was also a time during the completion of my project when this all seemed a big mistake and waste of time. It did not seem plausible that I would ever finish writing my thesis. I had lost both faith and will. It seemed preferable to work at a call centre than to invest any more effort into the project. Then came Dr. Rama Khokha. And everything changed.

The administrative staff at MBP (Annette, Chau and Merle). You guys are awesome! The support provided to me during some very complicated and difficult times is testimony to your skills and genuine concern for the students.

My parents were not in agreement with my pursuing higher studies, but they provided for me anyway, in every way possible. Their lack of expectations from me even today, gives me the freedom of tackling challenges without fear of failure.

My Sisters, Shruti and Kriti, who have picked up my fallen pieces in moments of crisis so many times, and handed them back to me. They refused to let me call myself a failure and that makes me believe I am not. They are my two best fans and advocates.

And lastly, my new husband, Rushil. I could do this, because it was the two of us. My family lives in a different country, my best friends have gone far away and without his financial,
emotional and domestic support, I might have taken that job at the call centre. He never pressed me to do more than I could, but he did not let me be comfortable with the idea of letting 3 years of effort and investment going to waste either. Thank you for the taking care of food, domestic duties, late night coffee, walking Bruce and me.
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## Abbreviations

- **5-Aza**  
  Aza-2’-deoxycytidine
- **ACH**  
  Active Chromatin Hub
- **ATCC**  
  American Type Culture Collection
- **AE**  
  Anti-Estrogen
- **AF-1**  
  Activation Function -1
- **AI**  
  Aromatase Inhibitor
- **AIB1**  
  Amplified in Breast 1. Also known as SRC-3 or TRAM-1
- **AP-1**  
  Activator Protein -1
- **AR**  
  Androgen Receptor
- **ATP**  
  Adenosine Tri-Phosphate
- **BC150**  
  Bio-Chemical 150
- **BCL2**  
  B-cell lymphoma 2
- **BIRC5**  
  Baculoviral IAP Repeat-Containing 5
- **BRCA1**  
  Breast Cancer 1 early onset
- **BSA**  
  Bovine Serum Albumin
- **cAMP**  
  Cyclic Adenosine Monophosphate
- **CREB**  
  cAMP Response Element Binding Protein
- **CBP**  
  CREB-binding protein
- **CCND**  
  Cyclin D
- **cDNA**  
  Complementary DNA
- **c-FOS**  
  Product of Cellular Oncogene FOS
- **ChIP**  
  Chromatin Immunoprecipitation
- **c-JUN**  
  Product of JUN proto-oncogene
- **c-MYB**  
  Proto-oncogene member of Myeloblastosis family of transcription factors
- **c-MYC**  
  A human proto-oncogene similar to the viral myelocytomatosis oncogene (v-Myc)
- **CO2**  
  Carbon Dioxide
- **CoREST**  
  Corepressor for REST (RE1 silencing transcription factor)
- **CRE**  
  cAMP response element
- **DBD**  
  DNA binding domain
- **DMEM**  
  Dulbecco/Vogt modified Eagle's minimal essential medium
- **DNA**  
  Deoxyribo nucleic acid
- **DNase**  
  Deoxyribonuclease
- **DTT**  
  Dithiothreitol
- **E2**  
  17-βestradiol
- **EDTA**  
  Ethylenediaminetetraacetic acid
- **Elk-1**  
  ETS domain-containing protein -1
- **EMT**  
  Epithelial-to-Mesenchymal transition
- **ERE**  
  Estrogen Response Element
- **ERα**
  Estrogen Receptor α
- **ESR1**
  Estrogen Receptor 1
- **EZH2**
  Enhancer of zeste 2
- **FBS**
  Fetal Bovine Serum
- **GATA3**
  A transcription factor characterized by their ability to bind the DNA sequence GATA
- **HEK 293T**
  Human Embryonic Kidney, transformed with the large T antigen
- **H3K4**
  Histone 3 Lysine (K) 4
- **H3K9Ac**
  Histone 3 Lysine9 Acetylation
- **H3K9me3**
  Histone 3 Lysine 9 trimethylation
- **HAT**
  Histone acetyl transferase
- **HCl**
  Hydrochloric acid
- **HDAC**
  Histone deacetylase
- **HDAC1**
  Histone deacetylase 1
- **HDAC2**
  Histone deacetylase 2
- **HER2 or ERBB2**
  Human Epidermal Growth Factor Receptor 2
- **HIF1**
  Hypoxia Inducible Factor 1
- **HMT**
  Histone Methyl transferase
- **HOXC13**
  Homeobox C13
- **HRP**
  Horse Radish peroxidase
- **Hrs**
  Hours
- **Hsp90**
  Heat Shock protein 90
- **IGF**
  Insulin-like growth factor
- **IgG**
  Immunoglobulin G
- **IL-6**
  Interleukin-6
- **IP**
  Immunoprecipitation
- **JmjC**
  C-terminal Jumonji domain
- **JMJD3B/KDM6B**
  Lysine (K) Specific Demethylase 6B
- **JmjN**
  N-terminal Jumonji domain
- **KAT2 or GCN5**
  Lysine (k) Acetyl transferase
- **KCI**
  Potassium Chloride
- **Kda**
  Kilo Dalton
- **KDM**
  Lysine specific demethylase
- **KDM4B or JMJD2B**
  Lysine (K) Specific Demethylase 4B
- **KDM4C or JMJD2C**
  Lysine (K) Specific Demethylase 4C
- **LBD**
  Ligand Binding Domain
- **LDL-r**
  Low-density lipoprotein receptor
- **LiCl**
  Lithium Chloride
- **LSD1**
  Lysine specific demethylase 1
- **MAP**
  Mitogen activated protein
- **MBD3**
  Methyl-CpG binding domain protein 3
- **MCF-7**
  Michigan Cancer Foundation 7
- **Med1**
  Mediator Complex Subunit 1
- **MgCl2**
  Magnesium Chloride
- **Mi-2 or CHD3**
  Chromodomain-helicase-DNA-binding protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>mM</td>
<td>Mili Molar</td>
</tr>
<tr>
<td>mQ</td>
<td>Mili Q</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTA-2</td>
<td>Metastasis associated protein 2</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NaAc</td>
<td>Sodium Acetate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear Receptor Corepressor 1</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>Nano Molar</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonyl phenoxypolyethoxylethanol – 40</td>
</tr>
<tr>
<td>NuRD</td>
<td>Nucleosome Remodeling Deacetylase</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>Optimal Minimum Essential Media</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin /Streptomycin</td>
</tr>
<tr>
<td>p160 or SRC</td>
<td>Steroid Receptor Coactivator</td>
</tr>
<tr>
<td>p300 or EP300</td>
<td>E1A binding protein p300</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Phenol Chloroform Isoamylalcohol</td>
</tr>
<tr>
<td>PGR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>pH</td>
<td>Power of Hydrogen</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant Homeo Domain</td>
</tr>
<tr>
<td>PI-3</td>
<td>Phosphoinositol -3</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PM-ERα</td>
<td>Plasma Membrane associate Estrogen Receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive Transcription Elongation Factor b</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>RbAp46</td>
<td>Retinoblastoma associated protein 46</td>
</tr>
<tr>
<td>REA</td>
<td>Repressor of Estrogen Receptor α activity</td>
</tr>
<tr>
<td>RNAPII</td>
<td>Ribonucleic Acid polymerase II</td>
</tr>
<tr>
<td>RNAPII S2P or RNAPII Ser2P</td>
<td>Ribonucleic Acid polymerase II phosphorylated on serine 2 of CTD</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>rt</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser2P or S2P</td>
<td>Serine2 phosphorylated</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective Estrogen Receptor downregulator</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulator</td>
</tr>
</tbody>
</table>
- Sin3: Switch Independent 3
- SMRT: silencing mediator for retinoid or thyroid-hormone receptors
- SP-1: Specificity protein 1
- Src: Sarcoma
- SRC1: Steroid Receptor Co-activator -1
- ssssDNA: Single strand salmon sperm DNA
- TAE: Tris base, Acetic acid, EDTA
- TAFIID: TBP Associated Factor II D
- Tam: Tamoxifen
- THR: Thyroid Hormone Receptor
- TE: Tris, EDTA
- **TFF1** or pS2: Trefoil Factor 1
- TNBC: Triple Negative Breast Cancer
- TSA: Trichostatin A
- VDR: Vitamin-D Receptor
- WST: Water soluble Tetrazolium
2. Introduction

2.1 Estrogen Receptor α (ERα)

2.1.1 Importance of ERα in breast cancer

Breast cancer is the 2nd most common cause of cancer related deaths in Canadian women; in Canada approximately 24,400 women will be diagnosed with breast cancer this year, and 5000 will die from it. Estrogen Receptor α (ERα) + breast cancer, accounting for about two thirds of all breast malignancies, remains the most prevalent cancer in women[1].

A vast amount of data has been gathered in support of estrogen being the major initiator and driver of ER+ breast cancer. An increase in ERα signaling has been thought to result from lapse at several points, including amplification of the ESRI gene [2], upregulation of transcription from the ESRI gene, reduced degradation of the product of transcription and increased translation from the mRNA, increased availability of co-factors of positive transcription, all leading to constitutive activation of cell signaling pathways, leading to proliferation, growth and evasion of apoptosis[3, 4]. In our lab [5] and others [6-8], many co-activators of the ERα signaling pathway have been selectively targeted / depleted and their inactivation has led to a crippled ERα signaling; this leads to reduction in breast cancer tumorigenesis in many tumor models like ERα+ breast cancer cell lines, patient samples, mice and xenografts. A vast number of ERα target genes, activated by stimulation with estradiol, have been implicated in promoting breast carcinogenesis. For example, processes like cell proliferation, apoptosis, DNA replication, DNA damage repair, genomic stability, cell adhesion, cell motility, cell metabolism and cell cycle, all have ERα target genes executing their regulation [9] [10].

This leads to the conclusion that ERα signaling plays a major role in the promotion and development of breast cancer and that ERα requires an extensive network of other protein partners to influence gene transcriptional pathways significant for tumorigenesis.

2.1.2 Breast Cancer can be divided into four molecular subtypes according to receptor status:

HER2 +/-

In HER2 positive breast cancer, the over expression of the HER2 receptor promotes tumorigenic behaviour. HER2+ tumors tend to be more aggressive than HER2- tumors, with worse prognosis.
for patients, and account for 10-15% of all breast tumors. HER2 positive breast cancers can be either positive or negative for ERα.

**Triple Negative Breast Cancer (TNBC)**
As the name implies, triple negative breast cancer or TNBC is marked by absence of all three receptors: ERα, PR and HER2. TNBC accounts for 10-15% of all breast tumors and is characterized by an aggressive nature and a higher metastatic rate.

**Luminal A**
This is the most dominant form of breast cancer, accounting for approximately 40% of breast malignancies. This luminal cancer is characterized by presence of estrogen receptor (ER) and progesterone receptor (PR), and lack of the Her2 receptor. Luminal cancers usually originate in the cells lining the inner lining (lumen) of the mammary ducts. Due to their receptor positive status, these cancers tend to be the easiest to treat, with best prognosis.

**Luminal B**
These breast tumors are very similar to the Luminal A subtype, originating in the luminal cells of the mammary ducts, except that they may be positive for the Her2 receptor and/or display high Ki67 in addition to the presence of ER and PR. These luminal tumors are distinguished from the Luminal A subtype in that they have a higher number of actively dividing cells and are of higher grade than luminal A. Women diagnosed with luminal B subtype typically display a poorer prognosis due to a number of factors like poor tumor grade and p53 mutations (~30%). The luminal B subtype accounts for about 30% of breast malignancies.

Recent genomic studies define breast cancer as not one, but several heterogeneous diseases. These studies classify breast cancer into more subtypes than just defined by the receptor status, based on molecular signatures like the genomic, epigenomic and transcriptome alterations of tumors, underlining the growing complexity and heterogeneity inherent in breast tumors. The breast cancer subtypes, therefore, are classified into ten variations, derived from transcriptomic and genomic data, providing more tailored therapeutic regiments and prognosis for patients[11, 12].
2.1.3 Treatment for ERα + breast cancer

Endocrine therapy is the core of treatment for hormone receptor positive breast cancer. These are targeted towards either reducing the levels of estrogen in a systemic way or modulating the activity of ERα by competing with estrogen for binding. Aromatase inhibitors (AI) inhibit the conversion of androgens into estrogens, and they are therapeutic in controlling the levels of estrogen available in the body. There are three AIs - letrozole and anastrozole (nonsteroidal), as well as exemestane (steroidal).

SERMs, or selective estrogen receptor modulators, are a group of drugs which modulate, i.e. alter, ERα activity but they are not pure antagonists. Tamoxifen and raloxifene are two SERMS which are used for management and treatment of breast cancer in the clinic. While tamoxifen has questionable role because of its effects on the endothelium and bone, raloxifene has been shown to be antagonistic in breast and uterine tissue, without risk of osteoporosis.

SERDS, or selective estrogen receptor downregulators, are a class of antiestrogens which are pure antagonists and work to destabilize ERα. Fulvestrant is one such SERD, and it increases degradation of ERα while at the same time completely deactivating it [13-15].

It is known that de novo resistance is most common in ERα+/PR- tumors. However, it has been observed that endocrine therapy may eventually cause the phenotype to become hormone receptor negative, from positive. Such resistance has been the subject of many studies and novel approaches, like targeting the mTOR/PI-3 kinase pathway, epigenetic pathways and growth factor pathways, are providing momentum to combating receptor positive breast cancer [16].

Several possible mechanisms have been proposed to explain resistance to tamoxifen in patients. The loss of ERα expression through aberrant epigenetic changes like methylation of the CpG islands and histone deacetylation of the ERα promoter would lead to extinguishing the target for tamoxifen [17, 18]. In a study by Fan et al.[19], it was shown that use of HDAC inhibitors like TSA (trichostatin A ) and SAHA [20] and DNMT-1 (DNA methyl transferase-1) inhibitors like AZA [19] restored ERα expression in ERα – cells (MDA-MB-435), restoring sensitivity to tamoxifen. There have also been mutant forms of ERα described which are no longer sensitive to tamoxifen [21]. Acquired resistance to tamoxifen also occurs because of cells losing sensitivity to tamoxifen while still expressing ERα [16].
Other possible mechanisms described for resistance to endocrine therapy are hypoxia, use of growth factor receptor pathways, and mutations in p53 [22]. Hypoxia has been shown to cause proteasome dependent degradation of ERα in ZR75 cells [23]. As evidence towards use of alternate signaling pathways, it was shown that EGFR (epidermal growth factor receptor) and HER 2 expression was increased in cells negative for ERα [24]. It was shown by Angeloni et al., that p53 upregulates ERα gene expression by controlling elements located upstream of the ERα promoter [25]. However, a large percentage of breast tumors are mutated for p53 [26], which cannot account for upregulated ERα gene expression.

2.1.4 ERα signaling

Estrogen Receptor is a member of the family of nuclear receptors, all of which share their major domains. It was one of the first nuclear hormone receptors to be characterized by Elwood Jensen, V. Craig Jordan and Jack Gorski in various complementary studies. These studies focused on discovering an estrogen binding factor which was present in tissues that responded to estrogen treatment with growth and differentiation [27, 28].

Estrogens are of three types, with the most potent form being 17 β-estradiol, which we will refer to as E2 [29]. They are called the female sex hormones, but in fact they have been shown to play an important role in many other tissues like the pituitary and the prostate glands. As perhaps the quintessential phenotype characterizing the role of ERα as a female sex hormone, ERα−/− mice show stunted ductal elongation during mammary gland morphogenesis [5]. But it was also observed by Omoto et al. that ERα−/− mice showed abnormal prostate development in terms of having long, distended ducts with minimal branching and very sparse periductal stroma [30]. A review by Couse et al. lists the various studies done in the beginning of ERα discovery, targeting the phenotypes of ERα−/− mice [31].

Estrogen Receptor (ER)α signaling is a primary determinant of mammary gland development under normal circumstances, and the main driver of ERα positive (ER+) breast cancer development [10]. ERα signaling can be characterized into three categories: ERE-mediated genomic signaling, non-ERE-mediated genomic signaling and non-genomic signaling [32, 33].

The ERα nuclear signaling pathway begins when ERα, sequestered in the cytoplasm by chaperone protein Hsp90 (heat shock protein 90), binds its ligand and changes conformation.
Upon this change in conformation, ERα is no longer sequestered by the chaperone protein and can translocate to the nucleus as a dimer. ERα gets recruited to the estrogen responsive elements (EREs) [34] often placed in enhancers of target genes, where it results in recruitment of the chromatin remodeling machinery and cofactors. The ligand-bound ERα can then play an activating or repressing role in transcription by modulating initiation or early elongation of target genes. Thus, ERα is a sequence-specific DNA binding protein which influences direct targets through activated-ERα (agonist bound activator) making direct contact with the genome at an ERE and recruiting p160 proteins [35, 36] and chromatin remodeling machinery [37] either directly or through recruitment of basal transcription factors.

Apart from recruitment of basal transcriptional machinery such as the RNAP II holoenzyme [38], the mediator machinery [39, 40], and TFIID [41], the other accessory protein complexes recruited by nuclear receptors like ER play a major role in mediating its signals. These proteins can belong to various subtypes, including histone modifying enzymes and ATP-dependent chromatin remodelling factors.

The mechanism described above is the classical action of ERα, through direct binding to DNA at EREs, and recruitment of transcription factors to the DNA to mediate transcription. The non-ERE mediated genomic action of ERα occurs through ERα binding DNA indirectly through other tethering proteins like the activating protein -1 (AP-1) [42], which activates expression of genes like ovalbumin, IGF (insulin growth factor)-1, collagenase, cyclin D1, etc [43]. ERα can also contact the DNA through other proteins like SP-1 and NFκB, where it activates LDL-R (low density lipoprotein receptor) and c-fos, or represses IL-6 (interleukin-6) expression [32].

The third, non-genomic mechanism of ERα action involves alternate signaling pathways involving G proteins, MAP kinases, phosphatidyl inositol -3 kinase (PI-3 kinase) etc. Through these alternate pathways ERα has been shown to aid in mobilization of intracellular calcium, stimulation of adenyl cyclase activity and cAMP production, and activation of the MAPK pathway (in breast cancer, endothelial, bone and neuroblastoma cells). It has been shown to participate in activation of the PI-3 kinase pathway in liver, endothelial and breast cancer cells. These non-genomic actions of ERα are mediated through ERα associated with the plasma membrane (PM), and these PM-ERα (plasma membrane associated ERs) can initiate protein-protein interactions with kinase domains of other proteins and be phosphorylated in turn,
enhancing their activation potential. PM-ERs can interact with G proteins and Src kinases, and were also found to have a role in activating membrane tyrosine kinase signaling, leading to MAPK activation. The signal transduction pathways translate the non-genomic effects of E2-ERα into genomic results. Activation of MAP kinase pathways by ERα leads to activation of CREB (cAMP response element binding protein), which in turn leads to expression of genes with a CRE (cAMP response element). MAPK activation also leads to activation of the AP-1 transcriptional complex and Elk-1, which is responsible for downstream transcriptional responses [32, 44].

*ERα signals via associated co factors*

All nuclear receptors mediate their genomic results through recruitment of various other proteins which play a role in either enhancing the basal levels of transcription or repressing basal transcription. Such proteins or co-factors are highly specific to the cell and the genomic environment to which ERα is recruited. The presence of response elements in the promoter or enhancer regions of genes, as well as the presence of such co-factors in the cell, direct specific recruitment of these proteins to ligand bound ERα. A comprehensive list of such factors can be found in Table 1, adapted from a review by Klinge *et al* [7].

ERα nuclear signaling is mediated by a complex interplay of cofactors (co-activators and co-repressors), which bind to liganded-ERα. The choice of a co-repressor versus a co-activator binding to the receptor relies upon the conformational changes induced in the receptor upon ligand binding. This is displayed in tamoxifen competing with estrogen to bind ERα, which changes the complex components of ERα signaling and hence alters gene transcription during treatment of ERα+ breast cancer patients [45-47].

ERα mediated transcriptional activation depends on an extensive network of nucleosome modifiers, histone mark writers, erasers and readers, among other things. To activate gene
Table 1A and B

### Table 1A

<table>
<thead>
<tr>
<th>Name</th>
<th>Information on co-activator</th>
<th>ERα interacting domain</th>
<th>Ligand required</th>
<th>Effect of co-expression with ERα on transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calmodulin</td>
<td>Intrinsic HAT activity</td>
<td>LBD</td>
<td>Agonist</td>
<td>Stimulates transcription and ERE binding</td>
</tr>
<tr>
<td>CBP/p300/p27</td>
<td>Also a cofactor for AP-1, c-myc, STAT1, E1A, p53 and Myo-D; helps recruit RNAPIII to the promoter</td>
<td>LBD</td>
<td>Independent of ligand although E2 enhances interaction</td>
<td>Stimulates transcription</td>
</tr>
<tr>
<td>PBP</td>
<td>Overexpressed in 50% breast tumors. The PBP gene is amplified in 25% of breast tumors</td>
<td>LBD</td>
<td>Agonist</td>
<td>Enhances transcriptional activity of E2</td>
</tr>
<tr>
<td>SRC-1 / NCoA-1</td>
<td>Intrinsic HAT activity. Interacts with cyclinD1.</td>
<td>AF-1 and AF-2 in LBD</td>
<td>Agonist required. Antagonist inhibits interaction</td>
<td>Stimulates ERα induced transcription</td>
</tr>
<tr>
<td>SRC2</td>
<td>Shares 40% sequence homology with SRC1</td>
<td>LBD</td>
<td>Agonist required. Antagonist inhibits interaction</td>
<td>Stimulates transcription</td>
</tr>
<tr>
<td>SRC3</td>
<td>Intrinsic HAT activity. Forms an activation complex with p/CAF and CBP/p300</td>
<td>LBD</td>
<td>Agonist</td>
<td>Stimulates transcription</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Chromatin remodeling complex. Human homologues are hSNF2α, hSNF2β or hbrm</td>
<td>LBD</td>
<td></td>
<td>Stimulates transcription</td>
</tr>
</tbody>
</table>

### Table 1B

<table>
<thead>
<tr>
<th>Name</th>
<th>Information on co-repressor</th>
<th>ERα interacting domain</th>
<th>Ligand required</th>
<th>Effect on ERα</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCoR</td>
<td>Suppressed antagonist-stimulated partial agonist activity of tamoxifen-ERα.</td>
<td>LBD</td>
<td>Tamoxifen</td>
<td>Enhanced the inhibitory effects of antiestrogens</td>
</tr>
<tr>
<td>REA</td>
<td>Small 37kDa protein</td>
<td>LBD</td>
<td>Agonist or tamoxifen</td>
<td>Inhibition of transcription</td>
</tr>
<tr>
<td>SHP</td>
<td>Orphan nuclear receptor that lacks a DBD</td>
<td>LBD</td>
<td></td>
<td>Overexpression reduced basal and tamoxifen stimulated gene expression.</td>
</tr>
<tr>
<td>SMRT</td>
<td>HDAC1 is associated with SMRT</td>
<td>LBD</td>
<td></td>
<td>No effect seen on E2 activity</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Mutation in BRCA1 leads to strong disposition to tumor development.</td>
<td>AF2</td>
<td></td>
<td>Overexpression blocks E2 stimulated reporter</td>
</tr>
</tbody>
</table>

The Tables 1A and 1B show a selected list ERα co-activators (A) and co-repressors (B) that have known effect on ERα mediated transcription. A more complete list is available in Klinge, 2000. These tables highlight the cofactors of ERα transcription relevant to the current study. CBP – cAMP response element binding protein binding protein. PBP – Penicillin binding protein. SRC- steroid receptor coactivator. NCoA-1 – Nuclear receptor coactivator-1. SWI/SNF- SWItch/Sucrose NonFermentable (human homologues are BAF- BRG1 associated factors). NCoR – Nuclear receptor corepressor. REA- Repressor of ERα activity. SHP- small heterodimer partner. SMRT – Silencing mediator of RAR (retinoic acid receptor) and TR (thyroid receptor). BRCA1 – breast cancer 1, early onset.
transcription in response to estrogen binding, histone acetyltransferases (HATs) like Src1, AIB1, get recruited to the ERα transcriptional complex [36, 38, 48, 49]. On the contrary, in response to tamoxifen binding, repressor complexes like NuRD (Nucleosome Remodelling and histone deacetylation) and NCoR (Nuclear Receptor Co-repressor), consisting of histone deacetylase activity, are recruited as multiprotein complexes to the ERα [47]. The important role played by epigenetics in supporting ERα signaling can also be observed in dependence of ERα on histone methyltransferases (HMTs) or demethylases (KDMs) like the MLL (mixed-lineage leukemia) family of proteins and the Jumonji family of proteins, respectively [50].

2.1.5 ERα domain structure and domain functions

ERα, like most nuclear receptors, is divided into six major domains (Figure 1). The N-terminal A/B domain is not conserved amongst nuclear receptors, and it harbors the ligand-independent transactivation function domain AF-1. The size of this domain is variable and a conserved secondary structure for this region has not been identified. This ligand independent domain can carry out transactivating roles in the absence of any ligand, and its activity is cell- and promoter-specific. The structure of the ER DNA binding domain (DBD) is characterized by a zinc finger-like motif with eight cysteines that comprise the tetrahedral coordination of two zinc ions. The “D” box in the ERα has been shown to participate in dimerization of the molecule. Moving towards the C-terminal end, the ligand binding domain (LBD) is a globular domain containing the hormone binding site. It also has a dimerization interface and sites for interacting with the corepressors and co-activators of ERα. The three dimensional structures of the LBDs in the nuclear receptor superfamily are very similar even without high sequence identity [51, 52].

The ligand binding pocket is surrounded by an antiparallel beta-sheet and an alpha helix called the H12 on two sides. The helix H12 has been shown to directly participate in the transactivation function of ERα and can adopt several conformations, depending on the structure of the ligand sitting in the ligand binding pocket of the receptor [51]. Upon binding of an agonist, the LBD of the protein adopts a rearranged conformation and this results in a specific recognition site for the helical NR (nuclear receptor)-box module present in nuclear co-activators. This co-activator binding site is characterized by a hydrophobic cleft formed by residues from the LBD. Nuclear co-activators consisting of a hydrophobic LXXLL motif can dock onto this hydrophobic cleft in the LBD of a nuclear receptor like ERα. These distinct conformations induced by binding of
**Figure 1**

![Diagram of ERα domains]

**Function**
- Transactivation
- Dimerization
- DNA binding
- Ligand binding
- Hsp 90 binding
- Co-Activator binding
- Co-repressor binding

**ERα major domains.** On top is a representation of the major domains present in the nuclear receptor ERα. Below is a description of each domain along with its major function. On top of the structure is a marking for the amino acids spanned by each domain. The N-terminal A/B domain is not well conserved among NRs and contains the autonomous transactivation function AF-1 (activation function -1 ). The size of the domain is extremely variable. No clear secondary structure can be identified in these regions.

The topology of ER DNA Binding Domains (DBDs) (C) is characterized by a zinc finger-like motif with eight cysteines that constitute the tetrahedral coordination of two zinc ions. Residues participating in the 'D box' have been shown to be involved in the dimerization interface.

The ligand-binding domain (LBD) (E): The LBD is a globular domain that harbors a hormone binding site, a dimerization interface (homo- and hetero-dimerization), and a coactivator and corepressor interaction function. Despite low sequence identity in LBDs of the NR superfamily, the three-dimensional structures of the LBDs are similar. The ligand pocket is closed on one side by an antiparallel beta-sheet and on the other by H12, known to be directly involved in the transactivation function AF-2 by mutagenesis studies, and for which several conformations (‘agonist’ or ‘antagonist’ conformations) have been evidenced.
agonists versus antagonists decide recruitment of co-activators versus co-repressors to the ERα transcriptional complex [53, 54].

2.1.6 Mammary Gland structure
Mammals derive their designation from the term ‘mammaries’ or mammary glands which distinguish them from other animals of the animal kingdom. These apocrine glands consist of the basic structure of an alveolus – a hollow cavity-lined with milk secreting cuboidal cells further surrounded by myoepithelial cells. Several alveoli cluster to form lobules which are connected to an opening in the nipple by a lactiferous duct. The hormone oxytocin causes contraction of the myoepithelial cells, which causes the secreted milk to be pushed out of the lumen of the alveoli into the lactiferous ducts. One lobule with a lactiferous duct forms a simple mammary gland. 10-20 simple mammary glands join to form a complex mammary gland which is situated in the breasts. In addition to the cluster of simple mammary glands, the mammary stroma is also a vital component of the mammary gland. The stroma consists of mammary epithelial cells (MECs), extra cellular matrix (ECM- consisting of myoepithelial cells and connective tissue), adipocytes, immune cells and fibroblasts [55] (Figure 2).

ERα expression in mammary epithelial cells
Not all MECs express ERα. In fact, only one third of luminal cells, out of all MECs, express ERα. In murine mammary gland, close to 60% of proliferating cells are devoid of any ERα expression. This was supported by findings showing that estrogens function in a paracrine fashion and that only epithelial ERα (but not stromal ERα) was required for normal mammary gland development in mice. Lack of ERα in luminal MECs led to stunted ductal elongation, side branching and alveogenesis [56].

2.1.7 Estrogen and Tamoxifen
The standard line of treatment used for treating ERα+ breast cancer is inhibiting binding of the receptor to its ligand estrogen. Estrogens are primary female sex hormones and are responsible for development of the mammary gland after puberty in female mammals. Estradiol (E2) is the most powerful estrogen in the body and it is produced by the ovaries. Inhibiting production of estrogen by using aromatase inhibitors (AI) or interfering with estrogen binding to its receptor are two of the ways in which receptor positive patients are treated. However, resistance to anti-
Figure 2: Structure of the Mammary Gland. The figure on the bottom right shows a cross-section of a lobule consisting of a periphery of myoepithelial cells, and an inner-lining of luminal cells, facing the lumen. The structure is surrounded by a basal or basement membrane which mediates the contact between the stroma and the myoepithelial cells. The structure on the top in the middle shows a lobule with a lining of luminal and myoepithelial cells, converting into a duct, which leads to a collecting duct and then to the nipple. Several lobules come together to form a mammary gland, and 10-20 mammary glands make up one breast (bottom left), which also consists of a fatty tissue, stroma, fibroblasts, immune cells and other supporting systems to host the glands.
estrogen or AI treatments are frequent with almost half the cases becoming refractory to therapy [16].

*Tamoxifen binding to activation function-2 domain of ERα*

As we see in Figure 3 the conformation adopted by the LBD, and specifically helix H12 of the ERα, is different depending on the ligand binding the receptor. This difference translates itself into the LBD or the AF-2 of ERα either being active, *i.e.*, conducive to recruiting and binding co-activators, or inactive (recruits co-repressors with HDAC activity). The approximately ninety degrees shift in position of helix 12 upon binding tamoxifen inactivates the AF-2 of the protein, but does not effect the AF-1. Upon binding tamoxifen, the H12 of LBD blocks the site for co-activator interaction in the hydrophobic groove, near \(\alpha\)-helices 3, 4 and 5, and prevents recruitment of co-activators to the ERα transcriptional complex. In this regard, tamoxifen and other modulators of receptor activity differ from complete antagonists like fulvestrant, which completely inactivate the ERα, inactivating both the AF-1 and AF-2 [51].

### 2.1.8 Tamoxifen biology

One of the main concerns which arise upon treating ERα+ breast cancer with tamoxifen is the agonistic activity displayed by tamoxifen in promoting endometrial carcinogenesis. Tamoxifen shows estrogenic activity in the uterus and bone. This is unlike the second – generation SERM raloxifen, which displays the beneficial estrogenic activity in the bone but is anti-estrogenic in the breast and the uterus [45].

There are several theories that attempt to explain this contradictory role that tamoxifen plays in the uterus. One explanation stems from the fact that tamoxifen binds ERα in the AF-2 domain (Figure 3) of the protein, where it specifically inactivates the activity of that domain, leaving the AF-1 domain still accessible to phosphorylation by growth factor signaling pathways and thus activation, in an environment where such factors are expressed [57, 58]. An associated hypothesis is the relative expression of different co-activators (Src proteins, p300, CBP etc.) and co-repressors (NCoR/SMRT, HDAC complexes like NuRD, etc.) in different tissues.

Experimental data have suggested an ability of tamoxifen-ligated ERα to bind co-activators [59]. Src (Steroid receptor co-activator) -1 is one such protein which is differentially expressed
Figure 3. Structure of ER bound to different ligands
Superposition of the 3D structure of ER LBD complexed with estradiol (green), raloxifen and tamoxifen (red and blue). This structural figure shows the positions taken by the helix 12 of LBD when different ligands are sitting in the LBD of ERα. The alpha helices are depicted as grey cylinders, except the H12, which is shown in color, bound to a ligand.

in the breast and uterine tissue [60]. Higher expression of Src-1 has been postulated to be one of the factors lending to the oestrogenic activity of tamoxifen in the uterus. An over-expression of Src-3 is believed to lead to tamoxifen resistance in the breast [61].

Secondly, it has been shown that E2-ERα and tamoxifen-ERα do not target the same genes. Estrogen can target genes with canonical EREs, half EREs and indirect targets through other transcription factors [62]. Tamoxifen-liganded ERα, owing to its different conformation, would target genes in a promoter context specific way, through the AF-1 domain in the N terminus of the protein as shown by [58]. Differential association of cofactors with tamoxifen-ERα versus E2-ER would result in activation of genes in a promoter and cell specific manner [63].

2.2 Role of epigenetics in breast cancer
Epigenetic changes can be described as stable, heritable changes in the genetic composition without an alteration in the DNA sequence. Several phenomena characterize epigenetic changes, such as histone modifications (methylation, phosphorylation, acetylation, ubiquitination, sumoylation etc.), chromatin structure alteration, DNA imprinting and DNA methylation. Control of expression of the ERα during therapy resistance has been shown to be epigenetic in nature [18]. Breast cancer cells have been reported to have abnormal genetic landscapes and aberrant expression of epigenetic modulators [64]. Use of various histone deacetylase (HDAC) inhibitors and DNA methyltransferase (DNMT) inhibitors have been shown to be therapeutic towards better prognosis for breast cancer patients [65].

2.2.1 Chromatin is made up of DNA wrapped around histone proteins
In order to fit the long strands of DNA into a cell nucleus 10 µm in diameter, the strands of genetic information need to be organized into higher order structures, such that the information contained in them can be accessed in an organized and efficient way. DNA is wound around an octamer of histone proteins consisting of 2 copies each of the four core histone proteins, H2A, H2B, H3 and H4, forming a nucleosome. Each nucleosome has 133 bps of DNA wound around it as well as a linker DNA between two nucleosomes, supported by histone protein H1. DNA wrapped in such a way around the histone proteins, also known as chromatin, can then assemble into higher order structures like the 30 nm chromatin fiber,
famously described as the “beads on a string” structure. These higher order structures materialize during cell replication, but remain in an open conformation before DNA replication [66-68].

2.2.2 **Histone proteins can be modified post-translationally**

The N-terminal tails of the histone octamer proteins are populated with basic residues like lysines and arginines, which form the basis of the interaction of the negative phosphate groups in the DNA backbone with positively charged histone proteins. The basic residues in the N-terminal tails can be modified to contain different modification moieties like phosphorylation, acetylation, ubiquitination, sumoylation, methylation. The modification of the histone tails not only affects the total charge on the protein but also its conformation. The result is an interruption of the interaction of the histone protein with its wound DNA, making the interaction tighter or looser, resulting in formation of heterochromatin or euchromatin respectively [68].

2.2.3 **Histone Methylation / Demethylation**

Where acetyl marks on the tails of histone proteins are usually activating, the effects of methyl marks on the tails of histone proteins are more context dependent. Moreover, histone methyl marks can be present in the form of mono-, di-, or tri-methyl marks, which provides an added level of complexity to this histone modification.

Enzymes responsible for histone methylation, called histone methyltransferases (HMTs), are part of the SET-domain containing superfamily of proteins [69]. The one exception to this rule is the DOT1L family, which methylate H3K79. The SET domain proteins are responsible for transferring methyl marks onto histone 3 lysines 4, 9, 27 and 36 and histone 4 lysine 20 [70]. For example, members of the Suv39h family are responsible for methylating H3K9 (Suv29h1 and G9a) [71]. Polycomb group chromatin regulator enhancer of zeste (EzH2) methylates H3K27, another mark for repressed transcription [72]. These two histone methyl transferases had first been discovered in *Drosophila melanogaster* along with the trithorax group chromatin regulators (trx) that control the expression of HOX genes. The methyl mark on H3K9 is recognized by heterochromatin protein 1 (HP1) via a chromodomain, which leads to formation of transcriptionally silent heterochromatin [73].

The H3K4me (histone 3 lysine 4 methyl) marks are usually indicative of an actively transcribed gene. The H3K4me3 is usually present at promoters of actively transcribed genes, the H3Kme2
in the body and the H3K4me1 in the 3’ region (tail). This mark is usually conferred upon the
gene by the MLL (mixed-lineage leukemia) - family of proteins, which act in concert with ERα
and other cofactors of ERα signaling [74]. Another example of an active-gene methylation mark
is the tri-methyl mark on H3K36, or H3K36me3. It has been shown that this methylation mark
is deposited on the gene body, post-passage of the RNAP II on that gene, by Seta2 as a way of
preventing transcriptional activation from cryptic / “fake” transcription start sites along the body
of the gene [75, 76].

Examples of methylation marks indicating repressed gene transcription are H3K27me3,
deposited by EZH2, in addition to H3K9me3 deposited by Suv39h1 near the promoter and
enhancer of the gene. The H3K9me3 mark has been shown to have contradictory roles,
indicating an active gene in a study by [77] Vakoc et. al., where it was shown that H3K9me3 and
heterochromatin protein 1 (HP1) were present at regions of all active genes examined. It was
further shown that the presence of both factors was dependent upon the presence of an active
RNAPII. According to current data, it might be safe to conclude that the H3K9me3 is activating
when present in the coding regions and repressive when present in the non-coding or regulatory
regions of the gene [78].

2.2.4 Histone Lysine Demethylases (KDMs)

Lysine specific demethylase (LSD)-1 was the first histone demethylase to be discovered, which
removes mono- and di- methyl marks from H3K4 and H3K9. Also known as KDM (lysine
demethylase)1, LSD1 has been shown to act as both a co-repressor [79] (associated with NuRD
and demethylating H3K4me2/me1) and a co-activator [80] (associated with androgen receptor
and demethylating H3K9me2/me1). Following the discovery of LSD1, a superfamily of histone
demethylases (Figure 4) was discovered when Tsukada et a. [81] suggested that the JmJc
catalytic activity / domain in certain proteins could play the role of demethylating methylated
histones.

Also known as 2-oxoglutarate-Fe(II)-dependent deoxygenases, because these enzymes utilize α-
ketoglutarate and Fe(II) as cofactors, the first JmJc (C-terminal Jumonji domain) containing
**Figure 4.** Histone Lysine demethylases from the JmjC family. The names, synonyms, substrate specificities, and domain structures of the proteins are provided. The list of synonyms is not exhaustive, but due to space limitations, only the most relevant are provided. Putative oncoproteins are in red and putative tumor suppressors are in green. (JmjC) Jumonji C domain; (JmjN) Jumonji N domain; (PHD) plant homeodomain; (Tdr) Tudor domain; (Arid) AT-rich interacting domain; (Fbox) F-box domain; (C5HC2) C5HC2 zinc-finger domain; (CXXC) CXXC zinc-finger domain; (TPR) tetra-tricopeptide domain; (LRR) leucine-rich repeat domain; (TCZ) treble-clef zinc-finger domain.
proteins with known demethylase activity were named the KDM2 cluster, consisting of KDM2A and KDM2B, responsible for demethylating H3K36me2/me1.

Subsequent research led to the discovery of different mono- or di- methylated lysines on histones (Figure 4), and mostly it was observed that members of one cluster followed a pattern and retain specificity in demethylation of methyl-lysines. For example, several histone demethylase enzymes have been known to be able to remove the methyl marks from mono- and di-methylated H3K9, namely LSD1 and JMJD1 (KDM3) cluster members. However, only one demethylase has been shown to be capable of demethylating H3K9me3, and it belongs to the JMJD2 (KDM4) cluster of Jumonji family of histone demethylases [82, 83].

**JMJD2B /KDM4B**

JMJD2B first finds mention in a 2006 study by Fodor et al., where it was shown to antagonize H3K9me3 at a pericentric heterochromatin region [84]. This approximately 150 KDa protein contains 1064 amino acids. This protein was found to be a direct target of hypoxia inducible factor – 1 (HIF-1) such that HIF-1 binds to exclusive recognition sites in the gene coding for JMJD2B [85]. As a corollary, cancer cells were shown to express higher levels of JMJD2B [5], possibly due to activation of the JMJD2B promoter by HIF-1 under hypoxic conditions.

The Figure 5 shows the structure of the JMJD2B protein. The catalytic JmjC domain is responsible for mediating contacts with ER [5] and also for the demethylase activity of the protein [84]. The JmjN domain has been known to be obligatorily present with the JmjC domain. The PHD and tudor domains of the protein may mediate interactions of the protein with DNA or motifs in other proteins.

The central role that JMJD2B plays in ERα signaling is evident in the study by Shi et al. [50], where it was shown that JMJD2B coordinates the mutually exclusive demethylation of H3K4 and H3K9, a pattern conserved from yeast to humans. JMJD2B mediated demethylation of H3K9me3 during activated ERα signaling was shown to be a pre-requisite for MLL2 mediated methylation of H3K4 to H3K4me3, and ERα – mediated transcriptional activation. JMJD2B also plays a role in mediating the hypoxic response in cells [85], where it is upregulated under hypoxia in an ERα-dependent manner. The JMJD2B protein has been called the master regulator of ERα signaling by authors of the study [86], where it was observed that the H3K9me3
Figure 5. Structure of the Jmjd2b protein. The figure shows the domain structure of the Jmjd2b protein with the catalytic JMJC domain in green towards the C terminus of the N terminal JMJD domain (in red). The other two domains of note in the structure are the PHD domain and the Tudor domain, both of which have been implicated to have a role in recognizing different DNA motifs. The PHD fingers recognize trimethylated lysines and the Tudor domain recognizes methylated histones.
demethylase was required for transcription of the ERα gene by facilitating co-activation and recruitment of the GATA3 pioneering factor for ERα gene transcription. Since JMJD2B is an ERα target gene itself [5], it forms a feed forward loop to promote target gene transcription.

In our previous study, JMJD2B has been shown to be required for estrogen induced upregulation of one third of estrogen responsive genes. JMJD2B is upregulated in response to estrogen in ER+ breast cancer cell lines, and its depletion in mouse xenografts led to smaller tumors. It was shown to be required for estrogen induced removal of the H3K9me3 mark and the recruitment of ERα and RNA polymerase II (RNAP II) at the enhancer / ERE or the c-MYB gene [5].

2.2.5 Estrogen Receptor α utilizes the epigenetic machinery

There have been reports of ERα co-activators like SRC1, AIB1, and p300/CBP easing access to DNA through their resident histone acetyltransferase (HAT) activity [36, 87]. ERα also interacts with histone deacetylating machinery to repress transcription by recruiting HDACs in multi-protein complexes to the target gene [47]. LSD1 is frequently found to be downregulated in breast cancer, in its capacity as demethylase of H3K4me2 [88]. EzH2 methylates H3K27 and is a binding partner of REA (repressor of ERα activity) [89] and thus was shown to be a negative regulator of ERα signaling.

Trimethylation at H3K4 is correlated with ERα transcriptional activity, and is mediated by mixed lineage leukemia (MLL) 1-4 [90]. Recruitment of MLLs by ERα has been shown in the context of the HOXC13 promoter [91]. As it relates to breast carcinogenesis, there have been studies providing positive correlation for interaction of the MLLs in ER+ breast cancer [92]. Such correlation has also been observed for other ERα cofactors like the members of the Jumonji family, JMJD2B and JMJD3 [65]. JMJD3 provides estrogen dependent regulation for the anti-apoptotic BCL2 gene, by removing the H3K27me3 marks deposited by EZH2 under estrogen signaling [93].

The focus of our study is the H3K9me3 mark. The H3K9me3 (histone 3 lysine 9 tri-methyl) mark in the enhancer of a gene is indicative of repressed transcription and is conferred by SET-domain containing proteins [94, 95], though conflicting studies show that the H3K9me3 mark is also indicative of transcribed regions. This mark has been shown to be removed during active ERα signaling by JMJD2B, a member of the Jumonji family of proteins and the only protein
known so far which is capable of demethylating the tri-methyl H3K9. Given the complexity of regulation that the H3K9me3 mark can provide to ERα signaling, it becomes important to study the modulation of this mark and its role in mediating effects of ERα agonists and antagonists.

2.2.6 Tamoxifen and Histone modifications

Tamoxifen signaling has been shown to recruit cofactors which possess histone modifying capacity; for example, the NCoR /SMRT complex recruited by tamoxifen bound ERα [47] consists of a homolog of yeast Sin3 and HDAC3, which has a deacetylating activity. Long term tamoxifen exposed cells have been shown to have dramatically different epigenetic landscapes compared to parental cells [96]. Tamoxifen alters ERα signaling in the breast tissue through recruitment of different cofactors and targeting different promoters. Its inhibition of the ERα signaling pathway was shown to be dependent on epigenetic modifiers as well. Epigenetics has been shown to play a role in acquired tamoxifen resistance as well, as demonstrated by Fan et al., in a study where anti-estrogen (AE) resistant cells were sensitized to tamoxifen treatment by use of HDAC inhibitors [19].

This study utilizes an ER agonist and antagonist to manipulate ER signaling in order to study the process of a normal cell turning rogue. It serves to identify another checkpoint that addition of tamoxifen imposes on the tumorigenesis in a cancerous cell; this provides information about the mechanism of tamoxifen action and identifies JMJD2B as a possible target for resistance studies.

2.3 Rationale

It has been previously shown in our lab that JMJD2B expression in ERα+ breast cancer patients was one of the top fifteen highly expressed proteins. Using several breast cancer cell lines, it was concluded that JMJD2B expression in ERα+ breast cancer is quite high, as was deduced from an analysis of data on ONCOMINE [97] (Figure 6). It was also observed that JMJD2B expression was sensitive to and dependent on estrogen treatment, in ERα+ breast cancer cell lines [97]. This was not true for the other members of the Jmjd2 cluster of proteins. Therefore, JMJD2B was a natural choice for studying its role in ERα signaling under the influence of ERα ligands [5].

The protein JMJD2B had not been studied extensively before our lab discovered that JMJD2B interacts with ERα in the ER+ breast cancer cell line T47D, in an E2-dependent fashion. This
histone demethylase was inferred to be responsible for causing removal of the H3K9me3 mark at the enhancer of \textit{c-MYB} in the presence of E2, as in the absence of JMJD2B, the E2-dependent demethylation did not occur. In a similar experiment, it was also observed that the presence of JMJD2B was required in the cell for recruitment of ER\(\alpha\) and RNAP II to the enhancer of the \textit{c-MYB} gene, upon addition of E2. Knockdown of JMJD2B in mouse xenograft tumors resulted in formation of smaller tumors, compared to xenografts with wild-type cells. This study highlighted the role of JMJD2B in breast cancer progression under ER\(\alpha\) signaling, and led to the question of whether JMJD2B would be as affected by an ER\(\alpha\) antagonist as by an agonist [5].

The decision to test effects of tamoxifen in estrogen induced signaling comes from a requirement to replicate the state of a breast tumor under tamoxifen therapy, in that the tumor resides in an environment containing both estrogen and tamoxifen. Given the limitations of our model system, we could not replicate the exact ratios of the two drugs as in the breast. Additionally, tamoxifen has been shown to target the epigenetic machinery and other important ER\(\alpha\) co-activators to execute inhibition of ER\(\alpha\) signaling in breast cancer. Furthermore, these two ligands on the ER\(\alpha\) serve as two tools to manipulate ER\(\alpha\) signaling in order to better understand the mechanistic details of this vital pathway for therapeutic manipulation.
Figure 6. JMJD2B is overexpressed in ER positive breast cancer. This shows JMJD2B mRNA samples from 19 studies registered in the OMCOMINE database. Red bars represent JMJD2B mRNA levels in ER positive patient samples and blue bars indicate JMJD2B mRNA levels in ER negative samples. This figure is taken from Kawazu et al., 2011.
2.4 Hypothesis:

*Tamoxifen exerts its inhibitory effects on breast cancer cells by targeting the ERα-JMJD2B axis*

**Aims:**

1. Determine effects of tamoxifen on recruitment of ERα, JMJD2B and RNAPII at the ERE of an ERα target gene, *c-MYB*.
2. Determine if the recruitment above translates into changes in gene transcription.
3. Materials and Methods

3.1 Primers

**mRNA expression**

**c-MYB**

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**JMJD2B**

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**TFF1(pS2)**

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**Chromatin IP**

**c-MYB enhancer**

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3.2 Antibodies

Antibodies for Immunoblotting

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Antibodies for Chromatin IP

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3.3 Reagents

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<tr>
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3.4 Cells

MCF-7 and 293T cells were obtained from ACTT and cultured as per the instructions of the vendor. Cells were maintained at 37°C and 5% CO2.
3.5 Buffer Composition

BC150 Buffer: 20 mM Tris HCl pH 8, 0.1% NP-40, 10% glycerol, 150 mM KCl, 1 mM DTT

ChIP lysis buffer: 10 mM Tris HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP40

ChIP nuclear lysis buffer: 50 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS

ChIP elution buffer: 1% SDS, 100 mM NaHCO₃

ChIP wash Buffer 1: 0.1% SDS, 1% Triton x-100, 2 mM EDTA, 20 mM Tris pH 8, 150 mM NaCl

ChIP wash Buffer 2: 0.1% SDS, 1% Triton x-100, 2 mM EDTA, 20 mM Tris pH 8, 500 mM NaCl

ChIP wash Buffer 3: 0.25 M LiCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris pH 8

ChIP dilution Buffer: 0.5% Triton x-100, 2 mM EDTA, 20 mM Tris HCl pH8, 150 mM NaCl

3.6 Culture Conditions

MCF-7 cells were cultured in DMEM:F12 HAM (1:1) supplemented with 10% FBS (fetal bovine serum), L-glutamine and (1%) penicillin/streptomycin (P/S). MCF-7 cells were plated in DMEM media (phenol red-free media without steroids), supplemented with 10% (charcoal stripped) FBS, L-glutamine and P/S. Cells were starved in the steroid depleted medium for minimum 3 days (maximum 4 days), before estrogen or tamoxifen treatment.

3.7 Chromatin Immunoprecipitation Assay (ChIP)

Cells were plated at a density of 7.0 x 10⁶ cells / 15 cm plate.

3.7.1 Estrogen treatment

17β-estradiol (estrogen) was diluted in 100% ethanol and added to the medium directly to a final concentration of 10nM. Tamoxifen was also diluted in 100% ethanol and added directly to the
medium to a final concentration of 1 µM. The treatment was carried out for either 45 min or 4 hrs.

3.7.2 Crosslinking
The crosslinking of proteins to DNA was performed by adding formaldehyde to a final concentration of 1% to the culture. The plates were placed on a belly-dancer for mixing well and incubated at room temperature for 10 min.

Post-crosslinking, glycine was added to the medium to a final concentration of 0.2 M to neutralize the formaldehyde. The plates were incubated on a belly-dancer at room temperature for 5 min for proper mixing.

3.7.3 Harvest
The media was gently decanted from the plates, and the plates were washed twice with ice-cold PBS (phosphate buffered saline, pH 7.4). The cells were then scraped with a rubber policeman in cold PBS and the cell pellet was washed twice with ice cold PBS by centrifugation at 1100 rpm for 3 min. The supernatant was discarded and the procedure for wash was repeated.

3.7.4 Lysis of the cells and sonication of the nuclei
The washed pellet was resuspended in ChIP lysis buffer (supplemented freshly with protease inhibitors) and pipetted up and down ten times with a p1000. The lysate was then incubated on ice for 10 min. The lysate was then dounced 10-15 times with a B-dounce on ice, to aid release of nuclei. This lysate was collected back in the falcon tubes and subjected to centrifugation at 4150 rpm for 5 min at 4°C.

3.7.5 Lysis of the nuclear fraction
The supernatant from the above process, which consists of the cytosolic fraction, was discarded. The nuclei pellet was resuspended in ChIP nuclear lysis buffer (supplemented freshly with protease inhibitors) and pipetted up and down ten times. The nuclear lysate was incubated on ice for 10 minutes.

3.7.6 Sonication to fragment DNA
The nuclear lysate was then subjected to DNA shearing in a bioruptor. The sonication was carried out in 20 repetitions of 1 minute cycles. Each one minute cycle consisted of 30 sec of no
sonication and 30 sec of active sonication. Total of 6 sets of the 20 repetitions were applied. In between each 20 repetitions, the 1:1 ice : water slurry in the bioruptor was freshened to maintain the temperature of the sonication temperature at low.

Post sonication, the lysate was diluted 1:10 in ChIP dilution buffer (supplemented with protease inhibitors).

3.7.7 Measurement of genomic DNA fragment size
This diluted lysate was checked for fragment size of genomic DNA. 20 µL of the lysate was set aside for size assessment and mixed with 20 µL of TAE. The lysate was incubated with 1uL. Proteinase K at 56°C for 1 hr. 1x volume (40 µL) of phenol-chloroform-isoamylalcohol (25:24:1 in volume) (PCI) was added to the lysate to degrade protein and separate nucleic acid. Post vortexing to mix well and centrifugation at 14000 rpm for 5 min, the aqueous layer was placed in a fresh tube for agarose gel analysis.

After confirming that genomic DNA was fragmented to a size range of 500-1000 bps, the sample was considered suitable for chromatin IP.

3.7.8 Volume of Dynabeads used for IP
10 µL of protein A/ G beads slurry was used per 7.0 x 10⁶ cells for preclearing, i.e., for every 15 cm plate of cells, 10 µL of protein A/G beads slurry was used. For pulling down the protein-DNA complexes with antibody for each treatment, 20 µL slurry of Protein A/ G beads was used per antibody, per treatment (control, E2 or tamoxifen + E2) for (IP) precipitation of DNA with protein specific to the antibody.

3.7.9 Preparation of Dynabeads
Dynabeads are magnetic in nature. Beads were washed three times with IP dilution buffer in the cold room on a slow moving rotor for 5 minutes each. A magnarack (magnetic rack) obtained from Invitrogen was used for pelleting beads at the side of the tube. The beads were then blocked for one hr at 4°C in a cocktail of 10% BSA, 1% single strand salmon sperm DNA (ssssDNA), made upto 1 mL with IP dilution buffer. For every 130 µL of beads, 1 mL of the blocking cocktail was used. The beads were prepared for use by final 3 washes with ChIP dilution buffer after blocking.
3.7.10 Preclearing of chromatin
A preclearing step was performed before the IP to get rid of non-specifically binding protein-chromatin complexes. The samples for each treatment were incubated with beads slurry for 1 hour at 4°C on a rotor. For every 15 cm plate, 10 µL of original (undiluted) volume of the beads slurry was used. The beads were pelleted and the precleared supernatant was saved in fresh tubes.

3.7.11 Chromatin IP
Input to IP sample volume ratio was maintained at 1:3.00 for every independent experiment. From every 15 cm plate, sample was aliquoted into Input and IP against anti-IgG and three other antibodies (4 antibodies in total). For every IP, 2 µg of antibody was used per treatment. Samples were incubated with antibody overnight, at 4 °C on a rotor.

3.7.12 Antibody-Dynabeads conjugation
Post overnight incubation with antibody, 20 µL of beads slurry was added to each sample and incubated for 1 hr at 4°C on a rotor. The sample was then washed with ChIP wash buffers for 5 min each at 4 °C. The order of washes was: 2x ChIP wash buffer 1, 2x ChIP wash buffer 2, 2x ChIP wash buffer 1, 1x ChIP wash buffer 3, 1x TE. These wash buffers offer an environment of increasing stringency for the antibody-protein interaction, and help to eliminate non-specific binding.

3.7.13 De-crosslinking
After the washes, the beads were incubated with 260 µL of IP elution buffer at 65 °C overnight to reverse the crosslinks between the nucleic acid and protein. The input fractions were de-crosslinked by incubating them with SDS to a final concentration of 0.8% and incubating overnight at 65°C.

3.7.14 Phenol Chloroform DNA Extraction
The beads were removed and samples were incubated with 1µL Proteinase K at 37 °C for 1 hr and then subjected to 250 µL PCI (phenol-chloroform-isoamyl alcohol). After mixing well and centrifugation at 12000 rpm for 5 min, the top layer was collected in fresh tubes. To every sample, 2 µL of glycogen was added to enhance the efficiency of precipitation of DNA. One-tenths volume of 3M NaAc and 1-volume of isopropanol were added to the sample and mixed
well. The sample was subjected to flash freezing in dry ice and thawing at RT. Upon centrifugation at 12000, at 4 °C for 30 min, the supernatant was discarded. The DNA pellet was washed again with 75% ethanol and left to dry at room temperature for 20 min before reconstitution with 100 µL TE.

3.7.15 *Quantitative Polymerase Chain Reaction*

1µL of ChIP DNA sample was applied to qPCR. Primers were used at a final concentration of 0.5 µM. I used 5µL of AB SYBRGreen PCR master mix per 10 µL qPCR reaction and made the volume to 10µL with 3 µL of mQ (Nuclease free double distilled water). The cycling parameters for the qPCR were: Denaturation at 95°C for 15 sec. Annealing at 62°C for 30 sec. Extension at 72°C for 30 sec. The procedure for qPCR has been outlined below.

The results were represented as percentage of input, next to IgG Rabbit control.

3.8 *RNA preparation for gene expression*

3.8.1 *Culture conditions*

MCF-7 cells were plated in 10 cm plates at a density of 1.8 x 10⁶ cells/10 cm plate.

3.8.2 *Treatment*

Estrogen was diluted in 100% ethanol and added to the medium directly to a final concentration of 10 nM. Tamoxifen was also diluted in 100% ethanol and added directly to the medium to a final concentration of 1 µM. The treatment was carried out for 12-18 hrs.

3.8.3 *Harvest*

Plates were washed twice with ice-cold PBS and Trizol reagent was added directly to plates. Cells were incubated with Ttrizol reagent for 30 minutes on ice. Phase separation was achieved by adding 0.2x volume of chloroform. Samples were incubated on ice for 2-3 minutes post-mixing well.

Samples were centrifuged for 15 minutes 12000 rpm in the cold. The upper layer was transferred to a fresh tube and 0.5x volume of isopropanol was added to it. The samples were centrifuged at
12000 rpm for 10 minutes and the supernatant was discarded. The RNA pellet was washed once with 75% ethanol and RNA was reconstituted with RNase free water.

3.8.4 Reverse Transcription

1ug of RNA was applied towards reverse transcriptase to convert to cDNA in a 20 µL reaction volume using iScript by BioRad (#170-8840). Briefly, 1ug of RNA prepared as above, was incubated with 10 µL of 5x supermix and 1 µL of reverse transcriptase (RT) enzyme. The reaction was carried out using cycling parameters: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min.

This reverse transcribed DNA was diluted 1 in 5 in mQ and 1uL was applied towards qPCR.

3.9 Quantitative PCR (polymerase chain reaction):

For carrying out the qPCR reaction for the cDNA samples prepared above or the ChIP samples, I mixed 1 µL of cDNA (or DNA) with 5 µL of the SYBR master mix and 0.5 µM of forward and reverse primers. The volume was made upto 10 µL with RNase/DNase free water. Cycling parameters were same as above.

3.10 WST-1 cellular proliferation assay

WST-1 assay is a colorimetric, nonradioactive way of quantifying cellular proliferation. Briefly, the tetrazolium salt WST-1 can be cleaved to soluble formazan by a cellular mechanism residing at the cell surface. The amount of formazan formed gives a direct estimate of the number of metabolically active cells in the medium. (Roche)

MCF-7 cells were plated in 96-well plate at a density of 3.0 x 10^3 cells.

Treatment: Estrogen was added to fresh medium directly, to a final concentration of 10 nM. Tamoxifen was added to fresh medium directly to the medium, to a final concentration of 1 µM.

Cells were incubated with the control or experimental treatment for the indicated number of days. At the same time on the indicated day, the old medium with the drugs was removed and
fresh medium with 1/10 (v/v) WST-1 reagent was added to the plates. The readings were taken at 460nM and the measured absorbance is a direct indication of the number of viable cells.

3.11 Statistical Analyses
A Student’s T-test (one tailed) was used to note statistical significance. The most common trend observed was reported, when it was observed for atleast 3 or more independent experiments, except where otherwise noted. The ChIP data was presented as percentage of input (enrichment over input), alongside control antibody IgG (in grey).
4. Results

Our hypothesis is derived from the numerous studies outlining the role of tamoxifen in targeting the ERα epigenetic machinery to achieve inhibition of ERα dependent breast cancer. It has also been established that histone demethylase JMJD2B acts in the capacity of an ERα co-activator to remove tri-methyl marks from H3K9me3 and activate ERα dependent transcription in the presence of E2. Thus we formulated two aims:

1. To analyze the effect of tamoxifen on recruitment of JMJD2B and RNAPII S2P at an ERα target gene implicated in proliferation.

2. To determine if the above recruitment results in changes in ERα mediated gene transcription.

We began with testing the effects of tamoxifen on proliferation of ER+ breast cancer cells with the understanding that if tamoxifen were to exert any inhibitory effects on co-activators of ERα like JMJD2B, it should be reflected in proliferation of ER+ breast cancer cells.

4.1 Tamoxifen causes reduced cell proliferation in estrogen treated breast cancer cells

Regarding the effect of tamoxifen on breast cancer cells, we performed a cellular proliferation experiment on T47D cells (Figure7). Similar results were obtained in MCF-7 cells.

Steroid-deprived cells were plated in 96-well plates in quadruplicates and kept in medium supplemented with E2 (10 nM) and/or tamoxifen (1 μM) for 3 days. On the third day, the medium was replaced with one containing one-tenth (v/v) WST-1 reagent and the reading was taken at 480 nm using a spectrophotometer.

Using the WST-1 cellular proliferation assay, we observed an increase in cellular proliferation upon treating the cells with estrogen for 3 days. This increase is almost 7 times more than that observed with control. It was also evident that upon addition of tamoxifen to estrogen treatment cells, the increase in cellular proliferation was antagonized and dropped to approximately 0.5x of what was observed with control. This led us to conclude that tamoxifen is inhibitory to the proliferation of ERα+ breast cancer cells (Figure 7). Our findings are supplemented by experiments done in other studies (for example [98]), which also conclude that tamoxifen is inhibitory to the growth of ERα+ breast cancer cells.
Figure 7. Tamoxifen inhibits E2 induced proliferation in ER+ breast cancer cells. T47D cells were stimulated with either control, E2 or tam+E2 for 2 days. Cellular proliferation was assessed using WST-1 cellular proliferation assay 48 hours post addition of the ligands. Error bars represent the mean ± standard deviation of four replicates. * p=0.0266
4.2 Estrogen-activated ERα target genes are inhibited by tamoxifen

The observation that tamoxifen is inhibitory to proliferation of ER+ breast cancer cells led us to further investigate the causes of this reduction in proliferation. Tamoxifen has been known to cause downregulation of several genes pivotal for growth and proliferation. It is a known ERα antagonist and therefore we conducted a gene expression test to explore the state of ERα mediated transcription in the presence of estrogen and tamoxifen.

We treated ERα+ breast cancer cells (MCF-7 or T47D) with estrogen (10 nM) or estrogen and tamoxifen (1 μM) to observe if tamoxifen affects estrogen induced gene expression. We observed that ERα target gene expression increased after treatment of cells with estrogen for 12 hours. This was true for all four ERα target genes tested: c-MYB, JMJD2B, TFF1 and PGR. (Figure 8)

All four of the genes we tested for mRNA expression have been implicated in breast cancer carcinogenesis. c-MYB was a choice for our experiments by reason of the implication of JMJD2B observed in its transcription [5, 99]. Additionally, c-MYB, TFF1 and PGR have been shown to be have roles in proliferation and cell cycle. Our fourth candidate, KDM4B, as shown by Kawazu et al., also has a function in cell proliferation [100].

For all four of the ERα target genes tested, I observed the trend that tamoxifen antagonized the increase in gene expression caused by estrogen. The downregulation of estrogen-induced mRNA expression is more than 2 fold for c-MYB, TFF1, and KDM4B and approximately 3 fold for PGR. I concluded that the reduction in estrogen induced cell proliferation of ERα+ breast cancer cells by tamoxifen can be attributed, at least in part, to reduced estrogen induction of ERα target genes in the presence of tamoxifen (Figure 8).

These experiments were performed as a validation of previously known data; as such, not all of them were repeated the necessary three times in each cell line. The ones which were repeated gave a p-value of 0.11 (c-MYB in T47D) and 0.100 (JMJD2B in T47D). Both these p-values were from comparisons between treatment with E2 and Tamoxifen+E2. Statistically, these values do not appear significant, which is alluded to in the discussion.
**Figure 8. Tamoxifen inhibits mRNA expression of E2 responsive genes.** Steroid deprived T47D cells were stimulated with E2 or Tam+E2 for 18 hours. mRNA analysis was done on cell extracts with qPCR assay to recapitulated previous findings from the literature. Error bars represent the mean ± standard deviation of three replicates. 
*C-myb* (N=3 T47D, N=1 MCF-7), *JMJD2B* (N=3 T47D, N=1 MCF7), *TFF1/pS2* (N=1 T47d, N=1 MCF7), *PGR* (N=1 MCF7).
4.3 Tamoxifen inhibits estrogen induced recruitment of ERα, JMJD2B and RNAPII

In a previous study [101, 102] and our’s [5] we also observed the dependence of c-MYB expression on ERα signaling. When ERα signaling was disrupted by depletion of JMJD2B [5], c-MYB expression was reduced. The c-MYB gene showed the strongest response to estrogen treatments in our lab. Consequently, we chose the c-MYB gene as the ERα target most suitable for our study to detect effects of estrogen and tamoxifen on ERα signaling.

Following the above two observations that tamoxifen antagonizes estrogen induced transcription of ER target genes that play a pivotal part in tumorigenesis [103], especially c-MYB, we wanted to test if tamoxifen was capable of antagonizing the recruitment of transcription factors like ERα, JMJD2B and RNAPII to the enhancer of the ERα target gene c-MYB induced by the ERα ligand estrogen (E2). The presence of RNAP II on the promoter of a gene has been considered a mark of active transcription, and we also included it in our studies to provide affirmation of ongoing transcription [104].

To test our hypothesis that tamoxifen exerts its inhibitory effects on breast cancer cells by targeting the ERα-JMJD2B axis, we performed chromatin IP against ERα, JMJD2B and RNAPII S2P (RNA polymerase phosphorylated on Ser2 in its C-terminal domain) in MCF-7 cells treated with estrogen or estrogen plus tamoxifen. The S2P form of RNAPII is considered to be the actively elongating form, and thus we attempted to determine its recruitment instead of unphosphorylated RNAPII [105]. Effects of tamoxifen on estrogen induced recruitment of these factors to an ERE would help answer the question if the ERα mediated transcription at target gene gets disrupted because JMJD2B enrichment is stalled.

In panel A of Figure 9 the drawing shows the c-MYB gene and the placement of exons along its locus. The orientation 5' to 3' is from left to right with (1) denoting the first exon after the transcriptional start site of the gene. The primers used for the chromatin immunoprecipitation experiments surrounded the enhancer or the estrogen responsive element of the gene near the 3’ end (shown in red) in an intronic region.

ERα (Figure 9B left) : Upon treating the cells with 10 nM estrogen, we noticed a 3 fold increase in the recruitment of the ERα to the enhancer of c-MYB relative to control (EtOH) treated cells. This increase was noticed after a treatment of cells with estrogen for 45 min. Upon adding
Figure 9. Tamoxifen inhibits E2 induced recruitment of transcriptional activators to the c-MYB gene. (A) The figure shows the location of the ERE near the 3’ end on the c-MYB gene as shown previously [5]. (B) Steroid-deprived MCF-7 cells were stimulated with either control (EtOH), E2 (10nM) or Tam (1μM) + E2 (10nM) for 45 minutes. Cell lysates were assayed via chromatin IP for the recruitment of ER, JMJD2B and RNAPII S2P. Enrichment was assayed via qPCR and expressed as percentage of input (blue) alongside chromatin IP for antibody negative control (IgG, grey). (C) Similar to (B), MCF-7 treated with ER ligands, were tested for enrichment of the H3K9me3 mark via ChIP. Error bars represent the mean ± standard deviation of three replicates.
tamoxifen to estrogen treated cells, we noticed that the estrogen induced enrichment of ERα at the c-MYB ERE was inhibited. The treatment duration of 45 min agrees with the previous study in our lab, where maximal ERα enrichment at the c-MYB enhancer was shown to occur at 45 min. The p-value for comparison between E2 and Tam + E2 treatments was noted to be 0.058.

JMJD2B (Figure 9B middle): The same trend as for ERα was observed for recruitment of JMJD2B. The histone demethylase was recruited to the enhancer of c-MYB upon estrogen treatment, the enrichment being five times higher than control. However, the recruitment induced by estrogen was antagonized by addition of tamoxifen by approximately 4 fold. This indicates that tamoxifen not only inhibits estrogen-induced recruitment of ERα but also actively targets other proteins in the ERα signaling pathway. The p-value for comparison between E2 and Tam + E2 treatment was 0.099.

RNAP II S2P (Figure 9C right): There have been reports of paused RNAP II being present on gene promoters while genes are not being transcribed [106]. This trend is most evident in poised genes, which bear marks of both active and repressed transcription. To demarcate between the mere presence of RNAP II on the gene versus RNAP II actively elongating, we decided to test for enrichment of the form of RNAP II which is phosphorylated on the second serine of its C-terminal domain (CTD). This RNAP II S2P is the elongating form of RNAP II as reviewed by [105]. We observed that RNAP II S2P enrichment at the ERE increased 6 fold with addition of estrogen. However, the presence of RNAP II S2P at the ERE in presence of tamoxifen was reduced by 12 fold. The p-value for comparison between E2 and Tam + E2 treatments was 0.089.

From our examination of the literature, nobody has previously presented evidence of tamoxifen-mediated inhibition of recruitment of the H3K9me3 demethylase JMJD2B to an ERα target gene. I would also like to note that some of our results were not statistically significant (0.1> p-value >0.05), but the trend reported here was observed in at least 3 independent experiments.

Since we observed that the presence of JMJD2B is antagonized by addition of tamoxifen, we next examined the methylation status at the H3K9 site at the enhancer of c-MYB. If our hypothesis holds true, we expected to see reduced trimethylation levels at H3K9, but higher trimethylation levels upon addition of tamoxifen. In other words, tamoxifen addition should
inhibit estrogen-induced demethylation of H3K9me3 by virtue of inhibition of JMJD2B recruitment.

I observed that upon 45 min (Figure 9C) of treatment with estrogen, the methylation mark was high despite the high recruitment of JMJD2B to this site. Also, the addition of tamoxifen led to a decrease in the methylation levels on H3K9, when treated for 45 min. This was observed along with a p-value of 0.038 between E2 and Tam+E2 treatments.

Based on this, we concluded that the duration of 45 min is not enough for a change in methylation levels to take place to reflect the effects on gene transcription. Consequently, based on the previous findings from Kawazu et al. (2011), we performed a treatment for 4h.

In Figure 10 we observed that during a treatment with estrogen for 4 hrs, the H3K9me3 is demethylated. This is in parallel to our previous study where estrogen treatment for prolonged period of 4 hrs led to demethylation of H3K9me3. In addition to confirmation of that finding, we observed that addition of tamoxifen abrogated the estrogen induced demethylation of H3K9me3. The observation that estrogen caused demethylation of H3K9me3 and tamoxifen inhibited this demethylation, at 4 hrs, is in complete contrast to the effects of estrogen and tamoxifen observed at 45 min.

From these data, we concluded that the inhibitory effects of tamoxifen on breast cancer cells are propagated at the mechanistic level by antagonizing the estrogen induced recruitment of ERα, ER co-activator JMJD2B and RNAP II S2P to the enhancer of c-MYB. Lack of recruitment of JMJD2B leads to the development of a hypermethylated state at H3K9, which prevents transcription from taking place. At the cellular level, these changes result in reduced estrogen induced gene transcription from the c-MYB gene and reduced proliferation of breast cancer cells in the presence of tamoxifen.

This, to the limits of our knowledge, is the first evidence towards tamoxifen targeting ERα signaling by means of inhibiting the demethylation of H3K9me3, via inhibition of recruitment of JMJD2B. We also show that tamoxifen diminishes levels of estrogen-induced RNAPII S2P at the c-MYB enhancer.
Figure 10. Tamoxifen leads to enrichment of H3K9me3 mark at the c-MYB after 4 h treatment. Steroid-deprived MCF-7 cells were stimulated with either control (EtOH), E2 (10nM) or Tam (1μM) + E2 (10nM) for 45 min (left) or 4 h (right). Cell lysates were assayed via chromatin IP for the enrichment of H3K9me3 mark at the enhancer of c-MYB. The results were expressed as percentage of input (blue), alongside antibody negative control (IgG, grey). Error bars represent the mean ± standard deviation of three replicates.
An important role for JMJD2B is underlined by the results from our study, where we show that the anti-estrogen breast cancer treatment drug tamoxifen targets the JMJD2B – ERα signaling axis to exert its inhibitory effects. In the present study we show that tamoxifen exerts abrogatory effects on estrogen induced ERα signaling. Addition of tamoxifen to ERα+ breast cancer cells treated with estrogen led to slower cellular proliferation. We also observed a reduction in estrogen induced transcription from ERα target genes when tamoxifen was added to the system. Tamoxifen addition targeted estrogen induced JMJD2B recruitment to the ERE of the c-MYB and reduced it, in addition to inhibiting estrogen induced recruitment of ERα and RNAPII to the enhancer. We also observed that presence of tamoxifen led to inhibition of estrogen induced interaction of ERα and JMJD2B in vitro (data not shown and [5]).

Elucidation of one of the mechanisms of tamoxifen inhibition of ERα+ breast cancer growth, and the dependence of this inhibition on the JMJD2B – ERα signaling axis provides us with a focal point to expand our understanding of therapies in the face of tamoxifen resistance. In the future, with more exhaustive studies JMJD2B could prove to be a therapeutic target for overcoming tamoxifen resistance or for treatment of ERα+ breast cancer in addition to tamoxifen therapy.
5. Discussion

5.1 Revisiting the rationale

We began this project with the aim of exploring the ERα-JMJD2B signaling pathway in the context of breast cancer. Several researchers have shown that ERα signaling is inhibited by tamoxifen in the breast and thus it has been used in mechanistic studies to study ERα signaling.

It has been shown that tamoxifen targets not only ERα, but affects the behavior of ERα cofactors as well [47], differentially inhibiting or enhancing recruitment of different cofactors in different tissue types. On the other hand, JMJD2B, as studied in our laboratory, has been shown to be an important ERα co-activator. Such studies, highlighted earlier in the dissertation, form the foundation of my hypothesis which is “Tamoxifen exerts inhibitory effects on breast cancer cells by targeting the ERα-JMJD2B signaling axis”.

Authors of an early study showed that ERα stability is affected by addition of tamoxifen [107]. A previous study [47] showed that the recruitment of ERα and co-activators involved in ERα signaling was diminished when tamoxifen treatment was applied to breast cancer cells. Observing its anti-ERα action in the breast, tamoxifen was designated as having therapeutic potential in the treatment of ERα+ breast cancer. Whereas it can be used to treat all ERα+ breast cancers, virtually 50% of cases develop resistance to the drug. Despite extensive research already focused on the mechanisms of action of this drug, combating resistance still remains a challenge in the management of breast cancer patients.

Treatment of ERα + breast cancer has had numerous studies devoted to it, but the mechanisms of SERM action and resistance still remain incompletely understood. In the current study, I used a SERM, tamoxifen, which is also used as the first line of treatment against the ERα receptor, and ERα agonist estrogen, to understand their contradictory roles in ERα action in the breast. With the help of these two opposing drugs, we investigated the role of JMD2B in ERα mediated transcription in ERα + breast cancer cell lines.

It has been previously discovered in our laboratory that the histone demethylase JMJD2B acts as an ERα co-activator and that its interaction with the hormone receptor is E2 dependent. Histone demethylases and other epigenetic factors have been recruited by hormone receptors to activate or inhibit receptor mediated transcription. For example, androgen receptor (AR) signaling has
been shown to be enhanced by LSD1 (lysine specific demethylase 1) by Metzger et al. [80], the first histone demethylase to be discovered. LSD1 was shown to be present with AR in chromatin-associated complexes and to demethylate mono- and di-, methylated H3K9 and H3K4. LSD1 was also found to be a part of the NuRD (nucleosome remodeling and deacetylase) complex [79] in breast cancer cells. In the same study, it was shown to downregulate breast cancer metastatic potential and oncogenesis. This contradictory role of LSD1 is further complicated by its implication in promoting tumorigenesis in ERα (-) breast cancers in a study by Lim et al., where they discovered high levels of LSD1 in ERα (-) tumors [88]. They found that pharmacological inhibition of LSD1 led to growth inhibition of breast cancer cells, and lack of LSD1 also led to downregulation of various genes associated with proliferation.

Other members of the Jumonji family also play a role in hormone receptor-driven cancers; for example, Liu et al. and Wu et al. discovered that JMJD2C is amplified and overexpressed in more than one breast cancer cell line, especially aggressive basal-like breast cancer [108]. JMJD3B or KDM6B has been shown to be induced by the VDR (Vitamin D receptor) pathway, and knockdown of JMJD3B in colon cancer cells led to reduction of VDR mediated induction of mesenchymal and EMT markers [109]. All of these studies give us enough evidence to hypothesize that JMJD2B might have a significant role to play in ERα mediated transcription, and may be susceptible to inhibition by an ERα antagonist in the breast.

5.2 Summary of results

We were able to determine that tamoxifen caused a reduction in estrogen-induced proliferation of ERα+ breast cancer cells and that this could be at least partly attributed to a reduction in gene expression from ERα target genes responsible for proliferation. We chose one of those target genes, c-MYB, for further exploration. We were able to show that addition of estrogen resulted in higher recruitment of ERα, JMJD2B and elongating RNAPII to the c-MYB locus, after 45 min of treatment, and that this was diminished by addition of tamoxifen. This loss of JMJD2B results in diminished demethylation of the H3K9me3 mark, and thereby maintenance of a “closed” chromatin state at the enhancer of this gene. The addition of tamoxifen also results in diminished enrichment of elongating RNAPII and GCN5 [110] (histone lysine acetyl transferase) to this gene, the latter of which results in diminished acetylation of H3K9 (Figure 11 in appendix).
5.3 Tamoxifen inhibits cell proliferation through a decline in transcription from ERα target genes.

Through a WST-1 cell proliferation assay (Figure 7), I was able to show that tamoxifen indeed inhibits E2 induced cell proliferation in the ERα + breast cancer cell line, MCF-7. We duplicated results in another ERα + breast cancer cell line, T47D. To be able to understand this previously observed result better, I performed gene expression experiments in the two cell lines. The gene expression results led to the conclusion that tamoxifen inhibits mRNA transcription from ERα target genes (Figure 8), which are induced with E2 treatment. The inhibition of estrogen induced cell proliferation observed with tamoxifen treatment could be attributed to the inhibition of gene expression from the ERα target genes.

ERα pathways in breast cancer are a driving force for carcinogenesis. An excellent review of how E2 bound ERα drives expression of a plethora of downstream targets, most of which have activating roles in cell proliferation, growth, metastasis, genomic instability and other phenomena sustaining tumorigenesis was published [100]. Examples of these genes include BCL2, BIRC5 (survivin), c-MYC, c-FOS, c-JUN, ERBB2, and CCND. Early response genes in ERα signaling have been found to have roles in cell cycle, proliferation, DNA replication, anti-apoptosis, and transcriptional regulation, whereas late response genes are involved in pathways like DNA repair and recovery. Therefore, we can conclude that the inhibition of proliferation of the ERα + breast cancer cell line was due to tamoxifen interfering with the ERα signaling pathway. This has been shown in other studies as well, where treatment with tamoxifen led to an inhibition in expression of ERα target genes, which are induced with estrogen [100].

5.4 Tamoxifen inhibits transcription from ERα target genes by inhibiting co-activator (JMJD2B) recruitment to c-MYB

Studies have also shown that tamoxifen inhibits recruitment of co-activators to ERα target genes, which are otherwise recruited by ERα upon estrogen stimulation, and are required for positive transcription. Tamoxifen inhibited recruitment of the transcription factors, which are recruited with estrogen stimulation to ERα target genes. Indeed, tamoxifen caused recruitment of co-
repressor components like NCoR (nuclear co-repressor)/ SMRT (silencing mediator of RAR and THR) complex and NuRD (nucleosome remodelling and deacetylation complex) complex to ERα targets [47]. These co-repressor complexes usually consist of components with histone deacetylating activities, and also play roles in condensing chromatin. Of note here is that normal ERα recruitment on its target genes follows a cyclical pattern, as shown in [38]: its recruitment is highest at 30-45 min after stimulation with estrogen and falls thereafter, only to peak again at 100 min and so on. Considering the peak of ERα recruitment at 45 min as the gold-standard, since we also observed a similar pattern in our laboratory, we tested for ERα recruitment at the 45 min mark in our experiments.

Following the conclusion that tamoxifen inhibits recruitment of ERα co-activators to target genes, which are otherwise recruited under estrogen stimulation, we wanted to test if this applied to another co-activator of ERα, JMJD2B. In a 2013 study [86], the authors implied that JMJD2B is a master regulator of ERα signaling which has roles spanning from control of ERα gene expression itself, to interacting with pivotal protein partners and facilitating co-activator and ERα mediated transcription.

In our experiments, where we treated ERα + breast cancer cell line T47D or MCF-7 with estrogen and tamoxifen, we observed diminished recruitment of JMJD2B to the enhancer of the c-MYB gene, compared to when only estrogen was applied (Figure 9B). This led us to conclude that the JMJD2B-ERα axis, functional in the presence of estrogen, is disrupted by the addition of tamoxifen to the system, by inhibiting the recruitment of JMJD2B to the ERα target gene. JMJD2B is a histone demethylase which removes the tri-methyl mark from H3K9, activating transcription. The discovery of estrogen induced JMJD2B recruitment being inhibited by tamoxifen led us to test for the H3K9me3 mark on the c-MYB enhancer. After treating cells for 45 min with estrogen, the H3K9me3 does not reduce but rises (Figure 9C), whereas it diminishes with the addition of tamoxifen. This result is reversed at 4 hrs, where the H3K9me3 mark is low with estrogen and higher with tamoxifen (Figure 10). Repeated results showing the same pattern led us to conclude that tamoxifen caused a rise in trimethylation levels of H3K9me3 at 4 hrs.

This suggested that even though the recruitment of JMJD2B is affected by tamoxifen at as early as 45 min post-treatment, the H3K9me3 mark required more time to respond to the estrogen or tamoxifen treatment, and become diminished or re-established, respectively.
The model being formed here gives the impression that JMJD2B is required for ERα mediated transcription through its role in demethylating H3K9me3, and also as its putative role in recruitment of other co-factors of ERα transcription [86]. This role of JMJD2B is abrogated by tamoxifen by inhibiting estrogen induced recruitment of JMJD2B to the enhancer (Figure 9B). It is worth mentioning here that the process of methylation/demethylation requires more time to become established (4 hrs) than needed for recruitment of transcription factors (45 min). The lowering of the trimethyl H3K9 in the presence of tamoxifen and absence of JMJD2B (45 mins) (Figure 9C) at the enhancer suggests a temporary compensatory action played by other histone demethylases(s). Experiments to derive the other histone demethylating factor(s) substituting for lack of recruitment of JMJD2B will provide insight into this paradox. These substituting factors might be unable to play their role in demethylating H3K9me3 constitutively because, as we observe at 4 hrs, H3K9me3 levels fall with estrogen treatment and rise with tamoxifen (Figure 10).

We also observed that tamoxifen inhibited recruitment of ERα and RNAPII S2P (as previously noted) despite the presence of E2. Tamoxifen, thus, abrogated estrogen induced recruitment of JMJD2B, ERα and elongating RNAPII at 45 min of treatment. It also caused the estrogen induced demethylation of H3K9me3 to diminish, upon 4 hrs of treatment, thereby providing a possible explanation to the decline in transcript levels of the c-MYB gene.

In appendix Figure 11 we provide another example of tamoxifen targeting an epigenetic co-activator which is part of the ERα mediated transcriptional complex. GCN5 or KAT2 (Lysine (K) Acetyl Transferase) is responsible for depositing acetyl groups on H3K9 [111], resulting in an open chromatin conformation for active transcription. Though this experiment was performed independently only twice, it shows that tamoxifen inhibits recruitment of GCN5 to the c-MYB enhancer, which is otherwise recruited upon stimulation with E2 (Figure 11A). Notably, the H3K9Ac levels at the enhancer of this gene diminish with tamoxifen, possibly as a result of the absence of GCN5 (Figure 11B). This particular chromatin IP was performed only once, and further confirmation of this speculation is required, but this single experiment is in agreement with the hypothesis that tamoxifen targets epigenetic co-activators of ERα to inhibit breast cancer cell proliferation.
5.6 Model

The emerging model, within the scope of our project and those studies highlighted earlier, define JMJD2B as an ERα co-activator which is required for recruitment of ERα to the target gene enhancer. The addition of tamoxifen to a system stimulated with estrogen interferes with the ERα-JMJD2B signaling axis by inhibiting the recruitment of both factors to the target gene, in addition to RNAPII and GCN5. It also inhibits the demethylation of the H3K9me3 mark at the enhancer. I would like to take the speculations discussed above further, and propose a model for the JMJD2B role and tamoxifen action, inferred from previous literature.

It was shown in an unpublished experiment from our lab that a subunit of the mediator complex, Med1 interacts with JMJD2B according to a mass-spectrometry analysis. That the Med1 protein turned up as a hit in a JMJD2B interacting partners screen lends weight to the supposition that JMJD2B plays a more important role than just histone demethylation and opening the chromatin.

We suggest a model in which JMJD2B is responsible for recruiting ERα to the target gene, but it may not be sufficient for carrying out this role. Addition of tamoxifen can have side-effects on ERα which are unconnected to JMJD2B. JMJD2B can putatively make contacts with the Med1 (part of the Mediator complex), thereby aiding in DNA looping between the basal transcription machinery at the promoter and the enhancer, where the co-activators and ERα are present. A study in RNAPII stalling emphasizes the role played by ERα in recruiting P-TEFb (containing CDK9), which is responsible for relieving RNAPII pausing by phosphorylating DSIF, NELF and the polymerase at Serine 2 in its CTD [101]. This study provides proof of distant genomic sites coming together during the transcription of the c-MYB gene to form an active chromatin hub (ACH), as has been shown in [112], which explains how the promoter and enhancer regions could act in concert. Similar observations of polymerase pausing and DNA looping at the c-MYB gene have been made by other authors as well.

It will be useful to question whether the loss of JMJD2B in the cell results in lack of DNA-looping ability, and if this same phenotype is also visible upon treatment of wild type cells with tamoxifen. In other words, it will be important to answer the question if lack of JMJD2B has the same phenotype in ERα+ breast cancer cells as addition of tamoxifen.
5.7 Future Directions and Insights

It would provide more insight into the role of JMJD2B if a cell proliferation experiment is performed under the same treatments, but with a $KDM4B^{-/-}$ cell line derived from the parental MCF-7. A similar cellular proliferation experiment, though without testing for tamoxifen, was performed by Kawazu et al., which showed that as with tamoxifen addition, estrogen induced cellular proliferation of an ERα+ breast cancer positive cell line was reduced in the absence of JMJD2B. The lack of JMJD2B displaying a similar phenotype to addition of tamoxifen could suggest that the effects of tamoxifen on the cell may be resulting through inactivating the JMJD2B pathway, i.e., tamoxifen targets the same pathway as JMJD2B. The same study also performed a gene expression assay in the absence of JMJD2B, and obtained the same results as with addition of tamoxifen – the estrogen induced rise in gene expression from ERα target genes was reduced by the lack of JMJD2B, further strengthening the above implication that tamoxifen targets the JMJD2B signaling pathway.

Several studies have implied that another Jumonji family member JMJD2A may act as a histone demethylase for H3K9me3 in the absence of JMJD2B [113, 114]. However, it needs to be determined if it plays a role in ERα signaling in the presence or absence of JMJD2B. Other interacting partners of ERα from the Jumonji family in ERα+ breast cancer cell lines need to be established as well.

The rise in the H3K9me3 mark at 4 hrs of treatment also raises several interesting queries. An important experimental question here is the status of JMJD2B, ERα and RNAPII under estrogen and/or tamoxifen treatment at 4 hrs and various time points in between. The low-resolution method we utilized did not allow us to temporally identify the precise shift from methylated to demethylated (and vice-versa) with regards to the H3K9me3 mark.

It will be also informative to test for transcript levels under our conditions at various time points from 45 min to 4 hrs to answer the question: At what point during the inhibition of transcription factor recruitment, under tamoxifen treatment, does transcription collapse? Interestingly, Romano et al observed that tamoxifen affects transcription levels as early as 50 min [115], though this might be due to differences in culture conditions.
An interesting question arises when other non-phosphorylated forms of the RNAPII are considered. In our studies we tested for the Ser2 phosphorylated form on RNAPII only, as this is the actively elongating form. However, this does not discriminate between tamoxifen inhibiting recruitment of the polymerase to the locus or the phosphorylation of the polymerase once it has been recruited.

Another important avenue of research would be to test the recruitment levels of other cofactors, and the levels of H3K9me3 at other ERα target gene enhancers, such as TFF1, BRCA1, PGR, etc. It was shown in the Kawazu study that absence of JMJD2B inhibited the estrogen induced recruitment of RNAPII and ERα to the DNA, but if this applies to other ERα co-activators as well, then it will define the role of JMJD2B as pivotal in recruiting the transcriptional machinery to the target gene. The scope of the current project could be extended from researching one gene to an affirmation of similar results on other ERα targets that could help in providing a more generalized model of the role of JMJD2B in ERα signaling and the effect of tamoxifen on it.

It is also worthwhile to note that our experiments do not test for levels of the Histone 3 (H3) protein upon treatment with E2 or tamoxifen. As histone density is a major determinant of chromatin structure, and thus accessibility to the transcriptional machinery, it would be paramount to analyze those levels in similar chromatin IP experiments. Results from ChIP of H3 on c-MYB will also put the levels of the H3K9me3 mark in perspective, helping us realize if the methyl mark rises due to increase in histone density or is independent from it.

A very important direction for going further in this research would be to test for JMJD2B and ERα interaction in the presence of tamoxifen. This experiment can be done both in vivo and in vitro with the help of recombinant proteins. There can be two possibilities: either tamoxifen will inhibit the interaction or it will not. It is already known that tamoxifen alters ERα conformation, so either the change in conformation of ERα effects the interaction with JMJD2B or it does not. If the interaction is inhibited with tamoxifen, it would explain the rising levels of H3K9me3 mark at the enhancer, and also account for the diminishing transcription. If the interaction is maintained despite tamoxifen, then the conformation of the LBD of ERα may not play an important role in interaction with JMJD2B.

This raises the question of how does JMJD2B get recruited to the DNA itself and if it has a DNA binding domain. As shown in the Figure 5, the structure of JMJD2B contains two domains.
known for interacting with different histone motifs: the tudor domain and the PHD domains. It has not been shown, however, that JMJD2B indeed makes physical contacts with DNA. An electrophoretic mobility shift assay (EMSA) may provide helpful answers as to whether JMJD2B and which part of the protein, interacts with DNA. The mechanism of how JMJD2B contacts the DNA will also provide insight into whether or not it is dependent on ERα for interaction with the DNA or vice versa. Another way to assess this question is the test for recruitment of JMJD2B to an ERα target gene, in a parental ERα + cell line, treated with shRNA against ERα.

The previous study in our lab showed how JMJD2B was induced only in ERα+ breast cancer cell lines upon stimulation with estrogen. But does this mean that ERα is sufficient to cause induction of JMJD2B with estrogen stimulation? Another avenue of inquiry is the time of RNAPII and other basal transcriptional machinery loading at the promoter. There are two views when it comes to receptor activated transcription. One school of thought promotes the possibility that the polymerase is pre-loaded at the promoter, with the basal transcriptional machinery transcribing weakly or stalling. The binding of an activated receptor causes the transcription to shift into an ultra-efficient mode by providing support to the basal machinery at the promoter via the ATP dependent chromatin remodeling machinery and histone acetylase containing complexes which open up the chromatin allowing the RNAPII to escape pausing. The other view is that the loading of the activated HR occurs first, followed by recruitment of other cofactors like the ATP dependent chromatin remodeling machinery and histone acetylase containing complexes, which open up the chromatin at the enhancer and the promoter, with the help of the mediator complex. This removes steric hindrance for the bulky RNAP holoenzyme, and the advent of the transcriptional machinery results in transcription. In view of the promoter-escape paper [101] and the low levels of RNAPII already present at the c-MYB enhancer in the absence of E2, I favor the first opinion. However, this remains to be tested through a time-log chromatin IP, looking for the status of RNAPII and other basal transcription factors along the promoters of target genes.

One essential consideration for all of these experiments is their low level of significance. Most of the experiments reported here do not warrant a high level of credibility if assessed from the level of their significance and the high p-values. However it should be noted here that for chromatin IP experiments, the same trend was observed in 3 or more independent experiments. Even if the p-
values are high, it is worth more experimentation based on the same trend repeated in multiple repetitions, pointing to tamoxifen disrupting ER signaling via interfering with JMJD2B recruitment.

The mRNA expression data, even though previously validated in the literature, also turned up low significance values. This could only be attributed to human error due to initial optimization of conditions. The experiments in this study which could not be validated 3 independent times, like Figures 10, 11, and mRNA expression data, need to be validated. Completion of this work will require that these aims are achieved so that non-refutable conclusions can be established. Repeating these experiments multiple times will also provide a way to test for statistical significance more stringently, and perhaps improve it.

In conclusion, we have established an essential role for JMJD2B in ERα signaling and that it is a target of the inhibitory effects of the SERM tamoxifen. Tamoxifen may sever the interaction between ERα and JMJD2B; even if it does not, JMJD2B remains essential for ERα recruitment to the ERα target gene c-MYB. However, tamoxifen does affect recruitment of ERα and its associated machinery to the target gene. Whether or not JMJD2B plays the role of a master transcription factor remains to be investigated, but our studies point a way for JMJD2B to be an important therapeutic target for ERα (+) breast cancer. This is especially significant in light of the high levels of tamoxifen resistance observed in breast cancer patients.

The role of JMJD2B in signaling pathways of other hormone receptors will provide more insight into the roles of this histone demethylase, and the side effects of using it as a therapeutic target.
6. Appendix

Figure 11

Figure 11. Tamoxifen leads to inhibition of E2-induced acetylation of H3K9(B) due to inhibition of E2-induced recruitment of GCN5 (A). ERα+ breast cancer cells were treated with indicated drugs for 45 min and ChIP was performed. The antibody control (IgG) is shown in grey and the tested GCN5 antibody is shown in blue. Results are shown as enrichment over input. Error bars represent the mean ± standard deviation of three replicates.
7. References


87. <p>300 is a component of an estrogen receptor coactivator complex. PNAS-1996-Hanstein-11540-5.pdf>.</p>


