UNIVERSITY OF MIAMI

THE ROLE OF CLASS I HISTONE DEACETYLASES IN THE TRANSCRIPTIONAL REPRESSION OF ESTROGEN RECEPTOR IN RESPONSE TO HYPERACTIVE MAP KINASE SIGNALING

By

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Estrogen receptor (ER)-negative breast cancer is more aggressive and associated with both shorter disease-free and overall survival than ER+ breast cancer. While anti-estrogen therapies have greatly improved treatment for ER positive breast cancer, both de-novo and acquired resistance occurs. Anti-estrogen therapies are not effective in ER negative breast cancers, thus identifying mechanisms underlying lack of ER expression in ER- breast cancers is imperative. Hypermethylation of the ER promoter has been demonstrated to repress ER, and is found in ~ 25% of ER- breast cancers. Previously we demonstrated that hyperactivation of ERK1/2 MAPK (hMAPK) downstream of overexpressed EGFR or overexpression/amplification of Her2 represses ER protein and mRNA expression. Abrogation of hMAPK in ER-negative breast cancer cell lines and primary cultures causes re-expression of ER and restoration of anti-estrogen responses.

hMAPK-mediated ER mRNA repression involves repression of transcription. We found that the ER mRNA synthesis rate and ER promoter activity, assessed with run-on assays and ER promoter reporter constructs, respectively, were significantly reduced in hMAPK-MCF-7 cell line models, with little to no ER
expression, compared to control-transfected MCF-7 (coMCF-7) cells with high ER expression. Co-transfection of dnERK1/2 abrogated this repression restoring promoter activity to levels comparable with coMCF-7s but promoter sequences responsible for this hMAPK repression could not be isolated. Thus we hypothesized that epigenetic mechanisms may be involved in hMAPK mediated repression of ER transcription; however, ER promoter methylation is not exhibited in our hMAPK-MCF-7 models, nor in several breast cancer cell lines with hMAPK.

In this study, we performed an epigenetic compound screen in the ER negative SUM 149 breast cancer cell line, which exhibits hMAPK, had been previously demonstrated to re-express ER with MAPK inhibition, and which does not exhibit hypermethylation of the ER promoter. Three candidate compounds, all HDAC inhibitors, were identified, validated in individual luciferase assays, and evaluated with rtPCR and Western blotting to verify their ability to modulate ER expression and estrogen signaling. They were further validated in additional ER negative cell lines and clinical tumor samples lacking ER promoter methylation, as well. Finally, they have been used in our hMAPK-MCF-7 cells to demonstrate the role of hMAPK in histone deacetylation of the ER promoter.

We identified HDAC1, 2, and 3 as playing a role in ER transcriptional repression and as being modified, both in activation and expression, by ERK1/2 MAPK signaling. Additionally, histone acetylation at the ER promoter was increased in response to either MEK or HDAC inhibition. Simultaneous treatment
with a MEK and an HDAC inhibitor led to synergistic restoration of ER mRNA expression.

While HDAC inhibitors have been evaluated previously for their ability to sensitize anti-estrogen resistant ER positive breast cancers and re-express ER in ER negative breast cancer cell lines exhibiting ER promoter methylation, histone deacetylation of ER in ER negative breast cancers lacking ER promoter methylation has not been previously identified. Collectively, these data link histone deacetylation as an underlying mechanism in hMAPK-repression of ER mRNA suggesting this is a common epigenetic mechanism in the generation of ER negative breast cancer.
Dedication

I would like to dedicate this dissertation to my wonderful fiancé, Laura Parsons
Acknowledgements

There are so many people who have helped me survive this PhD. I would first like to thank my family for their never-ending encouragement and support over these past five years. Whether I needed to blow-off steam or needed someone to tell me that I was doing a great job (even if I wasn’t) they were there for me. I’d also like that thank my mentor Dorraya for her guidance and for believing in me and in my project when I was having trouble believing in myself. Thank you to my committee for supporting me through so many tough years with slow progress and for encouraging me to press on through it all. Thanks to Phil Miller for the advice, guidance, and comedic relief in the lab all these years. Above all, I want to thank Laura. She’s been there for me through all of my emotional breakdowns and no matter how things are going she makes me happy.

Thank you all.
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>AF</td>
<td>Activation factor</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein Kinase B</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>Cbp</td>
<td>C-terminal Src kinase-binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CoREST</td>
<td>REST co-repressor</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DT</td>
<td>Dissociated tumor</td>
</tr>
<tr>
<td>DUSP</td>
<td>Dual-specificity phosphatase</td>
</tr>
<tr>
<td>E2</td>
<td>17-beta estradiol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetate</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR/HER1</td>
<td>Human Epidermal Growth Factor Receptor 1</td>
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<tr>
<td>ER</td>
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<tr>
<td>ERBB2/HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
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<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<tr>
<td>ERUR</td>
<td>ER upstream region</td>
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<td>ERUBF</td>
<td>ER upstream binding factor</td>
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<td>E26 transformation specific</td>
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<td>GAPDH</td>
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<td>Histone deacetylase inhibitor</td>
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<td>hMAPK</td>
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<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
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<td>ICI</td>
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<td>IDC-NOS</td>
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<tr>
<td>Ig</td>
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<td>IGF-IR</td>
<td>Insulin-like Growth Factor 1 Receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IMEM</td>
<td>Improved modified essential media</td>
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<td>LBD</td>
<td>Ligand-binding domain</td>
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<td>MAPK</td>
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<tr>
<td>MEK</td>
<td>MAPKK; mitogen/extracellular-signal regulated kinase</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
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<tr>
<td>NCoR</td>
<td>Nuclear co-repressor</td>
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<tr>
<td>NSC</td>
<td>Non Silencing Control</td>
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<tr>
<td>NuRD</td>
<td>Nucleosome remodeling deacetylase</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Ribonucleic Acid interference</td>
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<td>RPPA</td>
<td>Reverse phase protein array</td>
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<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SCW</td>
<td>Sonication, ChIP, Wash buffer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl-sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
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<tr>
<td>SERD</td>
<td>Selective estrogen receptor downregulator</td>
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<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcript 3</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate buffer</td>
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<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween20</td>
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<tr>
<td>TDLU</td>
<td>Terminal duct lobuloalveolar unit</td>
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<td>Thr</td>
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<td>Tyrosine Kinase Inhibitor</td>
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<td>Trichostatin A</td>
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<td>TSS</td>
<td>Transcriptional start site</td>
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<td>UM-CTI</td>
<td>University of Miami Center for Therapeutic Innovation</td>
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CHAPTER 1: BACKGROUND

1.1 BREAST CANCER

1.1.1 Breast cancer incidence and mortality

Breast cancer is the leading cause of cancer death in women worldwide [1]. It is estimated that 1 in 8 women will develop breast cancer over the course of her lifetime (approximately 12%) and in 2013, an estimated 232,000 new cases of breast cancer were expected to be diagnosed [2]. Incidence rates for breast cancer in women have been dropping since the year 2000, and this drop is thought to be due to the reduced use of hormone replacement therapy (HRT) following publication of the Women’s Health Initiative study [3]. White women are more likely to develop breast cancer than black women in the United States; however, in women under 45, the risk of developing breast cancer is higher for black women compared to white [2]. Additionally, black women have a higher risk of dying from breast cancer than white women. The risk of developing breast cancer in Asian, Hispanic, and Native-American women is lower than that of black or white women [2].

Women who have a first degree relative (mother, sister, daughter) who has had breast cancer have double the risk of developing breast cancer themselves [4]. Gene mutations leading to breast cancer development account for approximately 5 to 10% of breast cancer incidence, and these commonly occur in BRCA1 and BRCA2 genes leading to a 55 to 65% chance of developing breast cancer prior to the age of 70 [4].
Many of the risk factors for breast cancer are factors over which women have no control. These include being a woman, growing older, and genetic predisposition. Less than 1% of all diagnosed breast cancers occur in men, and two out of three invasive breast cancers occur in women over the age of 55. However, a number of other breast cancer risk factors exist which women are able to control, such as being overweight, lack of exercise, cigarette smoking, and unhealthy diet [4]. Other factors that can reduce the risk of breast cancer include having a full-term pregnancy before the age of 30, breastfeeding (especially for longer than 1 year), and later age of first menstruation and early age of menopause. The common trait between each of these risk factors is the amount of estrogen to which a woman is exposed throughout her lifetime. Increased concentrations of circulating estrogens over time have been strongly linked to increased breast cancer risk [5]. Additionally, differentiation of mammary epithelium during pregnancy and subsequent breast-feeding is thought to reduce the number of epithelial structures vulnerable to malignant transformation [6].

It has recently been reported that women who have received radiation to the chest during childhood for the treatment of cancer have a much higher risk of developing breast cancer as an adult [7]. A study found that these women have a risk of breast cancer that is 20 times higher than the general population, and about 30% of the women in the study had developed breast cancer by the time they were 50 years old. The increased risk in these women appeared to be linked to the size of the area receiving radiation for the childhood cancer, which those receiving low dose radiation to a large area and those receiving high dose
radiation to the “mantle field” consisting of lymph nodes in the neck, chest, and armpits having extremely high breast cancer rates [7]. These women also experienced double the mortality rate due to breast cancer than average.

1.1.2. Breast cancer classification systems

1.1.2.1. Histopathological classification of breast cancer

Breast cancer is a heterogenous disease consisting of a growing number of recognized biological subtypes. Following a diagnosis of breast cancer, analyses must be performed in order to predict prognoses and responses to treatment of individual breast cancers. The histopathological method of breast cancer classification has been the traditional classification scheme for many years, and utilizes antibodies to known breast cancer markers as well as phenotypic characterization of the tumor and tumor cells [8]. The four main subtypes of breast cancer as defined by immunohistochemical analysis are determined by the expression of the estrogen receptor (ER), the progesterone receptor (PR), and ErbB2/HER2 [9]. These four subtypes include ER+/PR+/HER2+, ER+/PR+/HER2-, ER-/PR-/HER2+, and ER-/PR-/HER2- [9]. These classifications have both prognostic and therapeutic value, with the triple negative (ER-/PR-/HER2-) group faring the worst, while the ER+/PR+/HER2-fares the best. In instances where there is a lack of fresh tissue available to gene expression analyses, and/or gene analysis is cost prohibitive, immunohistochemical classification of breast cancer is readily available.

Breast cancer morphological analyses classify the majority of breast cancers (70-80%) as invasive ductal carcinomas- not otherwise specified (IDC-
NOS), while approximately 25% are characterized as lobular, tubular, medullary, or metaplastic [10]. Tumor grade additionally allows breast cancers to be stratified in a prognostically meaningful way. Tumor grade assesses tumor differentiation, such as tubule formation and nuclear polymorphisms, and proliferative activity, based on mitotic index [11].

1.1.2.2. Molecular classification of breast cancer

Molecular profiling of breast cancers has been made possible with expression analysis using microarray-based technology [12]. Clinically relevant molecular subtypes have been identified and provide insights into the molecular heterogeneity of breast cancers. The differences in gene expression patterns indicate basic alterations in the cell biology of the tumors and are associated with significant variation in clinical outcomes. From these analyses emerged 5 molecular subgroups of breast cancers identified by their unique molecular profiles: luminal A, luminal B, HER2 overexpressing, basal-like, and normal like [8]. These molecularly defined groups are able to predict clinical outcomes, with the luminal subtypes having the best outcomes and the HER2 positive and basal-like groups having the worst. While these classifications were determined based on expression of 426 genes, a more refined classification method has been established utilizing only 50 genes. The PAM50 assay uses these 50 genes to classify single breast tumor samples into one of the 5 intrinsic breast cancer subtypes which consist of luminal A, luminal B, HER-2 enriched, basal-like, and claudin-low [13, 14].
Additional molecular classifications systems of breast cancers have been created. For example, analysis of differentially expressed genes in ER negative breast cancers led to their classification into 4 molecular subgroups [15]. Other studies have identified additional intrinsic subtypes known as Claudin-low or mesenchymal-like breast cancers [16]. This subtype is characteristically triple-negative and carries a prognosis that is intermediate between the basal and luminal subtypes. These tumors also seem to be enriched for cells showing biological properties associated with mammary stem cells/tumor-initiating cells [12, 17-20].

Gene expression profiling has also been used to develop prognostic signatures for clinical use. Two of these have been approved and include Mammaprint, a microarray-based assay of the Amsterdam 70-gene breast cancer signature, and OncotypeDX, a PCR-based assay of 21 genes [21, 22].

Though these molecular subtypes were not created with histopathological subtypes in mind, they bear a strong resemblance. Luminal A breast cancers, as classified by molecular characteristics, are extremely similar to low-grade, ER/PR positive breast cancers. The HER2+ breast cancers by immunohistochemistry correspond to HER2 overexpressing/amplified breast cancers, while basal-like breast cancers are associated with immunohistochemical expression of p63, cytokeratin5/6, EGFR and c-kit, as well as often being ER/PR/HER2 negative [23].

Molecular classification techniques may not replace histopathological analyses of individual breast cancers for some time. In addition to the expense of
microarray analyses, this classification method incorporates gene expression from non-tumor tissue into that of the tumor cells. In this manner, traditional immunohistochemistry appears superior to microarray analysis, though the information garnered is not as substantial.

Breast cancer is a collection of several biologically different diseases. There are large-scale molecular differences between ER positive and ER negative breast cancers that reach far beyond the presence or absence of ER, as is the case with HER2 expression, as well. It is likely that additional molecular subtypes of breast cancer exist within or outside of the 5 molecular subtypes discussed here.

1.2 ESTROGEN RECEPTOR

Approximately 65-70% of breast cancers express the estrogen receptor, and the majority of these breast cancers depend on estrogen-dependent signaling for their survival and proliferation [1]. The advent of targeted therapy for breast cancers largely focuses on modulation of estrogen receptor signaling and has resulted in dramatic decreases in breast cancer mortality in recent decades. However, resistance to estrogen receptor modulation remains a significant problem and the mechanisms of such resistance are not yet completely understood.
1.2.1. Estrogen receptor structure and function

The biological actions of estrogens are mediated by binding to two estrogen receptors (ERs), ER\(\alpha\) and ER\(\beta\). The ERs are ligand dependent transcription factors, belonging to the nuclear receptor superfamily. The ERs contain six structural domains that are defined by the putative functions contained in each region [24] (figure 1-1). These include the highly conserved DNA-binding domain (DBD), which contains two zinc fingers that mediate DNA binding, and the ligand-binding domain (LBD), which mediates estrogen binding. There are also two domains with transcriptional activation functions (AFs). AF1 regulates transcriptional activation in response to phosphorylation and acts independent of ligand binding, whereas AF2 is ligand dependent, regulating transcriptional activation upon estrogen binding [25-27]. ER\(\alpha\) and ER\(\beta\) share a high degree of sequence similarity with the exception of the NH\(_2\) terminal domain, which is truncated in ER\(\beta\) [28]. The characterization of ER\(\alpha\) and ER\(\beta\) knockout mice have revealed distinct, non-redundant, roles for the two ERs [29] and ER\(\beta\) seems to have opposing effects on ER\(\alpha\) induced proliferation [30-32]. Despite this, both ER\(\alpha\) and ER\(\beta\) seem to have similar affinities for estrogen and bind the same DNA response elements [33]. As it is the better characterized of the two and the focus of this dissertation, the mechanism of ER\(\alpha\) activation will be discussed here and henceforth mention of ER will refer to ER\(\alpha\).

Binding of estrogen to ER results in a conformational change within the receptor that enables dimerization. Dimerization initiates binding of ER to estrogen response elements (ERE) located in the promoter region of estrogen-
regulated genes [34, 35]. Activation of estrogen target gene expression then occurs by recruitment of the general transcription machinery by the activated ER to the transcription start site (TSS) (Figure 1-2A). This is mediated by co-activator complexes, which associate with estrogen-bound ER dimer and tether the activated receptor to the basal transcription machinery [36]. Additional co-factors with histone modifying and chromatin remodeling activities are also recruited, which facilitate gene transcription by overcoming the repressive effect of condensed chromatin [37].

Figure 1-1. Schematic representation of human ERα and ERβ. Both receptors contain six functional domains, A-F. DBD = DNA Binding Domain, LBD = Ligand Binding Domain, AF-1 and 2 = Activating Function domains 1 and 2. The percentage amino acid similarity between ERα and ERβ is indicated for ERβ [38-40].
Figure 1-2. Models of estrogen signaling. A. The classical pathway of genomic estrogen signaling involves binding of estrogen to ER resulting in ER activation. This causes a conformational change, leading to ER dimerization and direct DNA binding to estrogen response elements (ERE) in the promoter region of estrogen target genes. Target gene expression is then modulated. B. The non-classical genomic estrogen signaling pathway involves activated ER binding to other transcription factors. In this context, target gene expression is modulated by indirect binding of ER to DNA. C. In this postulated model, estrogen binds ER in the membrane or cytoplasm. D. Activated ER mediates cellular responses by a non-genomic mechanism that involves modulation of second messenger activation. E. Alternatively, activated ER can stimulate intracellular signaling cascades that modify the transcriptional activities of nuclear ER [38].

Along with its role as a transcription factor for estrogen-regulated genes, ER can also act as a co-activator for other transcription factors in the nucleus, where it modulates gene expression at regulatory DNA sequences such as AP1, SP1, and upstream stimulation factor sites [41-44]. In this context, ER is tethered to the specific promoter complex by its interaction with other DNA bound transcription factors such as c-Jun or c-Fos (figure 1-2B). In addition to the well-studied transcriptional effects of estrogen signaling in the nucleus, there are also rapid effects that occur within minutes after the addition of estrogen [45]. These
effects appear to be mediated by ER located in the cytoplasm or plasma membrane (figure 1-2C). Activation of cytoplasmic/membrane-bound ER by the addition of estrogen results in second messenger activation leading to a rapid response that does not involve modulation of gene expression (figure 1-2D). It may also result in the activation of growth factor signaling that, in turn, enhances genomic nuclear ER signaling [46-49] (figure 1-2E).

Many of the genes regulated by estrogen signaling promote tumorigenic phenotypes, including increased cellular proliferation, inhibition of apoptosis, promotion of invasion, metastasis, and angiogenesis [50]. Of particular importance are cyclin D1 [51, 52], c-Myc [53, 54], and GREB1 [55], all of which are essential drivers of estrogen stimulated cellular proliferation and tumorigenesis.

Cyclin D1 binds to and activates CDK4/6, which phosphorylate the retinoblastoma protein (Rb), resulting in release of the E2F transcription factor, and progression through the restriction point within the G1 phase of the cell cycle [56-58]. Inhibition of cyclin D1, either with antibodies or by expression of the CDK4 inhibitor p16\textsuperscript{INK}, prevents estrogen stimulation of cellular proliferation and progression through the G1 checkpoint [59]. c-Myc is a proto-oncogene transcription factor that regulates the expression of a large number of target genes that promote cell growth and cell cycle progression [60, 61]. Similar to cyclin D1, inhibition of c-Myc prevents estrogen stimulated cell proliferation [62]. Conversely, overexpression of cyclin D1 or c-Myc can reinitiate cell cycle progression in anti-estrogen arrested cells [63]. GREB1 is an early response...
gene regulated by ER and was discovered to be a key regulator of estrogen-induced breast cancer proliferation [55]. Studies have shown that following GREB1 silencing, estradiol induces growth inhibition rather than proliferation of ER positive breast cancer cells. These results indicate that cyclin D1, c-Myc, and GREB1 are essential drivers of estrogen stimulated cellular proliferation.

1.2.2. Estrogen receptor as a prognostic indicator in breast cancer

Estrogen receptor expression is an important prognostic indicator of disease course, with ER positive breast cancers faring significantly better than ER negative [64-68]. Approximately 30-40% of breast cancers present as ER negative and these cancers are associated with a more aggressive phenotype and decreased disease-free survival. Studies have indicated that survival of patients with ER negative and node negative breast cancers is similar to that of ER positive, node positive cancer patients [69].

Estrogen receptor status and tumor status of progesterone receptor, an ER responsive gene, can predict disease free survival and overall survival and are significantly correlated with histologic subtype and degree of differentiation of individual tumors [70-73]. Furthermore, these cancers do not respond to anti-estrogen therapies such as selective estrogen receptor modulators (SERM), selective estrogen receptor downregulators (SERD), or aromatase inhibitors (AI). Approximately 60% of those cancers expressing ER respond to anti-estrogen therapies, and the response correlates with the degree of ER expression, with those expressing higher levels of ER being the most likely to respond [74]. The alternative therapy to anti-estrogens in breast cancers that are non-responsive is
chemotherapy, which results in far greater toxicity to the patients [75]. In addition to those cancers presenting as estrogen receptor negative, as many as 50% of initially ER positive breast cancers recur following remission as ER negative. Many ER positive tumors that are initially responsive to tamoxifen become resistant over the course of treatment and may lose their ER expression, as well [76-79].

1.2.3. Estrogen receptor as a therapeutic target in breast cancer

The gold-standard treatment of estrogen receptor expressing breast cancers is anti-estrogenic therapies. In the 1970s, a selective estrogen receptor modulator (SERM), tamoxifen, was first tested in breast cancer patients and became a widely utilized therapeutic [80]. Though additional SERMs are now present in the market, tamoxifen remains the most widely studied and prescribed anti-estrogen and has made a significant impact in the outcomes of endocrine responsive breast cancer patients [81]. As well as treating currently existing breast cancers, anti-estrogens have also been shown to prevent the occurrence of contralateral breast cancer, as well as prevent/reduce the severity of osteoporosis in post-menopausal women [82-84].

In addition to SERMs, the pure estrogen antagonist ICI 182,780 (faslodex/fulvestrant), a selective estrogen receptor downregulator (SERD), is another widely utilized anti-estrogen therapeutic. While tamoxifen exhibits estrogen agonist actions in endometrial tissue and may stimulate breast tumors/metastases that previously responded to it as an antagonist, faslodex
does not incur these responses [85]. Clinical trials demonstrated that faslodex is effective in some women following disease progression on tamoxifen treatment.

Though endocrine therapies have provided substantial improvements in the treatment options available to breast cancer patients, resistance, both de novo and acquired, remains a sizeable problem. While some underlying mechanisms leading to anti-estrogen therapy resistance have been uncovered, there are likely a variety of signaling/epigenetic modifications responsible for resistance in a majority of patient tumors [78]. It is thought that the lack of success in clinical trials may be due to the variety of resistance mechanisms and it is necessary to tailor interventions for individual patients on a case-by-case basis.

1.3 REGULATION OF ESTROGEN RECEPTOR EXPRESSION

1.3.1. Estrogen receptor expression in normal breast

The first study analyzing estrogen receptor expression in the normal breast was performed by Peterson et al in the late 1980s and found that only a small population of epithelial cells in the normal breast express ER [86]. These ER positive cells were seen exclusively in the breast parenchyma, consisting of the ducts and lobules, with no staining seen in the stroma of the breast. Approximately 7% of the epithelial cells in the ducts and lobules stained positively for ER and these cells were distributed as single cell entities surrounded by ER negative cells [86]. The majority of the ER positive cells were located in the lobules as compared to the interlobular-ducts and tended to be located in intermediate positions between basal epithelial and luminal epithelial
cells. These cells stained negative for CALLA, an antigen present at the lateral membrane of basal/myoepithelial cells in the breast, indicating that they lack the differentiated myoepithelial morphology.

While it has been shown that luminal epithelial cell proliferation and progesterone receptor expression in the normal breast is stimulated by estradiol, only a small percentage of these cells express ER [87]. Clarke et al examined the distribution of ER-expressing cells in the terminal duct lobuloalveolar unit (TDLU) as well as the distribution of proliferating cells. They found that there is almost complete dissociation between steroid receptor expression and proliferation markers in normal breast luminal epithelial cells (figure 1-3). However, they also found that this was not the case in human breast tumors, where proliferating cells were observed to express ER [87]. The proliferating cells in normal breast were found in close proximity to those cells expressing ER. These findings led the authors to propose that either ER is downregulated in breast epithelial cells passing through the cell cycle, or that proliferating cells and those expressing ER are distinct populations.

Shoker et al examined ER and Ki67, a proliferation marker, expression in normal pre-menopausal breast lobules and calculated the number of double-labeled cells that would be expected were the expression of each independent [88]. They found a strong inverse correlation (p = 0.0003) between the expression of ER and the expression of Ki67, indicating that they are not expressed independently of one another, but rather they are mutually exclusive. Additionally, this group saw that ER positive breast epithelial cells increase with
age and Ki67 positive cells decrease. This increase in ER positive breast epithelial cells with age could be due to reduced exposure to circulating estrogens as a woman ages and undergoes menopause, supporting the theory that estrogen exposure and stimulation of proliferation downregulates ER expression.

Figure 1-3. Estrogen receptor and Ki67 staining do not overlap in normal breast epithelial cells. Breast lobular epithelial cells were stained for ER (green) and Ki76 proliferation associated antigen (red/orange). Nuclei containing both ER and Ki67, if present, would appear yellow in color [87].

1.3.2 Estrogen receptor downregulation in response to activation

Eckert et al first analyzed estrogen receptor expression in response to estradiol treatment in ER positive breast cancer cells [89]. Peak nuclear estrogen receptor levels were seen within 1 hour following ER agonist treatment and a 50% loss of total cellular ER occurred between 2 and 5 hours post-treatment.
Additional studies have demonstrated an approximately 60% decline in ER protein expression within 6 hours following estradiol treatment, while mRNA expression decreased about 90% as compared to levels prior to treatment. This reduction in protein and mRNA expression persisted to 24-48 hours [90]. Prolonged exposure to estradiol (96-120 hour) resulted in the maintenance of depressed ER protein levels, though ER mRNA levels returned to normal pre-treatment values [91].

Additional studies found that reduced ER protein expression was apparent by 1 hour following estradiol treatment, and therefore determined that ER protein stability was being reduced in response to estradiol treatment [92]. Proteasomal protease inhibitors were found to prevent this rapid loss of ER protein, while lysosomal protease and calpain protease inhibitors were not effective. These studies were performed on both lactotrope cell line PR1 as well as ER positive breast cancer cell line MCF-7, indicating that this mechanism of ER protein downregulation in response to estradiol treatment may be a common regulatory pathway for ER [92]. Nawaz et al found that proteasomal inhibition in HeLa cells transfected with an expression plasmid for ER and a reporter plasmid containing estrogen response elements (ERE) and treated with estradiol prevented the loss of ER protein [93]. In vitro incubation of ER protein in the presence of ubiquitin activating (E1) and ubiquitin conjugating (E2) enzymes led to accelerated degradation of ER protein which was prevented by treatment of cells with a proteasomal inhibitor. Western blotting of these cells for ER following proteasomal inhibition revealed higher molecular weight bands when probed with
an ER antibody suggesting the accumulation of polyubiquitinated ER within these cells [93].

There continues to be controversy concerning the necessity of proteasomal turnover of ER for its transcriptional activity [94-98]. Nawaz et al hypothesized that ubiquitination and proteasomal degradation of ER may serve to dissociate the ER-containing pre-initiation complex [93]. ER binds to ligand complexes in the nucleus with a co-activator (SRC3/AIB1 in breast cancer cells) and with CSN5/Jab1 [99]. The phosphorylation of ER or SRC3/AIB1 leads to recruitment of the complex to estrogen-regulated promoters and upon transcriptional activation the promoter is cleared. It is thought that this promoter clearance may be facilitated through polyubiquitination of this complex leading to the export of ER from the nucleus, as it lacks a nuclear export signal [99]. However, the inhibition of nuclear exportation by leptomycin B was able to inhibit ER degradation and increase hormone-dependent transcription [99].

In contrast, Lonard et al found that treatment with a proteasomal inhibitor prevented ER transcriptional activation of estrogen responsive luciferase reporter construct in HeLa cells [97]. The proteasomal inhibition prevented estrogen induced transcription of the progesterone receptor, as well. One explanation presented to explain the conflicting results of studies analyzing the necessity of ubiquitination and proteasomal turnover of ER for its transcriptional activity relates to the duration of proteasomal inhibitor treatment [99]. Callige pointed out that the experiments leading to the conclusion that ER turnover has no effect on transcriptional activity inhibited proteasomal activity for 3 hours or less, while
those finding that ER turnover was necessary for transcriptional activity use treatment times of longer than 12 hours [99]. These longer treatment times may lead to ER transcriptional activity loss due to the altered expression of co-regulators rather than altered turnover of ER.

Though the repression of ER protein in response to estradiol treatment has been extensively studied, mechanisms involved in the repression of ER transcription in response to ligand binding are not fully understood. A 2009 study by Ellison-Zelski et al discovered that repression of ER transcription following ligand-induced activation of ER involves the recruitment of co-repressor complex Sin3a to the proximal ER promoter [100]. This co-repressor complex consists of a Sin3a scaffold protein as well as HDA1, HDAC2, RbAp46/RbAp48 (stabilize the complex to nucleosomes) and SAP18/SAP30 (stabilize the interaction between Sin3a and HDACs). Sin3a does not contain a DNA binding domain but is targeted to promoter through interactions with DNA-binding or adapter proteins [101, 102]. Ellison-Zelski et al discovered that activated ER binds its own promoter following ligand binding and recruits the co-repressor complex Sin3a to the proximal promoter. The presence of Sin3a at the proximal promoter overcomes activating factors, such as AIB1 and p300, present at both the distal and proximal promoter, leading to transcriptional repression. In response to estradiol treatment, levels of acetylated H3K14 were reduced at the ER promoter thus preventing RNA polymerase II binding and repressing ER transcription [100].
Additional studies on the dysregulation of ER transcription identified an enhancer site in the 5'-flanking region of the ER gene [103]. This enhancer is located in the region between -943 to -793bp upstream of the transcriptional start site (TSS) within the ER upstream region (ERUR). In ER positive breast cancer cells it is bound by an enhancer, the ER upstream binding factor (ERUBF1). ERUBF-1 was found to be a complex of 4 proteins and was shown to lead to 20-fold higher induction of an ER promoter luciferase reporter construct [103].

Bartella et al discovered that ectopic expression of ERβ reduced ERα mRNA and protein levels in ERα positive breast cancer cell lines [104]. Using a reporter vector containing the ERα promoter region, they found that overexpression of ERβ reduced ERα promoter activity. A series of 5' promoter deletion reporter constructs were used to identify which portion of the ERα promoter was responsible for this downregulation by ERβ and they found repression relied on the -1000 to +212bp region of the promoter [104]. An Sp1 site located at -245 to +212bp region of the promoter was found to be necessary for the ERβ mediated down-regulation of ERα promoter activity. A complex containing ERβ, Sp1, and the co-repressor complex NCoR was identified as binding to the Sp1 site and mediating this ERα transcriptional repression.

1.3.3. Estrogen receptor expression from ADH through IDC

The presence and distribution of estrogen receptor positive cells in normal breast and throughout the spectrum of in situ hyperplasia to invasive ductal carcinoma have been studied. As was mentioned previously, ER expression in normal breast tissue is present in a minority of cells and these cells are
distributed as single ER positive cells surrounded by a number of ER negative cells [87]. The number of ER positive cells in the normal breast increases with a woman’s age, a change that may be due to reduced circulating estrogen levels. With this increase in ER positive cells in the breast comes the tendency for them to accumulate in patches [105]. Almost all cells in an atypical ductal hyperplasia (ADH) of the breast express estrogen receptor, and because of the high number, expression is obviously in neighboring cells. Similar ER expression was seen in lobular in situ neoplasia (LIN) and low-grade ductal carcinoma in situ (DCIS), though staining was slightly weaker in LIN [105]. High-grade ductal carcinoma in situ express lower levels of ER and a substantial proportion of them (approximately 25%) are fully ER negative (figure 1-4) (Allred, 2004). Additionally, unlike normal breast epithelial cells in which the dividing cells and ER-expressing cells are distinct, the majority of dividing cells in breast cancer precursors are ER positive. Invasive breast cancers, similar to high grade DCIS, express a variable range of ER [106]. Approximately 65-75% of invasive breast cancers express ER, though the percentage of cells expressing ER can range from less than 1% to almost 100%.

It has been noted that many invasive breast cancers presenting as ER negative overexpress certain membrane growth factor receptors [106]. Epidermal growth factor receptor (EGFR), Human EGF receptor 2 (HER2), and fibroblast growth factor (FGF) have been implicated as alternative mechanisms of growth that are able to bypass the classical estrogen-induced mitogenic pathway.
1.4. MECHANISMS OF ER REPRESSION IN ER NEGATIVE BREAST CANCERS

In breast cancer, ER promoter methylation was the first discovered mechanism underlying the ER negative phenotype; however, it was discovered that this hypermethylation only exists in a minority (approximately 25%) of ER negative breast cancers [107]. Rather than mutations, deletions, loss of heterozygosity or polymorphisms within the gene, ER negativity has also been shown to be caused by upregulation of tyrosine kinase induced signaling pathways leading to the proteasomal degradation of ER and loss of ER mRNA [108-111]. Overexpression of EGFR and ErbB2/HER2 are associated with ER negativity and lead to activation of MAPK and PI3K/AKT signaling pathway
activation. Overexpression of EGFR is seen in 50% of breast cancers while HER2 overexpression/amplification is seen in approximately 20% and their expression is inversely correlated with ER expression.

Src, a proto-oncogenic tyrosine kinase, may play a role in the regulation of the estrogen receptor via the ubiquitination and proteasomal degradation pathway [112]. Src overexpression has been shown to increase the ubiquitination of ER leading to its proteasomal degradation and decrease ER protein levels when transfected into ER positive breast cancer cells, but does not affect ER mRNA levels [109, 112]. In addition, Chu et al have demonstrated that Src inhibition can increase estrogen regulated gene expression and is able to restore the anti-estrogen response. Proteasomal inhibitors have been shown to cause the re-expression of ER in cell lines and the inhibition of certain E3 ubiquitin ligases has done the same [93]. Proteasomal inhibitor MG132 is able to block the estrogen induced down regulation of ER and the expression of ubiquitin ligase E6AP is inversely correlated to ER expression [113].

1.4.1. Epigenetic control of estrogen receptor expression

Literature on the mechanisms leading to ER negativity in breast cancer cite ER promoter methylation and hyperactive growth factor signaling as the two known causes. The laboratory of Nancy Davidson was first in identifying methylation of ER promoter CpG islands as being associated with the lack of ER expression in breast cancer. Hypermethylation of CpG islands in the promoter regions of genes leads to silencing of the associated gene through the inhibition of transcription or via recruitment of chromatin remodeling complexes. Studies
from her laboratory in 1994 showed that ER and PR negative breast cancers lack or express low levels of ER mRNA [114]. They concluded that methylation of certain CpG islands present in the ER promoter is associated with ER negativity in breast cancer cell lines and tumors and that this may be the mechanism of transcriptional repression of ER in some breast cancers. They also discovered that the treatment of breast cancer cell lines containing hypermethylated ER promoters with the demethylating agent 5-aza-2'-deoxycytidine results in the loss of ER promoter methylation and re-expression of functional ER protein [107].

These studies led to increased interest in DNA methyltransferase inhibition and epigenetic modulation in general as possible therapeutic targets in ER negative breast cancer. Evidence of interactions between DNA methyltransferases and histone deacetylases led investigators to the discovery of histone deacetylation at ER promoters with methylated CpG islands (figure 1-5) [115]. Post-translational histone acetylation has been characterized as gene expression modulation leading to significantly increased transcription. These acetyl groups are added to histones by histone acetyltransferases (HAT), whereas histone deacetylases (HDAC) remove acetyl groups from histones leading to transcriptional repression. Treatment of ER negative cells with methylated ER promoters with HDAC inhibitors led to re-expression of ER, though to a lesser degree than is seen with DNMT inhibitors. The combination of a demethylating agent and an HDAC inhibitor led to significantly higher ER expression [115, 116].
Figure 1-5. Epigenetic regulatory factors of the ER promoter region. Representation of epigenetic silencing of ER in some ER negative breast cancers due to CpG island methylation and histone deacetylation. Image from ‘Epigenetics of estrogen receptor-negative primary breast cancer’ [117].

It was reported that approximately 25% of ER negative breast cancers exhibit hypermethylation of the ER promoter; however, all studies on epigenetic restoration of estrogen receptor expression have been performed using cell lines with methylated ER promoters raising questions concerning epigenetic control of ER expression in the absence of ER promoter methylation [107, 118-120].

1.4.2. Hyperactive growth factor signaling leading to loss of ER expression

Hypermethylation of the ER promoter provides a mechanism for the transcriptional repression of ER in approximately 25% of human breast cancers [107]. However, this loss of ER transcription, and therefore all ER expression in
these cancers, does not explain how these cancer cells are able to survive and proliferate following the loss of their primary mitogenic signaling. Upregulation of tyrosine kinase induced signaling pathways present an additional mechanism leading to the ER negative phenotype in breast cancer and likely plays a role in those breast cancers exhibiting methylation of the ER promoter as well.

A significant inverse relationship exists between the expression of erbB family growth factor receptors and estrogen receptor in breast cancer. Epidermal growth factor receptor (EGFR) is overexpressed in approximately 50% of ER negative breast cancers while HER2 is overexpressed or amplified in approximately 20% [121]. When ligand bound or overexpressed, these growth factor receptors dimerize and initiate downstream signaling cascades such as extracellular-signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling (figure 1-6) [122].
Figure 1-6. Representation of the ERK1/2 MAPK signaling cascade in response to activation by dimerized receptor tyrosine kinases. Activated MAPK signaling transmits cell growth and proliferation signals to the nucleus through ERK1/2 phosphorylation. ERK1/2 then regulates cell cycle progression and transcription through the phosphorylation of transcription factors and physical binding to gene promoters [123].

Establishing a causative link to the inverse association between overexpressed growth factor receptors and ER were the observations that stable transfection of growth factor signaling components led to both estrogen-independent growth and reduced ER expression in ER positive breast cancer cell lines [124-130]. Our lab has created a number of MCF-7 cell line models of hyperactive MAPK signaling by transfection with constitutively active c-erbB2 [126], ligand-inducible EGFR [128], and constitutively active forms of cRaf1 [124] and MEK1 [131]. This induction of hyperactive MAPK signaling leads to a reduction of ER expression and the inhibition of MAPK signaling through transfection of dominant negative ERKs or siRNA against ERK1/2, or through
pharmacologic MEK inhibition leads to the restoration of ER expression [131]. Ex vivo tissues and primary cultures from human ER negative breast cancers also exhibited re-expression of ER upon inhibition of MAPK signaling and subsequently responded to anti-estrogen therapies [132].

Our laboratory has discovered that hyperactive MAPK signaling leads to rapid destabilization of ER protein through ubiquitination and proteasomal degradation. Additionally, ER promoter activity is reduced and ER mRNA is destabilized in response to hMAPK. The mechanisms leading to the transcriptional repression of ER are poorly understood, despite previous efforts by our lab to uncover them. They investigated the transcription factor binding occurring at the ER promoter in the presence and absence of hMAPK. DNase hypersensitivity assays of the endogenous ER promoter were performed on our hMAPK MCF-7 (ca)Raf cell line model to determine if hyperactive MAPK signaling results in altered transcription factor binding at the ER promoter (figure 1-7). The assays were performed on (ca)Raf MCF-7 and control MCF-7 cells and revealed loss of numerous hypersensitive sites upon hMAPK signaling, suggesting altered transcription factor binding (i.e. enhancers). However, new hypersensitive sites appeared, indicating a gain of binding (possibly repressors). Loss of sites occurred around 200 bp downstream (+200) of the transcriptional start site (TSS), as well as 400 bp and 1000-1900 bp upstream (-400, -1000 to -1900), while gains occurred at approximately 800 bp upstream (-800) of the TSS and 600 bp downstream (+600).
Having observed transcriptional repression of ER and alteration of DNase I hypersensitive sites by hyperactive MAPK signaling, they then sought to identify the potential loss of enhancer binding or gain of repressor binding suggested by the DNase I hypersensitivity assay. This analysis was performed using ER promoter-luciferase reporter deletion constructs containing a luciferase gene that is expressed in response to ER promoter activation with regions of the ER promoter deleted. To evaluate the importance of these deleted promoter regions in the repression of ER promoter activity by hyperactive MAPK signaling, dominant negative (DN) ERK1 and 2 were co-transfected with the ER-promoter luciferase constructs and the ability of DN ERK1/2 to reverse repression of ER promoter activity was assessed across the promoter deletion series. ER
promoter activity is reduced in both ca(Raf)-MCF-7 and ca(erbB-2)-MCF-7 cells compared to control MCF-7s, and co-transfection of DN ERK1/2 increases promoter activity in the hMAPK cell lines but not control MCF-7s (figure 1-8). The promoter deletion series indicates that the entire -3500 to +210 region of the promoter is responsive to DN ERK1/2 reversal of promoter repression (figure 1-9). Only deletion from +210 to +135 abrogated the DN ERK1/2 effect. However, this construct was not transcriptionally active in either wild-type MCF-7 cells or the hMAPK MCF-7 cells. Therefore there was no activity for hMAPK to repress, nor repression for the dominant negative ERKs to relieve. These results indicate that until enough of the ER promoter is deleted, no specific ER promoter sequence exists that mediates the repressive action of hMAPK signaling on ER transcriptional repression. Collectively, these data suggest that chromatin structure or accessibility may be altered by hMAPK leading to the hypothesis that epigenetic mechanisms play a role in the transcriptional repression of ER in response to hMAPK signaling.
Figure 1-8. Dominant negative ERKs relieve the repression of ER transcription in MCF-7 hMAPK cell line models. ER promoter luciferase assays with control MCF-7 cells grown in estrogen depleted media (co-MCF7(E2)), (ca)Raf- and (ca)erbB2-MCF-7 cells transfected with an ER promoter luciferase reporter and either a control vector or dominant negative ERK expression vectors. Results are fold change relative to ERP-luc/vector of same cell line (normalized to 1).

Figure 1-9. DN-ERKs reverse losses of ER mRNA in response to hMAPK signaling even when regions of the ER promoter are deleted. (ca)Raf cells (black bars) and (ca)erbB-2 cells (clear bars) were transiently transfected with a full length ER promoter (-3500 to +230)-luciferase construct or the indicated deletion mutants (5' deletions in C and 3' deletions in D) in the presence of pCEP4L (vector) or equal amounts of dnERKs 1 and 2 (dnERKs). Cells were analyzed for luciferase activity 48 hrs after transfection.
1.5. SUMMARY

Although great success in the treatment of ERα positive breast cancer has been achieved with endocrine therapies, their effectiveness is limited by the significant number of breast cancers that do not express estrogen receptor, and thus are de novo resistant. The underlying mechanisms leading to the ER negative breast cancer phenotype remain incompletely understood. Further work is therefore required to uncover mechanisms suppressing ER expression in order to identify potential therapeutic targets for the treatment of ER negative breast cancer.

Currently, there are two main mechanisms established for the ER-phenotype. The first discovered was hypermethylation of the ER promoter, a permanent repressive mechanism seen in approximately 25% of ER negative breast cancers. Dysregulation of growth factor signaling results in a dynamic and reversible repression of ER protein and/or mRNA expression, and has been shown to play a role in a much larger fraction of ER- breast cancers [132, 133].

Studies have demonstrated that DNA methyltransferase and histone deacetylase inhibition are able to induce the re-expression of ER mRNA in ER negative cell lines exhibiting methylation of the ER promoter [107, 114, 118]. Our laboratory has demonstrated that inhibition of MAPK activity in several ER negative breast cancer cell lines without ER promoter hypermethylation and ER negative breast cancer primary cultures restores ER expression at both mRNA and protein levels. Mechanisms underlying MAPK-induced repression of ER expression include targeting of ER protein for proteasomal degradation, reduced
mRNA stability, and repression of transcription of ER mRNA that is seen as both a reduced rate of ER transcription and reduced ER promoter activity. Specific promoter sequences responsible for the MAPK action on transcription could not be identified, suggesting chromatin structure may be involved.

This thesis describes an epigenetic small molecule enzyme-modulator screen to identify modifiers of estrogen receptor transcriptional activity. We demonstrate the ability of HDAC inhibition to cause re-expression of ER mRNA in ER negative breast cancer cell lines lacking ER promoter methylation, establishing HDAC mediated transcriptional repression as a common mechanism in breast cancers with methylated or unmethylated ER promoter CpG islands. Additionally, we have identified HDAC-mediated transcriptional repression of ER as the downstream effector of hMAPK ER repression by showing that hMAPK-dependent hypoacetylation of ER promoter-associated histones contributes to the repression of ER transcription by hMAPK signaling.
CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Chemicals, Compounds, and Solutions

Compounds

The following compounds were purchased from the indicated manufacturers:

- U0126 (Upstate, 9903)
- DMSO (Sigma, D2650)
- PD90859 (Sigma, p215)
- 17B-estradiol (Sigma, E8875)
- CI994 (Sigma, C0621)
- SAHA (Sigma, SML0061)
- M344 (Enzo Life Sciences, 270-297)
- Selumetinib (AstraZeneca, AZD6244)
- Faslodex/Fulvestrant (Sigma, ICI182780)

Solutions

PBS: 137mM NaCl, 2mM KCl, 8mM Na2HPO4 1.5mM KH2PO4 in H2O. pH adjusted to 7.4 with HCl.

37% Formaldehyde (Sigma)

RIPA lysis buffer (Pierce): 50mM Tris pH 8.0, 150mM NaCl, 0.1% SDS, 0.1% deoxycholic acid (DOC), 1% TritonX-100, 50mM NaF, 1mM Na3VO4 and Halt protease and phosphatase inhibitor cocktails.

Gold lysis buffer: with protease inhibitors (Leupeptin, sodium orthovanadate, Aprotinin, Pepstatin, Pefabloc).
Western transfer buffer: 100 mL Tris-Glycine and 200mL methanol were made up to a final volume of 1L with H2O.
Western wash buffer: 100 mL 10X Tris buffered saline and 1mL Tween20 (BioRad) were made up to a final volume of 1L with H2O.
Western running buffer: 100 mL Tris-glycine-SDS made up to a final volume of 1L with H2O.
TE buffer: 10mM Tris-HCl (pH 7.5 or 8.0), 1mM EDTA.

**Compound library**

The library contained 60 epigenetic compounds at 60mM in 1.2% DMSO to be used at a final concentration of 10mM (0.2% DMSO) for screening purposes. Compounds included HDAC inhibitors, HDAC stimulators, histone lysine deacetylase inhibitors, HIF1a inhibitor, histone demethylase, histone acetyltransferase inhibitor, sirtuin inhibitors and sirtuin activators (Table 2.1).
Table 2-1. Compounds represented in the plate-based compound screen. Compounds include: HDAC inhibitors, HDAC stimulators, histone lysine deacetylase inhibitors, HIF1a inhibitor, histone demethylase, histone acetyltransferase inhibitor, sirtuin inhibitors (type III HDAC), etc.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Action</th>
<th>Compound</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichostatin A</td>
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<td>BIX-01294-3HCl</td>
<td>Histone methyl transferase inhibitor</td>
</tr>
<tr>
<td>2,4-Pyrindinedicarboxylic Acid</td>
<td>Histone demethylase inhibitor</td>
<td>Butyroactone 3</td>
<td>HAT inhibitor</td>
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<td>HAT inhibitor</td>
<td>CTPB</td>
<td>HAT inhibitor</td>
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<td>SIRT-2 inhibitor</td>
<td>Oxamflatin</td>
<td>HDAC inhibitor</td>
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<tr>
<td>BML-210</td>
<td>HDAC inhibitor</td>
<td>Sirtinol</td>
<td>SIRT inhibitor</td>
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<td>Apicidin</td>
<td>HDAC inhibitor</td>
<td>Suramin-6Na</td>
<td>SIRT1 inhibitor</td>
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<td>HDAC inhibitor</td>
<td>BML-278</td>
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<td>NCH-51</td>
<td>HDAC inhibitor</td>
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<td>Scriptaid Neg control</td>
<td>Ci-994</td>
<td>HDAC inhibitor</td>
</tr>
<tr>
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<td>DNA Me transferase inhibitor</td>
<td>NSC-3852</td>
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<td>SIRT2 inhibitor</td>
<td>CUDDC-907</td>
<td>P38K/HDAC dual inhibitor –</td>
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<td>CUDDC-101</td>
<td>HDAC/EGFR/HER2 Inhibitor –</td>
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<td>HDAC inhibitor</td>
<td>UNC1215</td>
<td>L3MBTL3 Domain Inhibitor –</td>
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<td>MI-2</td>
<td>Menin-MLL Inhibitor</td>
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<td>WDR5-C47</td>
<td>WDR5-MLL (SET1) Antagonist</td>
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</tbody>
</table>
2.1.2. Cell lines

Cell lines used in this study were established cell lines, MCF-7 cell line models created in the El-Ashry lab, and dissociated tumor lines created from primary breast tumors. The co-MCF-7, co-MCF-7/lt-E2, (ca)Raf-MCF-7, EGFR/MCF-7, and ErbB2-MCF-7 cell lines were created as previously described [124, 131]. The co-MCF-7 and co-MCF-7/lt-E2 cells were stably transfected with a control expression vector and differ only in that the co-MCF-7/lt-E2 cells are maintained in estrogen-depleted media. (ca)Raf-MCF-7 cells were selected from a clonal population of MCF-7 cells stably transfected with a constitutively active Raf construct containing a deletion of a regulatory domain (ΔRaf), resulting in constitutive activation of Raf and downstream MAPK signaling. ErbB2-MCF-7 cells were created through the stable transfection of a wild-type erbB2 expression vector, leading to overexpression of erbB2. This clone exhibited high constitutive autophosphorylation of the erbB2 receptors and activation of downstream pathways, including the MAPK signaling pathway. EGFR-MCF-7 cells were stably transfected to overexpress EGFR, leading to some constitutive activation of downstream MAPK signaling by autophosphorylation of receptors, but also inducible by EGFR ligands to hyperactivate MAPK signaling. Steve Ethier at the University of Michigan created the SUM cell lines used. SUM149 cells originated from an invasive inflammatory ductal carcinoma, are ER and PR negative, and overexpress active EGFR resulting in activation of MAPK signaling. SUM229 cells were taken from a pleural effusion, overexpress EGFR, and are also ER and PR negative. MDA-MB-231 cells are a triple-negative breast
cancer cell line. Cells were incubated in a 37°C, 5% CO₂ forced-air humidified incubator. SUM 149 and SUM 229 cells were grown in F-12 Nutrient Mixture (Ham) with 5% fetal bovine serum (Hyclone, SH30396.03), 10nmol/L insulin (Sigma, I9278), and 1ug/mL hydrocortisone (Sigma, H0888). (ca)erbB2-MCF-7 cells, (ca)Raf-MCF-7 cells, and EGFR-MCF-7 cells were grown in phenol red–free modified IMEM with L-glutamine, without gentamicin sulfate (Gibco; Life Technologies), with 10% charcoal-stripped fetal bovine serum (Hyclone, SH30068.03). EGFR-MCF-7 cells were treated with 10ng/mL EGF for 8 hrs prior to RNA collection. MCF-7 cells, MDA-MB-231 cells and dissociated tumor (DT) lines 13 and 22 were grown in phenol red-containing modified IMEM (Gibco; Life Technologies) with 10% fetal bovine serum. The dissociated tumor cell lines were created as described [132, 134].

2.1.3. Plasmids/Constructs

Luciferase reporter constructs:

- pGreenFire-ERE-EF1-Puro plasmid was purchased from System Biosciences. It contains genes for both ampicillin (bacterial) and puromycin (mammalian) resistance for selection purposes.

- ER promoter deletion constructs were generously provided by the laboratory of Dr. Ron Weigel [135].

- pRL-CMV plasmid purchased from Addgene for use as a transfection control in combination with a firefly luciferase vector.
• pGL4-CMV-fLuc plasmid purchased from Promega for use as a positive luciferase expression control. Contains ampicillin and neomycin resistance genes.

• pGL2-NONluc- plasmid created by Dorraya El-Ashry with luciferase expression vector run off of a MMTV minimal promoter with scrambled estrogen response elements. Negative control for ERE-luciferase vector.

CMV-deltaMEK expression vector was created by the El-Ashry lab and used to overexpress constitutively active MEK leading to hyperactivation of MAPK.

pMG2.G was a gift from Dr. Ward and contains an ampicillin resistance gene for bacterial selection. The plasmid is used for packaging viral particles in 293T cells and encodes the VSV-G envelope protein.

psPAX was a gift from Dr. Ward and contains an ampicillin resistance gene for bacterial selection. The plasmid is used for packaging viral particles in 293T cells and encodes packaging proteins.

Constructs were propagated in One-Shot Top10 chemically competent E. coli (Invitrogen) grown in LB broth containing 1mg/mL ampicillin (Sigma).

2.1.4. Antibodies

Westerns blots. Primary antibodies: ERα (Sp1, abcam), vinculin (VLN01, abcam, ab80665), HDAC1 (Invitrogen, 49-1025), HDAC2 (Invitrogen, 51-5100), HDAC3 (abcam, ab16047), pHDAC1 (phospho-S421, abcam), pHDAC2 (phospho-S394, abcam), pHDAC3 (phospho-S424, abcam), acetyl-histone-H3 (Lys9, Upstate cell signaling solutions), p44/42 MAPK (ERK1/2) (Cell signaling technology, 9102L), P-p44/42 MAPK (T202, Y204, Cell signaling technology, 9101L). Secondary

Chromatin immunoprecipitation (ChIP) antibodies: ChIPAb+ HDAC1 (Millipore, 17-10199), ChIPAb+ HDAC2 (Millipore, 17-10237), ChIPAb+ HDAC3 (Millipore, 17-10238), anti-acetyl-H3 (Millipore, 06-599).

2.2. METHODS

2.2.1. Viral packaging and transduction

Viral packaging was performed by transfecting LentiX 293-T cells (Clontech) with the lentiviral vector of interest, a packaging plasmid (psPAX2), and an envelope plasmid (pMD2.G) using Lipofectamine 2000 (Invitrogen, Life Technologies). 293-T cells were plated at approximately 60% confluence in phenol-red free IMEM with 10% cFBS and no antibiotic added. Cells were co-transfected with 10ug pGreenFire-ERE-luc, 7ug psPAX2, and 3ug pMD2.G in 300uL OptiMEM with 40uL Lipofectamine 2000 per T75 flask. On day 1 post-transfection, transfection solution was removed and replaced with 15mL 10% cFBS-containing phenol-red free IMEM. Viral supernatants were collected and media replaced on days 2, 3, and 4 post-transfection and were centrifuged for 10 minutes at 3000 x g to remove 293T cells. Polybrene was added to a final concentration of 4ug/mL. Viral supernatants were used to transduce cell lines. Virus not immediately used for transduction was stored in aliquots at -80C. Beginning one week post-transduction, cells were selected and maintained in puromycin-containing media.
2.2.2. Compound screening

Prior to screening, SUM149 cells transduced with the pGreenFire-ERE luciferase vector were plated at various cell densities in opaque white 384-well plates to determine the cell/well number resulting in optimal consistency of luciferase expression across the plate. It was determined that 10,000 cells per well would be used for screening. Additionally, the estradiol concentration leading to maximal luciferase expression was determined to be 10nM through the use of MCF-7 cells transduced with the ERE luciferase reporter. SUM149 cells were plated in 384-well plates at 10,000 cells per well 24 hours prior to compound library addition. Compounds were added at a 1:6 dilution and wells not receiving compounds were treated with equivalent DMSO. 10nM estradiol was added to the plates 24 hours post-compound addition and luciferase assays performed at 48 hours post-compound addition. The average and standard deviation of luciferase expression of cells in the 264 wells treated with DMSO and estradiol but not receiving compound was determined and expressed as a baseline to which expression of treated wells would be compared. Three standard deviations above and below the baseline provides the cutoff expression values leading to p<0.01 for luciferase expression changes. Those compounds with luciferase expression greater than 3SD above the baseline were considered compounds of interest and investigated further.

2.2.3. Luciferase assays

Luciferase assays were performed using the Britelite luciferase assay system (Perkin-Elmer). The reaction of D-Luciferin leading to the production of light is
shown below. Britelite reagent was added to cells and media at a 1:1 ratio and plates were mixed and centrifuged. Plates were read with a Perkin Elmer Envision Multilabel Plate Reader at the UM-CTI.

2.2.4. siRNA transfection

ON-TARGETplus SMARTpool siRNA against HDAC1, HDAC2, and HDAC3 were purchased from GE Healthcare, Dharmacon RNAi and Gene Expression.

Target sequences are as follows:

ON-TARGETplus Human HDAC3 (8841) siRNA
- AACAAGAUCUGUGAAUUG
- GGAAUGCGUGAAUAUGUC
- GCACCCGCAUCGCAGAAUCA
- AAAGCGAUGUGAGAAUUA

ON-TARGETplus Human HDAC2 (3066) siRNA
- GCGGAUAGCUUGUGAUGAA
- GCAAAAGAAAGCUAGAAUUG
- GAUAACAUGUCUGAGUAUA
- GAUCGUGUAAGACGGUAU

ON-TARGETplus Human HDAC1 (3065) siRNA

- ACUAUGGUCUCUACCGAAA
- GCAAGUAUUAUGCUGUUAA
- CCGGUCAUGUCCAAAGUAA
- CCACACGCAUGACUACAUU

Reverse transfection of siRNA was performed with Lipofectamine RNAiMAX (Invitrogen, Life Technologies). SMARTpool siRNAs were resuspended in Dhharmacon siRNA buffer to a concentration of 20uM. Transfections were performed giving a final siRNA concentration of 50nM per siRNA. Lipofectamine RNAiMAX was used at 7.5uL per well in solution with the siRNA and OptiMEM. 500uL transfection solution was added to 6-well plates to which 1.5mL cell suspension was added with appropriate cell numbers to achieve 60-80% confluence upon attachment. Cells were harvested for protein and RNA extraction at 96 hours post-transfection.

2.2.5. Western blot analysis

Cells were washed with cold PBS and harvested on ice in RIPA buffer (Pierce, Thermo Scientific) supplemented with 1x Halt protease inhibitor cocktail, 1x Halt phosphatase inhibitor cocktail, and 1X leupeptin. When blots were to be probed for estrogen receptor, lysis was done with Gold Lysis buffer supplemented with 1X Aprotinin, 1X Pepstatin, 1X sodium orthovanadate, 2X Leupeptin, and 1X Pefabloc. Lysates were centrifuged at 10,000 x g for 10 minutes at 4°C to
remove insoluble material. The lysates were kept on ice at all times. The protein concentration of the lysates was determined by the bicinchoninic acid (BCA) method (Pierce, Thermo Scientific). Protein samples (20ug) were combined with 1X Laemmli sample buffer (BioRad) with added beta-mercaptoethanol (EMD Millipore) and incubated for 5 minutes at 95°C. Following incubation, samples were immediately returned to ice. Proteins were separated on 10% SDS-PAGE gels (Criterion, Bio-Rad) and transferred to PVDF membranes overnight in a 4°C cold-room at 30volts using the Bio-Rad Criterion Blotter system. Protein transfer quality was assessed with Ponceau stain membranes were cut according to molecular weight markers to allow for the probing with multiple antibodies simultaneously. Membranes were blocked in tris-buffered saline containing 0.1% Tween 20 (TBST), 5% bovine serum albumin (BSA, Sigma); incubated with primary antibody in TBST, 5% BSA, overnight at 4°C. Membranes to be probed for estrogen receptor were blocked in TBST with 3% non-fat dried milk powder (BioRad). Membranes were washed with TBST 3 times for 10 minutes per wash on a rocking platform and incubated with the appropriate secondary antibody in TBST, 5% BSA for 60 min at room temperature. Membranes were again washed 3 times for 10 minutes per wash on a rocking platform. Chemiluminescent detection was accomplished using SuperSignal West Pico chemiluminescent substrate or ECL (Pierce, Thermo Scientific) following the manufacturer's protocol. If a membrane was to be probed with a different antibody, the previous antibodies were stripped from the membrane by incubating it in Restore Western Blot Stripping Buffer (Thermo Scientific) for 20 minutes followed by a rinse in
TBST. The membrane was then blocked in the appropriate blocking buffer and probed for additional proteins.

2.2.6. DNA manipulation

2.2.6.1. DNA transformation

One-Shot Top10 cells were thawed on ice and 2uL plasmid solution pipetted into the vial of competent cells with stirring. The vials were incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds and immediately placed back on ice. 250uL room-temperature S.O.C media was then added to each vial and the vials were shaken horizontally at 37°C for one hour at 225rpm. Bacterial solution was spread onto LB-agar plates containing 100ug/mL ampicillin and plates were incubated overnight at 37°C.

2.2.6.2. Isolation of plasmid DNA

Bacterial colonies grown on LB-agar plates containing 100ug/mL ampicillin were incubated in 5mL L-broth with 1mg/mL ampicillin overnight at 37°C in a shaking incubator (200 rpm). Cultures were used to inoculate 300mL L-broth and were incubated overnight at 37°C in a shaking incubator. Plasmid was extracted using the GenElute HP Endotoxin-free Plasmid Maxiprep kit (Sigma) according to the manufacturers instructions.

2.2.6.3. Transient transfection of plasmid DNA

Transient transfections were performed using Lipofectamine LTX and Plus reagent (Invitrogen, Life Technologies) according to manufacturer’s protocol. All MCF-7 based cell lines were reverse-transfected, whereby the transfection solution is plated prior to the addition of cell suspension.
2.2.6.4. Isolation of genomic DNA

Genomic DNA was extracted from cells using the QIAamp DNA blood mini kit (Qiagen) according to manufacturers instructions. DNA was eluted in 50uL H2O and stored at -20C.

2.2.6.5. Estrogen receptor promoter methylation assay

Incubated 250ng isolated DNA sample with the restriction enzymes (methylation sensitive, methylation dependent, or a combination of the two) and incubated in a thermal cycler at 37C for 6 hours followed by heat inactivation for 20 minutes at 65C. RT-PCR was performed with the samples with primers specific for the ER promoter CpG island 112843 (Chr6: 152,128,822 to 152,129,771). Ct values were exported into the Epitect Methyl II PCR Array Microsoft Excel based data analysis template resulting in gene methylation statuses as percentages of methylated and unmethylated fractions of input DNA.

2.2.6.6. Polymerase chain reaction (PCR)

PCR reactions were performed following ChIP assays for visualization of DNA fragments of interest. For each 20uL PCR reaction, 7uL DNase free H2O, 1uL of 10uM primers (forward and reverse), 10uL AmpliTaq Gold 360 Master Mix (Invitrogen, Life Technologies), and 2uL ChIP DNA sample. Samples were denatured at 94C for 2 minutes, then underwent 50 cycles of denaturation of 94C for 30 seconds, annealing at 60C for 30 seconds, and extension at 72 for 1 minute per kb of PCR product, followed by a final extension period of 5 minutes at 72C.
2.2.6.7. **Agarose gel electrophoresis**

1-2% agarose gels were made up in 1X TAE buffer with 0.5ug/mL ethidium bromide (EtBr, EMD Millipore). DNA samples were mixed with 1X DNA loading buffer and samples loaded in the gel in 1X TAE buffer with 1Kb Plus DNA ladder for size determination. The gels were run at 100-120 volts and visualized with a BioRad UV transilluminator.

2.2.7. **RNA manipulation**

2.2.7.1. **RNA isolation from cells**

RNA was extracted from adherent cells in tissue culture plates using Trizol reagent (Invitrogen, Life Technologies) followed by scraping of the cells from the plate. Phenol/chloroform extraction followed by isopropanol precipitation of RNA was used according to manufacturer’s instructions (Invitrogen, Life Technologies). RNA concentration was determined on a spectrophotometer (Nanodrop, ThermoScientific) following DNase treatment by measuring UV absorbance at 260nm and 280nm.

2.2.7.2. **DNase treatment**

Following RNA extraction, samples were resuspended in 17uL nuclease-free H2O with 2uL baseline-ZERO DNase buffer (Epicentre) and 1uL baseline-ZERO DNase. Samples were incubated at 37C for 30 minutes. 2uL baseline-ZERO DNase stop solution was added to each sample and samples were incubated for 15 minutes at 65C to inactivate DNase.
2.2.7.3. RNA to cDNA conversion

RNA samples were adjusted to 200ng/uL with nuclease-free water. 20uL reactions were made up as follows (reagents from Applied Biosystems): 2uL 10X reverse transcription buffer, 0.8uL 25x dNTP mix (100mM), 2uL 10x reverse transcription random primers, 1uL MultiScribe reverse transcriptase, 4.2uL nuclease-free water, and 10uL sample. Reactions were loaded in 96-well PCR plates and sealed with optical adhesive seals (BioRad). Thermal cycler settings were 10 minutes at 25C, 120 minutes at 37C, and 5 minutes at 85C followed by a hold at 4C. Samples were stored at 4C if they were to be used within 24 hours or at -20C for long-term storage.

2.2.7.4. Real-time PCR

Real-time PCR was performed with with Power SYBR green PCR master mix (Applied Biosystems) to determine relative quantification of genes of interest in samples. Plates were run on a 7900HT Fast Real-Time PCR System with 384-well block module (Applied Biosystems). Thermal-cycling conditions were as follows: 10 minutes at 95C, 40 cycles of 15 seconds at 95C (denaturation) and 60 seconds at 60C (anneal/extension). Samples were run in triplicate and results normalized to GAPDH and expressed as fold-change relative to untreated samples. Results are the average and standard deviation of three independent experiments.

Primers used in RT-PCR:

- GAPDH forward (5'-CACCAGGGCTGCTTTTAACTCTGGTA-3')
- GAPDH reverse (5'-CCTTGACGGTGCCATGGAATTTGC-3')
2.2.8. ChIP assays.

ChIP assays were performed using buffers from the Millipore Magna ChIP HiSens kit (17-10460) according to manufacturer’s instructions. Cells were formaldehyde-fixed followed by quenching with 1X glycine. They were collected in 1mL PBS and pelleted by centrifugation for 5 minutes at 800 x g at 4C. The cell pellets were then resuspended in nuclear isolation buffer and incubated on ice for 20 minutes with vortexing at 5-minute intervals. Samples were again centrifuged for 5 minutes at 800 x g at 4C, supernatant removed, and resuspended in 500uL SCW (sonication/ChIP/wash) buffer. Sonication was performed in a (sonicator make and model?) and each sample was sonicated for 8 repetitions of 15 seconds at 60% power followed by 45 seconds rest. Microcentrifuge tubes containing samples were kept submerged in ice water during sonication. Samples were then centrifuged at 10,000 x g at 4C for 10 minutes to remove insoluble material. 20uL of sample was proteinase K treated and electrophoreosed through 1.2% agarose gels to evaluate DNA fragment size. Magnetic protein A/G beads were incubated with 2-4ug antibody for 4 hours on a rotating platform at 4C. Tubes were then placed on a magnetic separator and supernatant containing unbound antibody was removed. 450uL SCW buffer and 50uL fragmented chromatin samples were added to the beads, tubes were

- ERα forward (5'-CCACCAACCAGTGACCATT-3')
- ERα reverse (5'-GGTCTTTTCGTATCCACCTTC-3')
- PR forward (5'-TGAAGCATCAGGCTGATT-3')
- PR reverse (5'-CTTCCATTGCCCTTTAAA-3').
parafilm and incubated overnight on a rotating platform at 4°C. Samples were then washed and ChIP Elution buffer and proteinase K was added. To elute the samples, beads were incubated at 65°C for 2 hours followed by 95°C for 15 minutes and allowed to cool to room temperature. Using a magnetic separator, supernatant was removed from the beads. RT-PCR was performed to quantify the presence of DNA fragments of interest. Results are the average and standard deviation of three independent experiments. Results were analyzed as treated vs. untreated within each cell line with one-tailed, unpaired student T-tests.

ChIP primers for RT-PCR:

- SimpleChIP ESR1 promoter primers (Cell Signaling, 9673S)
- SimpleChIP GAPDH promoter primers (Cell Signaling).

2.2.9. Oncomine analysis of breast cancer gene expression

The Oncomine cancer gene expression database was used to examine associations between genes of interest and breast cancer grade, biomarker status, or expression of additional genes. The associations are expressed as fold-change between groups and p-values are reported.

2.2.10. General statistical analysis

Statistical analysis was performed with Microsoft Excel with mean and SD of three separate triplicate experiments. All p-values were calculated using 1-sided Student t-tests unless stated otherwise. The cut-off for statistical significance was set at p<0.05.
 CHAPTER 3: IDENTIFICATION OF EPIGENETIC COMPOUNDS LEADING TO THE ABROGATION OF ESTROGEN RECEPTOR TRANSCRIPTIONAL REPRESSION

Studies have suggested that ER negative breast cancers arise from ER positive cells that have ceased to express the estrogen receptor. ER is highly expressed in all atypical ductal hyperplasias, benign breast lesions with significant premalignant potential [136]. ER expression decreases as atypical ductal hyperplasia progresses to ductal carcinoma in-situ and further to invasive breast cancer, with 75% and 60-70% of these lesions expressing ER, respectively. By contrast, because some ER negative cells exist in all premalignant lesions, it is possible that these cells are the precursors of ER negative breast cancers. If these cells were to become independent of the estrogen-induced paracrine support from their ER positive brethren and achieve a growth advantage, they could presumably progress to ER negative breast cancer [106].

The first mechanism found to play a role in the repression of ER expression is hypermethylation of the ER promoter and this hypermethylation is found in approximately 25% of ER negative breast cancers [107]. The 5’ promoter region of the estrogen receptor gene contains five CpG islands, and the methylation of two of these islands strongly correlates to ER negativity [107, 114]. Following treatment with the demethylating agent 5-aza-2’-deoxycytidine, ER expression in ER negative breast cancer cells with hypermethylated ER promoters is restored. Importantly, the ER that is expressed is also functional [118].
More recently, hyperactivation of tyrosine kinase induced signaling pathways has been shown to lead to ER repression. Overexpression of epidermal growth factor receptor (EGFR) occurs in approximately 50% of breast cancers while overexpression or amplification of Her2 is seen in approximately 20%. Importantly, EGFR and Her2 overexpression is inversely correlated to ER expression [137]. We previously demonstrated that hyperactivation of ERK1/2 MAPK (hMAPK) downstream of overexpressed EGFR or overexpression/amplification of Her2 represses ER protein and mRNA expression [131, 138]. This hMAPK-mediated ER repression involves the repression of transcription, decreased mRNA stability, and decreased protein stability through proteasomal degradation. Stable transfection of wild-type c-erbB-2 [126], ligand inducible EGFR [128], and constitutively active c-Raf1 [124] and MEK1 led to hyperactivation of ERK1/2 MAPK signaling to levels seen in ER negative breast cancer cells. This hMAPK signaling resulted in downregulation of ER expression to levels seen in ER negative breast cancers that was reversible through the use of pharmacologic MEK inhibitors or dominant negative ERKs [131, 139]. Similarly, in a number of ER negative breast cancer cell lines and primary cultures developed from patient tumor samples showing hyperactive MAPK signaling, hMAPK inhibition resulted in the re-expression of ER and sensitization to anti-estrogen therapies [132].

Our overall goal is to define the mechanism of hMAPK-mediated repression of ER transcription. Although we were able to demonstrate hMAPK repression of the ER promoter, we were not able to define specific promoter
sequence elements underlying this regulation. Thus, we hypothesized that epigenetic modification may contribute to hMAPK dependent regulation of the ER promoter. To identify the epigenetic pathways involved in this regulation, we performed a small molecule screen utilizing epigenetic enzyme inhibitors and activators in breast cancer cells with hMAPK and without ER promoter methylation. We found that HDAC inhibitors are able to reverse the repression of ER mRNA expression.

3.1. ER PROMOTER METHYLATION AND EPIGENETIC STUDIES OF ER RE-EXPRESSION

Epigenetic events are defined as heritable changes in gene expression without the alteration of DNA sequence [140]. There have been numerous investigations into the mechanisms of estrogen receptor loss in breast cancer, and many of these investigations have resulted in the identification of epigenetics as playing a role in the transcriptional control of ER expression. The laboratory of Nancy Davidson was first in identifying methylation of ER promoter CpG islands as being associated with the lack of ER expression in breast cancer. A 1994 Davidson paper notes that ER and PR negative breast cancers lack or express extremely low levels of ER mRNA [114]. Methylation of CpG islands present in the ER promoter is associated with ER negativity in breast cancer cell lines and tumors and that this may be the mechanism of transcriptional repression of ER in some breast cancers. Methylation of these CpG islands may impede recruitment of basal transcription factors and transcriptional co-activators. A later study discovered that approximately 25% of ER negative breast cancers exhibit hypermethylation of the ER promoter. Studies also found that the treatment of
breast cancer cell lines containing hypermethylated ER promoters with the demethylating agent 5-aza-2'-deoxycytidine results in the loss of ER promoter methylation and re-expression of functional ER protein [107].

These studies led to increased interest in DNA methyltransferase expression and epigenetic modulation in general as possible therapeutic targets in ER negative breast cancer. Evidence of interactions between DNA methyltransferases and histone deacetylases led investigators to the discovery of histone deacetylation at ER promoters with methylated CpG islands [115]. Treatment of ER negative cells with methylated ER promoters with HDAC inhibitors led to modest re-expression of ER while the combination of a demethylating agent and an HDAC inhibitor enhanced this re-expression [115, 116, 141].

Interestingly, while it was reported that approximately 25% of ER negative breast cancers exhibit hypermethylation of the ER promoter, all of the studies on epigenetic restoration of estrogen receptor expression have been performed using cell lines with methylated ER promoters (table 3-1).
<table>
<thead>
<tr>
<th>Paper title</th>
<th>Author</th>
<th>Cell lines studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of methyl-CpG binding proteins and histone deacetylase 1 from the estrogen receptor alpha (ER) promoter upon reactivation in ER-negative human breast cancer cells</td>
<td>D. Sharma, N.E. Davidson</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha negative breast cancer cells</td>
<td>X. Yang, N.E. Davidson</td>
<td>MDA-MB-231, MDA-MB-435</td>
</tr>
<tr>
<td>ER alpha negative breast cancer cells restore response to endocrine therapy by combination treatment with both HDAC inhibitor and DNMT inhibitor</td>
<td>J. Fan, Z.M Shao</td>
<td>MDA-MB-435</td>
</tr>
<tr>
<td>The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen</td>
<td>E.R. Jang, J.S. Lee</td>
<td>Hs578T, MDA-MB-231</td>
</tr>
<tr>
<td>Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes</td>
<td>D. Sharma, P.M. Vertino</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>Histone deacetylase inhibitor LBH589 reactivates silenced estrogen receptor alpha (ER) gene expression without loss of DNA hypermethylation</td>
<td>Q. Zhou, N.E. Davidson</td>
<td>MDA-MB-231, MDA-MB-435</td>
</tr>
<tr>
<td>Arsenic trioxide restores ERα expression in ERα-negative human breast cancer cells and its treatment efficacy in combination with tamoxifen in xenografts in nude mice</td>
<td>W.J. Zhang, L.X. Wang</td>
<td>MDA-MB-435</td>
</tr>
<tr>
<td>Functional activation of the estrogen receptor-α and aromatase by the HDAC inhibitor entinostat sensitizes ER-negative tumors to letrozole</td>
<td>G.J Sabnis, A.M. Brodie</td>
<td>MDA-MB-231, Hs578T, SKBr3</td>
</tr>
<tr>
<td>Epigenetic reactivation of estrogen receptor-α (ERα) by genistein enhances hormonal therapy sensitivity in ERα-negative breast cancer</td>
<td>Y. Li, T.O. Tollefsbol</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>Reactivation of Estrogen Receptor α by Vorinostat Sensitizes Mesenchymal-Like Triple-Negative Breast Cancer to Aminoflavone, a Ligand of the Aryl Hydrocarbon Receptor</td>
<td>K. Stark, J. Li</td>
<td>MDA-MB-231, Hs578T</td>
</tr>
</tbody>
</table>

Table 3-1. Published papers concerning the epigenetic re-expression of the estrogen receptor in ER negative breast cancers. All cell lines utilized in these studies are known to possess hypermethylated ER promoters [120, 142-150].
3.2. ANALYSIS OF ER PROMOTER METHYLATION IN SUM CELL LINES, HMAPK-MCF-7 CELL LINE MODELS, AND DISSOCIATED TUMOR CULTURES

Preliminary data from our lab indicates that epigenetic mechanisms could play a role in the repression of ER transcription in response to hMAPK signaling; however, methylation of the ER promoter was not seen in our MCF-7 cell line models that lost ER expression following induction of hMAPK. The lack of ER promoter methylation in these cells remained with continued passaging of the cell line models [151]. Due to the lack of information concerning ER transcriptional repression in ER negative breast cancers lacking ER promoter methylation, we wished to identify cell lines meeting this criteria and that we could utilize for studies of additional epigenetic mechanisms repressing ER transcription. EpiTect Methyl-profiler assays were performed on a number of ER negative primary breast cancer samples and cell lines. This assay utilizes methylation sensitive or dependent restriction enzymes followed by RT-PCR with primers for the detection of the CpG island of the ESR1 promoter. MDA-MB-231 cells, widely known to exhibit methylation of the ER promoter, were used as a positive control for the assay while ER positive MCF-7 cells were a negative control. The SUM149 and SUM229 cell lines show completely unmethylated ER promoters as do the dissociated tumor (DT) lines 13 and 22, and our hMAPK-MCF-7 cell line models (caRAF-MCF-7, caErbB2-MCF-7) (figure 3-1).
Figure 3-1. ER negative cell lines and tumor samples lacking ER promoter methylation. Graph shows ESR1 promoter methylation status in a number of primary tumor cell lines, SUM cell lines, and engineered hyperactive ERK1/2 MAP kinase MCF-7 cells analyzed by the Epitect Methyl-II PCR assay (Qiagen). MDA-MB-231 serves as a positive control for ESR1 promoter methylation while MCF-7 cells are the negative control. Red boxes identify those cell lines utilized for screening and validation studies.
3.3. EPIGENETIC COMPOUND LIBRARY SCREENING OF SUM149 CELLS

3.3.1. Development of a lentiviral ERE-luciferase reporter vector

To identify small molecule inhibitors that are capable of inducing ER re-expression, we generated an estrogen receptor reporter. A pGreenFire lentiviral reporter vector containing 4 tandem estrogen response elements inserted into a multiple cloning site upstream of a minimal CMV promoter was utilized as a read-out for an epigenetic compound library screen (figure 3-2).

Figure 3-2. Plasmid map of System Biosciences pGreenFire lentiviral reporter vector. 4 tandem EREs were inserted into the multiple cloning site prior to the minimal CMV promoter upstream of GFP and luciferase genes. In the absence of ER activation, there is no expression of GFP or luciferase.

Lentiviral reporters provide a benefit over commonly used plasmid-based transcriptional reporter vectors, which can often skew transcriptional network reporting due to their episomal nature. A lentiviral reporter integrates into the host genome and is able to become structured into the chromatin leading to more accurate transcription activation signals.
faithful transcriptional activity reporting. The ERE-reporter vector contains a minimal CMV (mCMV) promoter upstream of destabilized GFP and firefly luciferase expression constructs. The tandem estrogen response elements upstream of the mCMV promoter recruit activated estrogen receptor and lead to transcription of the reporter constructs (figure 3-3).

Figure 3-3. pGreenFire Lenti-Reporter. Schematic of pGreenFire Lenti-Reporter (System Biosciences) in the presence or absence of activated transcription factor binding.

### 3.3.2. Validation of luciferase reporter vector functionality

Stable transductants of MCF-7 and SUM149 cell lines were utilized in luciferase assays following treatment with estradiol and/or the selective estrogen receptor down-regulator (SERD) faslodex to evaluate the ability of the reporter to quantify estrogen receptor activity. Significant differences between the luciferase expression of the ER positive and negative cell lines were visible (p<0.001) as well as significant responses to estradiol in the ER positive cell line (p<0.001) that were abrogated by the addition of faslodex (figure 3-4). These experiments confirmed the ability of the reporter to distinguish the presence or absence of ER
in ER positive and negative cell lines and ER positive cell lines that have been treated with an ER down-regulator.

![Graph](image1.png)

Figure 3-4. ERE-luciferase reporter vector is able to accurately report ER activity. MCF-7 and SUM149 cells stably transduced with the pGreenFire ERE-luciferase reporter vector. 24 hr treatment with ethanol vehicle, 10nM estradiol, 100nM faslodex, or estradiol + faslodex. Y-axis is relative luciferase units (RLU). **p<0.001.

To ensure that the ERE-luciferase reporter was acting as an accurate proxy for target gene expression, we then performed RT-PCR for progesterone receptor expression following the same treatments as in figure 3-4. The results of the RT-PCR mirror those of the luciferase assay, ensuring accurate measurement of ER activity by the reporter (figure 3-5).
Figure 3-5. rtPCR for progesterone receptor confirming that the reporter accurately represents ER target gene expression. Samples were normalized to GAPDH and results are expressed as fold change based on Mcf7 vehicle control cells (no estradiol).

Next, the reporter transduced cell lines were transfected with siRNA prior to treatment with estradiol to evaluate the effects of siRNA transfection on the reporter expression and the efficacy of siRNA in gene knockdown in these cells. The transfected siRNA included a non-silencing control siRNA, siRNA against GFP, which is essentially an additional non-silencing control due to the fact that GFP expression is not being measured in this assay, and siRNA against luciferase, which is being measured in the assay and therefore the knockdown of the gene is quantifiable and illustrates successful transfection. While the siRNA transfections of the transduced cell lines appear to reduce baseline luciferase
expression, induction following estradiol treatment is unchanged and the luciferase gene knockdown achieved was in the range of 80-90% (Figure 3-6).

Figure 3-6. Reporter transduced cells exhibit high transfection efficiencies and retain ability to measure functional ER following siRNA transfections. Left panel. Relative luciferase units (y-axis) in 24 hr vehicle versus estradiol treated (10nM) transduced ERE-reporter MCF7 and SUM149 cell lines. 24 hr prior to treatment, cells were transfected with non-silencing control (NSC) siRNA, siRNA against GFP (siGFP), or siRNA against luciferase (siLUC). Right panel. Same figure, zoomed in to visualize SUM149 response to treatments. Notice difference in scale of y-axis (relative luciferase units).

3.3.3. Preliminary plate-based variability studies in cell lines to be screened

When developing an assay to test the effects of certain drugs, it is necessary to first measure how well the assay works in terms of the ability of the test to measure changes in a determined output reading. Zhang et al created a method for quantifying the quality of such assays by defining a ‘separation band’ between a range of likely normal values and positive controls [152]. Virtually all of the background values will be less than a threshold defined as the mean of the background values plus three standard deviations of the background values.
Similarly, the ‘hits’ of the assay should lie above the threshold located at three standard deviations of the positive control values below the mean of the positive control values. The separation band is the area between the two threshold values and the dynamic range of the assay is defined as the values spanning from the mean of the background values to the mean of the positive control values. The quality of the assay can be described by the Z-score, which is the result of the separation band value divided by the dynamic range value (figure 3-7).

Figure 3-7. Visual depiction of Z-score calculations. Values defining the separation band and dynamic range values necessary to calculate a Z-score that quantifies the quality of a drug treatment assay.

Reporter vector transduced MCF-7 cells were used to calculate the Z-score of the assay, as they express endogenous estrogen receptor that will cause reporter expression when activated by estradiol treatment. The wells treated with estradiol act as the positive controls for Z-score calculations while wells treated with the estrogen receptor down regulator fulvestrant are the baseline (figure 3-8). Three plates of MCF-7 cells were treated in this manner.
and the resulting luciferase assays produced Z-scores above 0.5, defining the assay as excellent quality.

Figure 3-8. Representative heat map of relative luciferase values in reporter-transduced MCF-7 cells. Left two columns were treated with 10nM estradiol for 24 hrs; right two columns were treated with 100nM fulvestrant (ICI-182,780); middle 8 columns untreated. Highest 3% of luciferase values are bright red while lowest 10% are blue. Z-score = 0.56.

Because the identification of candidate compounds leading to the re-expression of ER in the ER negative cell line SUM149 would involve increased luciferase expression in response to estradiol treatment, it was first necessary to analyze the natural variation of luciferase expression between wells on a plate not treated with compound (figure 3-9). This was done three times over three different days to ensure that there was no day-to-day variation in luciferase values.
Figure 3-9. Representative heat map of relative luciferase values in reporter-transduced SUM149 cells. Cells were plated at 10,000 cells per well 24 hours prior to the luciferase assay. Upper 3% of relative luciferase values are red while lower 10% are blue. Cells were untreated and luciferase values had an average of 2,443 and a standard deviation of 189.7. Values were similar for each of the three plates.

3.3.4. Compound library treatment and results

An epigenetic compound library screen was performed using an ER negative breast cancer cell line lacking ER promoter methylation (SUM149) stably transduced with the ERE luciferase construct. The compound library contained 60 unique compounds with known epigenetic enzyme activity modulation (table 2-1). In order to activate re-expressed ER, plates were treated with either ethanol vehicle or 10nM 17b-estradiol and screens were performed in duplicate to ensure reproducibility of results. Of the 384-wells on the plate, 120 received compound while the remaining wells received 0.2% DMSO, equivalent to the DMSO present in the compound treated wells. These DMSO-treated wells served as a baseline value for luciferase expression and can be seen surrounding the treated wells in the heat map (figure 3-10). These untreated wells are expressed as 100% luciferase activity in the scatter plot depicting the results of one run of the screen (figure 3-11). Duplicate screens provided identical results in terms of compounds that were identified as significantly
increasing luciferase expression in the SUM149 cells. A standard method for hit
selection in screening is to evaluate hits that lead to a result that is outside of 3
standard deviations from the mean of untreated cells, indicating that a result is
significant at p<0.01. Of the 14 compounds leading to the largest increases in
luciferase expression, 12 were HDAC inhibitors and all of them produced highly
significant luciferase expression values. Additionally, many of the HDAC
inhibitors identified were class I specific HDAC inhibitors affecting mainly HDACs
1, 2, and 3.

Figure 3-10. Representative heat map showing luciferase values of reporter-transduced
SUM149 cells following 48hour treatment with epigenetic compound library (10uM for
each compound). Cells were treated with 10nM estradiol for the final 24 hours prior to
the luciferase assay. Black line outlines wells that received compound treatment. Red
wells are those with luciferase values in the upper 5% of all values on the plate.
Figure 3-11. Graphic representation of luciferase expression in response to compound + estradiol treatment. Expression is shown as percent of baseline expression, as calculated from untreated wells of the compound plate. Three standard deviations from the mean gives a range of 71-129% of baseline leading to a number of candidates providing a statistically significant increase in luciferase expression, the highest expressers being HDAC inhibitors.

3.4. VALIDATION OF HISTONE DEACETYLASE INHIBITORS IDENTIFIED IN THE EPIGENETIC SMALL MOLECULE SCREEN

3.4.1. Reporter and cell viability assays with individual HDAC inhibitors

Dose response curves were performed with the compounds identified in the screen as having enhanced ER signaling, visualized through increases in luciferase expression. These dose response curves served to confirm that increased luciferase activity occurs with compound plus estradiol treatment. Additionally, they evaluated the effects on cell proliferation and viability by through the luciferase assays with viability assays, and determined the optimal dosing for follow up experiments.

Because the cells are stably transduced with the reporter vector and thus the vector replicates with the cells as part of their chromosomal DNA, the compounds may alter the total luciferase expression in a well of the plates by
increasing the total cell number. Therefore, a compound that increases proliferation will increase luciferase expression while not necessarily increasing estrogen receptor signaling per cell. To assess this, it is necessary to normalize luciferase values to cell viability in an assay that could be performed on the same cells on which the luciferase assay would be performed immediately after. The CellTiter-Fluor cell viability assay by Promega was used in the dose response experiments to provide a normalization value for luciferase expression. The assay consists of a cell-permeable substrate that is cleaved by an intracellular protease into a fluorescent moiety. This protease is active only in intact, viable cells and so the fluorescent output in a well directly correlates to cell number.

The dose response curves included compound treatments in 2x dilutions ranging from 39nM to 10uM and 10nM estradiol was added to all wells 24 hrs post-compound treatment. Large increases in luciferase expression were seen mainly in the micromolar doses of compound (figure 3-12) and, while the number of cells in each well decreased at higher concentrations of compound (generally beginning around 350nM) for the majority of the drugs (figure 3-13), luciferase values per cell continued to increase.
Figure 3-12. Dose response curves in reporter-transduced SUM149 cells treated with three of the HDAC inhibitors identified in the compound screen. Cells were treated with compound for 48 hours at doses ranging from 39nM to 10µM. Estradiol was added to the cells 24 hours prior to the luciferase assays. DMSO is shown as a vehicle control. Y-axis is relative luciferase units (RLU).
Figure 3-13. CI-994 does not lead to a loss in cell viability with escalating doses. Cell-titer Fluor viability assay (Promega) performed on reporter-transduced SUM149 cells following 48-hour treatment with escalating doses of four HDAC inhibitors identified in the epigenetic small molecule screen. Drug doses ranged from 19.5nM to 10uM.

3.4.2. RT-PCR for ESR1 mRNA in cell lines following treatment with HDAC inhibitors

We evaluated HDAC inhibitor effects on ER mRNA in additional ER negative cell lines containing hMAPK signaling and lacking ER promoter CpG island methylation. RT-PCR was performed on SUM149 and SUM229 cells and one of our primary tumor cultures (DT22) following 48-hour treatment with 3 HDAC inhibitors (figure 3-14). With the exception of the SUM229 response to SAHA, each of the ER negative cell lines responded to the HDAC inhibitors with significantly increased ER mRNA expression (p<0.01). As has been reported previously, the ER positive MCF-7 cells showed a reduction in ER mRNA in response to the HDAC inhibitors [153, 154]. To determine whether these
compounds could overcome the transcriptional repression resulting from hMAPK signaling, we treated our hMAPK-MCF-7 cell models with the three compounds, which were able to induce significant ER re-expression in these cell lines, as well (p<0.05) (figure 3-15).

Figure 3-14. ER mRNA expression increases following 48 hour HDAC inhibitor treatment in hMAPK cell lines lacking ER promoter methylation. ER mRNA levels in each of 4 different cell lines (MCF-7 representing ER+ cells) following 48 hr treatment with class I HDAC inhibitors SAHA, CI994, and M344. Results presented as mRNA fold change relative to vehicle DMSO treated cells of the same cell line. *p<0.05, **p<0.01, ***p<0.001.
Figure 3-15. ER mRNA expression increases following 48 hr HDAC inhibitor treatment in hMAPK-MCF-7 cell line models lacking ER promoter methylation. ER mRNA levels in (ca)Raf-MCF-7 and (ca)ErbB2-MCF-7 cells following 48 hr treatment with class I HDAC inhibitors SAHA, CI994, and M344. Results presented as mRNA fold change relative to vehicle DMSO treated cells of the same cell line. *p<0.05, **p<0.01, ***p<0.001.

3.5. SUMMARY

Previous findings in our laboratory have suggested that epigenetic mechanisms may be responsible for the repression of ER transcription in response to hyperactive MAPK signaling. We have observed reduced ER mRNA levels, reduced ER promoter activity, and loss of multiple DNase hypersensitive sites in induced hMAPK MCF-7 cell line models when compared to control MCF-
7 cells. However, despite this evidence of hMAPK signaling affecting the ER promoter, we were not able to identify specific sequences in the promoter that are responsible for this repression. This evidence led to our hypothesis that epigenetic mechanisms may play a role in transcriptional repression of ER in response to hMAPK signaling.

To investigate the possibility of epigenetic alterations affecting ER transcription, we first needed to determine the ER promoter CpG island methylation status of our hMAPK cell line models. Over time with multiple passages, these cells maintain unmethylated ER promoters, as do a number of additional ER negative cell lines with hMAPK signaling. Interestingly, epigenetic mechanisms of ER transcriptional repression have not previously been investigated in ER negative cell lines lacking ER promoter methylation, and because 75% of ER negative breast cancers possess unmethylated ER promoters, the investigation into such mechanisms is warranted.

An epigenetic small molecule screen was used to determine the possible epigenetic mechanisms behind ER transcriptional repression by hMAPK in an unbiased approach. A lentiviral luciferase reporter provided a read-out for the screen, as the luciferase enzyme is expressed in response to ligand activated estrogen receptor homodimers binding estrogen response elements in the reporter to drive transcription.

The small molecule screen identified histone deacetylase inhibitors as the predominant class of epigenetic compound able to induce ER re-expression. Of the 14 compounds producing the highest luciferase expression in the reporter-
transduced SUM149 cells, 12 of these compounds were HDAC inhibitors. Follow-up studies on the HDAC inhibitors identified SAHA (vorinostat), CI994 (tacedinaline), and M344 (ondansetron) as leading to the largest increases in ERE-luciferase expression, as well as ER mRNA expression in the reporter-transduced SUM149 cells and additional ER negative breast cancer cells lacking ER promoter methylation. Additionally, these HDAC inhibitors were able to significantly increase ER mRNA expression in our hMAPK-MCF-7 cell line models, thus linking histone deacetylation to hMAPK signaling as a possible mechanism behind ER transcriptional repression. This mechanism that has been so clearly linked to ER promoter methylation in ER negative breast cancers and is suggested to be operating here in the absence of methylation and the presence of hMAPK. In fact, there are links between MAPK signaling and both histone deacetylation and promoter methylation, so this data suggests a new area for HDAC mediated repression of ER in breast cancer.
HDAC inhibitors have been investigated for the re-expression of estrogen receptor in ER negative breast cancers with methylated ER promoter CpG islands for some time. Promoter methylation and histone deacetylation are thought to be linked due to evidence of direction interaction between HDACs and DNA methyltransferases [155]. Literature on the mechanisms leading to ER negativity in breast cancer cite ER promoter methylation and hyperactive growth factor signaling as the two known causes. While hyperactive growth factor signaling leads to downstream mechanisms causing destabilization of ER protein and mRNA as well as reduced ER transcription, ER promoter methylation may be a mechanism downstream of additional factors leading to ER negativity. Similarly, it seems that histone deacetylation at the ER promoter may be responsible for repressing ER transcription in response to upstream signaling pathways.

To better understand actions of the histone deacetylase inhibitors identified in the epigenetic small molecule screen in leading to increased ER mRNA expression, we needed to understand which histone deacetylases were being affected by the compounds identified and which of them are playing a role in the repression of ER transcription. To accomplish this, investigated the HDAC specificity of the three HDAC inhibitors most effective in our ER negative cell lines lacking ER promoter methylation. Because two of the compounds identified specifically inhibit the class I HDACs, specifically HDAC1, HDAC2, and HDAC3,
and lead to ER mRNA expression that is of equal or increased levels as compared to the pan-HDAC inhibitor, SAHA, these are the enzymes on which we chose to focus in our investigation of ER transcriptional repression.

4.1. HDAC SPECIFICITIES OF IDENTIFIED COMPOUNDS

Recently, much attention has been given to histone deacetylase inhibitors for the treatment of solid and hematologic malignancies. Two protein families with histone deacetylase activity have been identified and consist of those that depend on a zinc ion in the catalytic site for deacetylation activity, which are class I and II histone deacetylases, and those that require NAD for catalytic activity, the sirtuins [156-158]. Because aberrant histone acetylation has been associated with well-characterized cellular oncogenes and tumor suppressors, pharmacologic agents able to modify HDAC activity have been of interest.

Early HDAC inhibitors identified included short-chain fatty acids such as sodium butyrate and valproic acid, hydroxamic acids consisting of SAHA, trichostatin A (TSA) and oxamflatin, synthetic benzamide derivatives (MS-275 and CI994), and cyclic tetrapeptides, such as trapoxin and apicidin [158, 159]. SAHA (suberoylanidile hydroxamic acid) is a second-generation HDAC inhibitor that induces growth arrest, differentiation and/or apoptosis and is FDA approved for the treatment of cutaneous T-cell lymphoma. It is marketed by Zolinza under the name Vorinostat and is the first HDAC inhibitor to be FDA approved [158-160]. While SAHA is said to be a “canonical pan-HDAC inhibitor”, this specifically refers to only class I and II HDACs and does not include the sirtuins. It inhibits class I and II HDACs at nanomolar concentrations, with an IC50 of less than
86nM. In addition to cutaneous T-cell lymphoma, SAHA has been evaluated in a number of other malignancies, including prostate, bladder carcinoma, and myeloma [161-163]. Studies performed with colon and breast cancer cell lines have shown reactivation of expression of genes leading to growth arrest and differentiation/apoptosis [164].

The synthetic benzamide derivatives consist of structurally diverse agents that contain a benzamide moiety [158]. These drugs are able to enter the catalytic sites of HDACs and bind the zinc that is necessary for catalytic activity. Tacedinaline (CI994) is a member of the synthetic benzamide derivative family of HDAC inhibitors and was initially developed as an anticonvulsant agent. A randomized double-blind phase II study has been performed in pancreatic cancer patients comparing gemcitabine treatment to gemcitabine with CI994 treatment; however, no difference in patient outcomes was seen [165]. While the mechanism of action of CI994 is not yet completely understood, it is known to inhibit HDAC1, HDAC2, and HDAC3 at micromolar concentrations.

Ondansetron (M344) combines two classes to HDAC inhibitor drugs: it is a benzamide analog of the hydroxamic acid HDAC inhibitor trichostatin A [166]. M344 has shown promise as an antiproliferative agent in a wide range of cancer cell lines, including endometrial, ovarian, medulloblastoma, neuroblastoma, and rhabdoid [166-168]. In additional cancer cell lines, M344 was able to increase the response to radiation therapy [169]. When ER positive breast cancer cell line MCF-7 was treated with M344, it led to significant reductions in proliferation at as little as 1uM concentrations with maximal inhibition of 65% at 10uM [170].
While these drugs have previously shown promise as cancer therapeutics, their effects on steroid hormone expression in breast cancer have not been analyzed. We have identified these three distinct HDAC inhibitors as having the ability to restore ER mRNA expression in ER negative breast cancers lacking ER promoter methylation. Due to the efficacy of CI994 in restoring ER mRNA expression, as well as the specificity of this drug for HDACs 1, 2, and 3, we hypothesize that these HDACs are responsible for transcriptional repression at the ER promoter.

4.2. CLASS I HDAC EXPRESSION AND ACTION IN BREAST CANCER

Due to the specificities of the HDAC inhibitors identified in the epigenetic small molecule screen, we focused our attention on HDAC1, 2, and 3 for our
studies concerning the transcriptional repression of ER in response to hMAPK signaling. As histone deacetylation has been of great interest as a therapeutic target in cancer treatment, these particular HDACs have been studied intensely and information exists concerning their actions in cancer, and more specifically in breast cancer as they relate to the estrogen receptor. The class I HDACs form the catalytic cores of megadalton protein complexes that are involved in chromatin remodeling and gene repression [172]. Four of these complexes have been identified and characterized and include CoREST, NuRD, and sin3 which contain an HDAC1-HDAC2 dimer as a core, and the NCoR complex which contains HDAC3.

Studies of epigenetic mechanisms of ER transcriptional repression with ER negative cell lines with hypermethylated ER promoters have localized HDACs to the ER promoter with DNA methyltransferases and methyl binding proteins. HDAC1 has been discovered to bind the ER promoter in MDA-MB-231 cells in concert with DNMT1, DNMT3b, MeCP2, MBD1, and MBD2. While treatment with trichostatin A led to a low level of ER re-expression in this cell line, the DNMT and methyl-binding proteins remained bound to the ER promoter. Additional treatment with a DNMT inhibitor was necessary to facilitate promoter demethylation and higher degree of ER re-expression [144].

Overexpression of HDAC1 has additionally been shown to lead to loss of estrogen receptor expression in MCF-7 cells [141]. Overexpressed HDAC1 is able to interact with the AF-2 domain of ER to suppress ER activity, but also appears to mediate the loss of ER transcription in ER positive breast cancer
Malacuso, et al showed HDAC1 binding at the ER promoter in MDA-MB-231 cells as a part of a multimolecular repression complex consisting of pRb2, p130, E2F3/5, SUV39H1, and DNMT1 [173]. SUV39H1 is a histone methyltransferase and pRb2/p130 a cell cycle regulatory protein. This complex is hypothesized to regulate ER expression by altering local chromatin structure.

While HDAC1 has been seen to physically interact with the ER promoter, HDAC2 and HDAC3 are associated with ER negativity and clinicopathological indicators of disease progression [174]. Expression of HDAC1, 2, and 3 was analyzed in tissue microarrays of 238 human breast cancers and correlated with clinicopathological parameters, including hormone receptor status. Interestingly, given the data previously discussed, HDAC1 was expressed more highly in ER positive tumors than in ER negative tumors [174]. HDAC2 and HDAC3 were both significantly associated with negative hormone receptor status, as well as higher histological grade and less differentiated tumors [174]. A similar study performed 8 years prior analyzed HDAC1 and 3 expression in tissue microarrays of 200 human breast cancer samples and also saw the positive correlation between HDAC1 expression and ER positivity [175]. However, Krusche et al additionally saw a positive correlation between HDAC3 and ER positivity, in contrast to the more recent study.

Macaluso, et al also studied HDAC1 binding at the ER promoter in MCF-7 cells and found a similar complex containing pRb2/p130, E2F4/5, HDAC1, SUV39H1 [173]. However, the complex at the ER promoter in MCF-7 cells contained the histone acetyltransferase p300 rather than the DNA
methyltransferase DNMT1 [173]. These very similar complexes lead to differential promoter activities, the DNMT1-containing complex leading to methylation of the ER promoter in the MDA-MB-231 cells and the p300-containing complex leading to histone acetylation and active ER transcription in the MCF-7 cells. Somehow, in the presence of both a histone deacetylase and a histone acetyltransferase, histone acetylation prevails and the complex leads to active ER transcription. It is possible that, following overexpression of HDAC1 in this cell line, the fine balance between histone acetylation and deacetylation is skewed such that ER becomes transcriptionally repressed. Such overexpression of HDAC1 may not be a natural occurrence, hence the association of HDAC1 expression with ER positivity; however, overexpression of HDAC2 and 3 may lead to similar losses of ER expression in MCF-7 cells due to a comparable mechanism.

4.3. CLASS I HDAC EXPRESSION AND RELATIONSHIPS WITH BREAST CANCER BIOMARKERS AND/OR PROGNOSIS

We have identified HDAC1, 2, and 3 as potentially influencing transcriptional activity of the ER promoter. Due to the specificities of the HDAC inhibitors that are best able to induce ER re-expression in ER negative cell lines lacking ER promoter methylation, we investigated these class I HDACs further in order to determine the extent of their involvement in ER transcriptional control. In order to determine if these HDACs are clinically relevant in our investigation of ER negative breast cancers, we studied their expression in a number of clinical breast cancer datasets.
Oncomine is a web application that integrates and unifies high-throughput cancer profiling data across a number of cancer types and subtypes. Relationships between genes, cancer characteristics, and prognosis can be analyzed for insight into the biology and regulation of these cancers. We have utilized Oncomine to investigate the expression of HDAC1, 2, and 3 in relation to biomarker expression and clinical parameters in breast cancers.

We first analyzed the correlation between HDAC1, 2, and 3 expression and ER expression or triple negativity (ER-/PR-/HER2-), as previously mentioned studies utilizing tissue microarrays have produced conflicting results. Significant correlation was seen between overexpression of HDAC2 mRNA and ER negativity in breast cancers (figure 4-2). Similarly, high HDAC2 mRNA expression is correlated with triple negativity in breast cancers, as well (figure 4-3).

Figure 4-2. HDAC2 expression is significantly associated with ER negativity in breast cancer mRNA datasets. Oncomine data from the two largest breast cancer datasets provided, the (left) TGCA breast dataset and (right) Curtis breast dataset showing HDAC2 mRNA expression in ER positive and ER negative breast cancers.
Figure 4-3. HDAC2 expression is significantly associated with triple-negativity in breast cancer mRNA datasets. Oncomine data from (left) TCGA breast dataset and (right) Curtis breast dataset showing HDAC2 mRNA expression in ERBB2/ER/PR negative breast cancers and those with other biomarker statuses.

Neither HDAC1 mRNA nor HDAC3 mRNA levels were significantly correlated with ER negativity or triple negativity in Oncomine breast cancer datasets. Of note, while the tissue microarray analyses of HDACs 1 and 3 found that HDAC1 is significantly associated with ER positivity and had conflicting results concerning HDAC3, the dataset analyses indicate that there is no difference in the expression of HDAC1 mRNA between ER positive and negative breast cancers (figure 4-4). They also show that HDAC3 mRNA level is significantly associated with ER positivity (figure 4-5), in agreement with Krusche, et al. It is important to remember that these dataset analyses are based on mRNA levels of HDAC1 and 3, while the tissue microarray data is based on protein expression. It is possible that mRNA and protein expression levels of these HDACs differ.
Figure 4-4. HDAC1 expression is not associated with ER positivity or negativity in breast cancer mRNA datasets. Oncomine data from (left) TCGA breast dataset and (right) Curtis breast dataset showing HDAC1 mRNA expression in ER positive and ER negative breast cancers.

Figure 4-5. HDAC3 expression is significantly associated with ER positivity in breast cancer mRNA datasets. Oncomine data from (left) TCGA breast dataset and (right) Curtis breast dataset showing HDAC3 mRNA expression in ER positive and ER negative breast cancers.
Additionally, we looked at the expression of HDAC1, 2, and 3 mRNA in invasive ductal breast cancer versus normal breast and over increasing tumor grade. Each of HDACs 1, 2, and 3 is significantly overexpressed in invasive ductal breast carcinoma as compared to normal breast, with HDAC2 showing the largest correlation in terms of both fold-change and p-value (figure 4-6). HDAC1 and HDAC2 are also positively correlated with increasing tumor grade, as well (figure 4-7). Oddly, while HDAC3 associates with invasive ductal breast carcinoma as compared to normal breast, its expression is inversely related to tumor grade (figure 4-8). None of HDACs 1, 2, or 3 were related to tumor stage in any of the breast cancer subtypes analyzed.

Figure 4-6. HDAC1, 2, and 3 expression is significantly associated with invasive ductal breast carcinoma as compared to normal breast, with HDAC2 being the most strongly associated. Oncomine data from the Curtis breast dataset showing HDAC2 expression in normal breast and invasive ductal breast carcinoma.
Figure 4-7. HDAC1 and 2 are significantly associated with tumor grade in invasive ductal breast carcinoma datasets. Oncomine data from the (left) Hatzis breast dataset and (right) Curtis breast dataset showing HDAC1 and HDAC2 expression in differing tumor grades, respectively.

Figure 4-8. HDAC3 expression is inversely correlated with tumor grade. Oncomine data showing HDAC3 mRNA expression over increasing tumor grade in the Curtis breast dataset.
Using the CBioPortal funded by Memorial Sloan Kettering Cancer Center, we were able to analyze cellular protein expression in breast cancers with varying HDAC1, 2, and 3 mRNA expression levels. When sorting tumors by those over-expressing a class I HDAC mRNA at 1.5 standard deviations above the mean versus those that don’t, we could analyze protein expression associated with high HDAC expression. Both ER total protein and ER phosphorylation were inversely associated with HDAC2 overexpression (figure 4-9). Similarly, progesterone receptor (PR) protein expression was negatively correlated with HDAC2 expression (figure 4-10).

Figure 4-9. ER protein and expression and phosphorylation are significantly reduced in HDAC2 over-expressing breast cancers. RPPA data from the TGCA dataset in cBioPortal showing ER protein expression (left) and ER phosphorylation (right) in breast cancers with HDAC2 mRNA levels greater than 1.5 standard deviations above the mean for all breast cancer samples (altered), or less than 1.5 standard deviations above the mean (unaltered).
Figure 4-10. PR protein expression is significantly reduced in HDAC2 over-expressing breast cancers. RPPA data from the TCGA dataset in cBioPortal showing PR protein expression in breast cancers with HDAC2 mRNA levels greater than 1.5 standard deviations above the mean for all breast cancer samples (altered), or less than 1.5 standard deviations above the mean (unaltered).

4.4. SILENCING OF CLASS I HDACS IN ER NEGATIVE CELL LINES AND TUMOR SAMPLES LACKING ER PROMOTER METHYLATION

Because the class I HDAC inhibitors led to the greatest increases in ER mRNA expression in the ER negative cell lines lacking ER promoter methylation, and due to our observations and those of others concerning expression of these HDACs in ER positive and ER negative breast cancers, we sought to determine the effects of individual class I HDAC knockdowns. We performed these knockdowns in two of the SUM cell lines with hMAPK and lacking ER promoter methylation and in our primary tumor line DT22, which is similarly hMAPK and lacking ER promoter methylation. We also performed the knockdowns in the
MDA-MB-231 cell line as a representative of those ER negative breast cancer cells exhibiting ER promoter methylation.

In the SUM229 and SUM149 cells, almost 100% HDAC knockdown was achieved when silencing them individually; however, when HDAC1 was silenced, HDAC2 levels exhibited a compensatory increase, and similarly when HDAC2 was silenced, HDAC1 levels increased. Interestingly, these compensatory increases in expression did not appear to occur with HDAC3 nor with HDACs 1 and 2 when HDAC3 was silenced (figure 4-11). As a result, HDAC2 knockdowns appear to be less effective when combined with knockdown of HDAC1 and vice versa.

Each of the HDAC knockdowns, whether single or in combination, led to increases in ER mRNA expression in the SUM229 and SUM149 cell lines. While HDAC3 knockdown led to the greatest increases in ER mRNA expression, the individual knockdowns of both HDAC1 and 2 did lead to ER expression increases, and the combined knockdown of all three HDACs led to the greatest expression increase.
Figure 4-11. Class I HDAC knockdown leads to increased ER mRNA expression in SUM229 and SUM149 cells. Western blots and rtPCR for ER mRNA of SUM229 and SUM149 cells untreated, mock transfected or transfected with non-silencing control siRNA, siRNA to HDAC1, siRNA to HDAC2, siRNA to HDAC3, siRNA to both HDACs 1 and 2, siRNA to both HDACs 2 and 3, siRNA to both HDACs 1 and 3, or siRNA to HDACs 1, 2, and 3. Probed for HDAC1, HDAC2, HDAC3, and vinculin (loading control). rtPCR results presented as mRNA fold change relative to media only.
It is known that HDACs 1 and 2 are highly homologous (83% identical) and functionally redundant. These HDACs are able to homo- or heterodimerize as components of co-repressor complexes [176]. As was evident in our individual siRNA mediated knockdown of HDAC1 or 2, these proteins compensate for one another in the event that expression of one is lost, with no change in overall cellular HDAC activity [176]. It is likely that although HDAC1 mRNA and protein expression are not related to ER negativity, the ability of HDAC1 to compensate for the loss of HDAC2, which is highly correlated with ER negativity and aggressive tumor behavior, leads to the necessity of combined HDAC1 and 2 knockdown in order to abrogate their repression of ER expression. Unfortunately, it is also difficult to achieve complete knockdown of both HDAC1 and HDAC2 simultaneously. Maximal recommended concentrations of siRNA for each HDAC1 and 2 were used in these experiments, and while those doses were sufficient for knocking down the HDACs individually, they were not sufficient for knocking them down when their expression has increased in compensation for attempted knockdown of the other. Additionally, it is more difficult to transfec larger amounts of siRNA into the cells, and the combinations of maximum concentrations of each HDAC siRNA leads to totals of 100 to 150nM siRNA to be transfected.
The DT22 primary breast tumor cell line also exhibits hMAPK signaling and lacks a methylated ER promoter. We performed the HDAC1, 2, and 3 knockdowns in these cells, as well (figure 4-12). The compensatory increase in HDAC1 following attempted knockdown of HDAC2 is highly visible in these cells, even in the absence of complete HDAC2 knockdown. It is unknown why the HDAC2 knockdowns in these cells were incomplete, but possible explanations include higher endogenous HDAC2 expression or increased stability of existing HDAC2 protein. Similarly, complete HDAC3 knockdown was not achieved in these cells, though HDAC3 protein expression was decreased to a further extent than that of HDAC2. It is likely that the reduced ER mRNA increases in the DT22 cells as compared to the SUM229 and SUM149 cell responses are due to the lack of similar reductions in HDAC2 and 3 protein expression.

Figure 4-12. Class I HDAC knockdown leads to increased ER mRNA expression in DT22 cells. Western blots and rtPCR for ER mRNA of DT22 cells with treatments listed in figure 4-10. Probed for HDAC1, HDAC2, HDAC3, and vinculin (loading control). rtPCR results presented as mRNA fold change relative to media only.
The MDA-MB-231 cells, as expected, did not exhibit increased ER mRNA expression with any of the class I HDAC knockdowns (figure 4-13). Again, compensatory increases were seen between HDAC1 and 2, though the difficulty in the MDA-MB-231 cells came with knocking down HDAC1. Full knockdown of HDAC1 was never achieved in this cell line, though a large percentage of HDAC1 was silenced in the absence of simultaneous HDAC2 knockdown. We predict that the extent of HDAC inhibition we have achieved from siRNA silencing of HDAC1, 2, and 3 is not to the extent that can be achieved with an HDAC inhibitor. We have seen, and it has been reported, that an increase in ER mRNA expression in MDA-MB-231 is seen in response to HDAC inhibitor treatment. However, without pharmacologically demethylating the ER promoter in this cell line, the ER mRNA increases are modest and over 10-fold reduced from those seen with combined DNMT and HDAC inhibition. Thus, the lack of increased ER mRNA in the MDA-MB-231 cells in response to silencing of HDAC1, 2, and 3 was expected.
Figure 4-13. Class I HDAC knockdown does not lead to increased ER mRNA expression in MDA-MB231 cells. Western blots and rtPCR for ER mRNA of MDA-MB-231 cells with treatments listed in figure 4-10. Probed for HDAC1, HDAC2, HDAC3, and vinculin (loading control). rtPCR results presented as mRNA fold change relative to media only.

4.5. SUMMARY

Through the use of an epigenetic small molecule screen, we have identified histone deacetylase inhibitors as the predominant class of epigenetic compound able to induce ER re-expression. Twelve HDAC inhibitors in the compound screen were able to induce luciferase expression greater than 150% of the mean luciferase expression in untreated cells. Follow-up studies on the HDAC inhibitors identified SAHA (vorinostat), CI994 (tacedinaline), and M344 (ondansetron) as leading to the largest increases in ERE-luciferase expression, as well as ER mRNA expression in the reporter-transduced SUM149 cells and additional ER negative breast cancer cells lacking ER promoter methylation. Additionally, these HDAC inhibitors were able to significantly increase ER mRNA
expression in our hMAPK-MCF-7 cell line models, thus linking histone deacetylation to hMAPK signaling as a possible mechanism behind ER transcriptional repression. To identify which HDACs and/or HDAC classes are responsible for the repression of ER transcription in the absence of ER promoter methylation and in response to hMAPK signaling, we studied the specificities of the HDAC inhibitors identified, as well as the expression and reported activities of those HDACs predicted to be involved. Additionally, we silenced each of these HDACs alone and in combination to evaluate their contributions to ER transcriptional repression.

According to chemoproteomic profiling of SAHA and CI994, they affect HDACs 1, 2, 3, 6 and HDACs 1, 2, and 3, respectively at 10uM [171]. While there is little information available on the compound M344 (Ondansetron), it is reported to affect HDAC6 most strongly, but also inhibits HDAC1, 2, and 3 at 10uM [171]. In preliminary studies, we have identified CI994 as leading to the largest increases in ER mRNA expression, and as this compound affects only HDAC1, 2, and 3 at the 10uM dose used in the preliminary studies, we have decided to investigate these HDACs further in our analysis of ER transcriptional repression by hMAPK.

HDAC1 has been seen to physically interact with the ER promoter as part of large multi-protein complexes in both ER positive and ER negative cell lines [173]. Interestingly, the difference between promoter repressive and enhancing actions of these complexes comes from whether they include a DNA methyltransferase or a histone acetyltransferase. Associations between HDAC2
and ER positivity and prognostics of poor disease outcome have been seen in tissue microarray studies of HDAC expression in breast cancer samples [174, 175]. These studies also show a correlation between HDAC1 and ER positivity in breast cancers, and have produced conflicting results concerning the association of HDAC3 and ER expression.

Our studies with mRNA datasets confirm the association between HDAC2 and ER negativity as well as high tumor grade in breast cancers; however, no association is seen between HDAC1 and ER expression, though it is similarly correlated with high tumor grade. These datasets indicate a slight positive correlation between ER positivity and HDAC3 mRNA expression. Each of the class I HDACs is more highly expressed in invasive ductal breast carcinoma than normal breast.

When silencing HDAC1, 2, and 3 in ER negative breast cancers with hMAPK and lacking ER promoter methylation, we saw significant increases in ER mRNA expression. The knockdown of all three HDACs simultaneous produced the largest increases in ER mRNA expression, even in the presence of incomplete knockdowns.

We saw compensatory increases in HDAC1 when HDAC2 was silenced, and increases in HDAC2 when HDAC1 was silenced. These HDACs are highly homologous and carry out compensatory actions when the expression of one is lost. This compensatory action may explain why silencing of HDAC1 contributes to increased ER mRNA expression though HDAC1 is not associated with ER negativity by mRNA or protein expression. HDAC1 is able to compensate for the
loss of HDAC2, which is associated with ER negativity, and thus the abrogation of both is necessary to optimally restore ER mRNA expression.
Our previous studies utilizing our hMAPK mRNA signature indicates that hyperactive MAPK signaling is present in the majority of ER negative breast cancers, and the ability to restore ER expression in several ER-negative breast cancer cell lines and primary culture models implicates hMAPK signaling as the driver of ER loss in a significant number of ER-negative breast cancers [109, 132, 138]. hMAPK signaling has been shown to repress the transcription of ER, although the mechanisms of this repression have not been elucidated.

Prior studies demonstrated that DNA methyltransferase and histone deacetylase inhibition are able to induce the expression of ER mRNA in ER negative cell lines exhibiting methylation of the ER promoter [107, 114, 118]. We have demonstrated the ability of HDAC inhibition to cause re-expression of ER mRNA in ER negative breast cancer cell lines lacking ER promoter methylation, establishing HDAC mediated transcriptional repression as a common mechanism between breast cancers with methylated or unmethylated ER promoter CpG islands. Additionally, the ER negative cell lines lacking ER promoter methylation that we have utilized in these studies exhibit hyperactive MAPK signaling and the abrogation of this hMAPK signaling leads to the restoration of ER expression in these cells [132]. This data implicates alterations in histone acetylation at the ER promoter as a mechanism leading to ER transcriptional repression in response to hMAPK signaling.
There have been multiple studies reporting links between ERK1/2 MAPK signaling and modulation of chromatin conformation and histone deacetylases. Continuous Ras signaling in NIH3T3 fibroblasts by expression of mutated forms of H-Ras, K-Ras, or N-Ras leads to transformation of these cells [177]. This transformation is associated with decreased expression of the CBP/p300 histone acetyltransferase in response to constitutive Ras signaling, leading to a global shift toward hypoacylation. This loss of CBP/p300 expression occurred mainly through proteasomal degradation, though mRNA levels of CBP/p300 were reduced, as well.

In mouse embryonic fibroblasts, Ras induced transformation led to the epigenetic loss of C-terminal Src Kinase-binding protein (Cbp) [178]. Cbp expression levels were inversely correlated to MEK and Akt activity and inhibition of MEK and PI3K suppressed inhibition of Cbp expression. Transformation did not affect Cbp mRNA stability, transcriptional activity of the Cbp promoter, or DNA methylation at the Cbp promoter CpG islands; however, treatment with HDAC inhibitors or siRNA-mediated knockdown of HDAC1 and 2 restored Cbp expression. This study concluded that Cbp downregulation in a subset of cancer cells is mediated by epigenetic histone modifications downstream of oncogenic MAPK/PI3k pathways.

HDAC4 is one of the class II HDACs, which shuttle between the nucleus and cytosol whereas the class I HDACs are predominantly nuclear at all times. Activation of the Ras-MAPK pathways resulted in a significantly increased percentage of C2C12 myoblast cells that expressed HDAC4 in the nucleus [179].
The authors found that ERK1/2 was able to associate with and phosphorylate HDAC4 in vitro. Additionally, nuclear HDAC4 in these cells with activated MAPK signaling was physically associated with kinase activity, leading the authors to hypothesize a physical interaction between nuclear HDAC4 and activated ERK1/2 leading to the phosphorylation and activation of HDAC4.

In addition to these studies presenting evidence of MAPK signaling altering the expression and/or activation of modulators of histone acetylation, there have been reports of HDAC inhibition affecting ERK1/2 signaling, as well. Decreased EGFR expression in colorectal cancer cells has been noted in response to HDAC inhibition [180]. HDAC inhibitor treatment of K-Ras mutant colorectal cancer cell lines HCT116 and SW480 led to loss of EGFR transcription. Interestingly, this transcriptional repression of EGFR in response to HDAC inhibitor treatment was discovered to be due to histone hypoacetylation at the EGFR promoter. The efficacy of HDAC inhibitors in blocking both HDAC activity and abrogating hMAPK signaling in colorectal cancer cells makes it a highly attractive potential therapeutic.

Studies on cardiomyocyte hypertrophy have revealed the HDAC dependent repression of DUSP5, an ERK1/2 phosphatase [181]. Following HDAC inhibition in cardiomyocytes using either pan- or class I HDAC inhibitors, the authors saw decreased ERK phosphorylation, as well as increased DUSP5 mRNA and protein expression. They also discovered decreased DUSP5 expression in response to MAPK pathway activation by phenylephrine, indicating
that HDAC inhibition de-represses DUSP5 from suppression induced by MAPK signaling, and that this repression of DUSP5 is mediated by class I HDACs.

Due to these reports of histone deacetylase activity and expression being affected by MAPK signaling, as well as alterations in MAPK signaling by HDAC inhibitors, we wished to determine the link between hyperactive MAPK signaling in breast cancer and its effects on histone deacetylases. We were specifically interested in hMAPK interactions with class I HDACs and their control of ER transcription.

5.1. HMAPK EFFECTS ON CLASS I HDAC PHOSPHORYLATION AND EXPRESSION

5.1.1. Effects of constitutively active MEK overexpression on class I HDACs in MCF-7 cells

To begin to understand the relationship between hyperactive MAPK signaling and HDAC control of the ER promoter, we first investigated the alterations in HDAC1, 2, and 3 expression and phosphorylation in an ER positive cell line when made to exhibit hyperactive MAPK signaling. We transiently transfected MCF-7 cells with a constitutively active HA-tagged MEK construct expressed downstream of a CMV promoter. Western blotting for HA demonstrated successful transfection and expression of the construct, and increased phosphorylated ERK1/2 ensured activation of MAPK signaling by the ca-MEK (figure 5-1). In response to this induced hMAPK signaling in the MCF-7 cells, estrogen receptor expression was reduced and phosphorylation of HDAC1, 2, and 3 were increased.
### 5.1.2. Phosphorylation and expression of class I HDACs in response to short term MEK inhibition

Because we hypothesized that hMAPK is leading to transcriptional repression of ER through control of HDAC activation or expression, we analyzed the phosphorylation and overall protein expression of HDACs 1, 2, and 3 in response to MEK inhibition. (ca)Raf MCF-7 cells were treated with the three HDAC inhibitors identified in the compound screen, as well as three specific MEK inhibitors (UO126, AZD6244, and PD98059). The HDAC inhibitors, which are known to bind to the zinc-containing catalytic sites of histone deacetylases, do

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Figure 5-1. Constitutively active MEK transfection leads to reduced phosphorylation of HDAC1, 2, and 3 in MCF-7 cells. Western blot of control MCF-7 cells and MCF-7 cells transfected with a CMV-caMEK construct. Cells were harvested 48 hours after transfection. Westerns were probed for vinculin (loading control), ER, phosphorylated ERK1/2, HA-tag, phosphorylated HDAC1, 2, and 3.
not affect the HDAC phosphorylation states, but lead to an increase in overall acetylated histone H3 in the chromatin. Interestingly, the MEK inhibitors led to decreased phosphorylation of HDACs 1, 2, and 3, with no decrease in overall HDAC protein expression at these time points (figure 5-2).

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Figure 5-2. Pharmacologic MEK inhibition results in decreased phosphorylation of HDACs 1, 2, and 3. Western blot of caRAF-Mcf7 cells treated with DMSO, one of 3 different HDAC inhibitors, or one of three different MEK inhibitors for 30 minutes or 1 hr. Probed for vinculin (loading control), acetylated histone H3 (AcH3), phosphorylated HDAC1, and phosphorylated HDAC2.

Eight-hour HDAC inhibition and MEK inhibition led to similar results in SUM229 and DT22 cells. The MEK inhibition, while not affecting class I HDAC protein expression, led to decreased HDAC1, 2, and 3 phosphorylation (figure 5-3).

Following this observation that short term MEK inhibition leads to reduced phosphorylation of HDAC1, 2, and 3, we investigated whether ERK1/2 could directly phosphorylate these HDACs. We analyzed the protein sequences of each HDAC and identified possible ERK1/2 phosphorylation sites. ERK1 and 2
are proline-directed kinases, meaning that they preferentially phosphorylate serine and threonine residues neighboring proline residues (figure 5-4) [182]. The preferred phosphorylation consensus sequence for ERK1 and 2 is Pro-Xxx-Ser/Thr-Pro, with the Xxx is a neutral or basic amino acid; however, Ser/Thr-Pro sites may be phosphorylated, as well. HDAC4 and HDAC6 have previously been shown to physically interact with ERK1/2 and the ERK phosphorylation site in HDAC6 has been identified [179, 183]. This ERK phosphorylation site in HDAC6 is located at serine 1035, which consists of a proline-threonine-serine-proline site. While there are a number of Ser/Thr-Pro sites within the amino acid sequences of HDAC1, 2, and 3, there are no Pro-Xxx-Ser/Thr-Pro sites, nor do any of the Ser/Thr-Pro sites correspond to known activating phosphorylation sites in the HDACs [184-186]. There are a number of predicted, yet still unvalidated, phosphorylation sites in each of these HDACs and it is possible that these serines and threonines neighboring prolines could be activating phosphorylation sites in HDAC1, 2, and 3. It is also possible that phosphorylation of these HDACs by hyperactive MAPK signaling is not mediated directly by ERK1/2 but through downstream effectors.
Figure 5-3. Eight hour MEK inhibition leads to reduced phosphorylation of HDAC1, 2, and 3 with no change in overall protein expression. Western blot of SUM229 and DT22 cells following 8 hour treatment with an HDAC inhibitor (10μM CI994) or a MEK inhibitor (10μM AZD6244).
Figure 5-4. Known phosphorylation sites in HDAC1, 2, and 3 do not correspond with ERK1/2 phosphorylation consensus sites. Amino acid sequences of HDAC1, 2, and 3 with ERK1/2 partial consensus phosphorylation sites highlighted in yellow and known HDAC activating phosphorylation sites highlighted in green.
5.1.3. Phosphorylation and expression of class I HDACs in response to long term MEK inhibition

Following long term (48 hour) MEK inhibition in the SUM229 and SUM149 cell lines, total protein expression of HDACs 1, 2, and 3 were decreased (figure 5-5). In the MDA-MB-231 cells, protein expression of HDACs 2 and 3 was reduced, while in the DT22 primary tumor culture, only HDAC2 protein levels were decreased by MEK inhibition while HDAC1 expression appeared to increase in this cell line in response to MEK inhibition. These decreases in total HDAC1, 2, and 3 protein expression were not apparent at 30 minutes, 1 hour, and 8 hours post-MEK inhibitor treatment (figure 5-2, 5-3), although decreased HDAC phosphorylation was apparent. Additionally, long term HDAC inhibition in the ER negative cell lines lacking promoter methylation led to decreased phosphorylation of ERK1/2. Similar to the reduced HDAC expression with MEK inhibition, shorter treatment times with the HDAC inhibitor did not produce this reduction in ERK phosphorylation, which may indicate that these are indirect effects of the treatments.

Because we witnessed decreased ERK1/2 phosphorylation following 48 hour treatment of cells with an HDAC inhibitor, but did not observe this effect with shorter HDAC inhibitor treatments, we hypothesized that expression of an ERK1/2 modulator may be increased following HDAC inhibition. Sarkar et al similarly noted this decreased in ERK1/2 phosphorylation following treatment with a pan-HDAC inhibitor in prostate cancer cells [187]. They were not able to determine the mechanism by which HDAC inhibition reduced ERK activation, but
stated that their preliminary results did not suggest a direct inhibitory effect by the HDAC inhibitors. This led us to consider increased DUSP5 expression, as occurred in cardiomyocytes following class I HDAC inhibition [181]. There are multiple dual-specificity phosphatase (DUSP) proteins that de-phosphorylate tyrosine and serine/threonine kinases, with 11 classified as mitogen-activated protein kinase phosphatases [188]. Ferguson et al analyzed the expression of 9 of these DUSP proteins, specifically those known to affect ERK1/2 MAPK signaling [181]. Of these 9 DUSPs, only DUSP5 mRNA expression was significantly induced by a class I HDAC inhibitor (figure 5-6A). DUSP5 expression was reduced through activation of ERK signaling by phenylephrine, and this reduction was rescued by both a pan-HDAC inhibitor as well as a class I specific HDAC inhibitor, MGCD (figure 5-6B/C). DUSP5 expression was similarly rescued by the abrogation of ERK1/2 MAPK signaling by MEK inhibitors (figure 5-6D). These findings led the authors to hypothesize that ERK1/2 phosphorylation leads to class I HDAC activation and deacetylation of the DUSP5 promoter, leading to DUSP5 silencing. The inhibition of class I HDACs is able to relieve the transcriptional repression of DUSP5 by activated MAPK signaling, leading to DUSP5 expression and phosphatase activity against ERK1/2. These events lead to a positive feedback loop whereby DUSP5 expression further decreased its own transcriptional repression by MAPK through class I HDACs (figure 5-7).
Figure 5-5. 48-hour MEK inhibition leads to reduced total protein expression of HDAC1, 2, and 3. 48-hour HDAC inhibition leads to reduced ERK1/2 phosphorylation in ER negative cell lines lacking ER promoter methylation. Western blots following 48-hour treatment with an HDAC inhibitor (10uM CI994) or a MEK inhibitor (10uM AZD6244) in SUM229, SUM149, DT22, and MDA-MB-231 cells. Blots were probed for vinculin (loading control), phosphor-ERK1/2, total ERK1/2, total HDAC1, 2, and 3, and acetylated histone H3 (acH3).
Because we have seen a reduction in ERK1/2 phosphorylation in response to class I HDAC inhibition and therefore sought to determine if this DUSP5-ERK phosphorylation feedback loop could be relevant to ER negative breast cancers. We first analyzed clinical breast cancer datasets in Oncomine and saw that DUSP5 expression is significantly positively correlated with ER positivity in breast cancer (figure 5-8). DUSP5 overexpression is also negatively associated with ER/PR/HER2 negativity and tumor grade in breast cancers (figure 5-9).

We next investigated DUSP5 protein expression in our ER negative cell lines lacking ER promoter methylation so see if this mechanism is present. Following treatment with either a class I HDAC inhibitor or a MEK inhibitor for 48 hours, we saw that DUSP5 protein levels were increased (figure 5-10). However, this increase in DUSP5 expression in response to HDAC or MEK inhibition was not seen in the MDA-MB-231 cells, nor was reduced phospho-ERK in response to HDAC inhibition. MDA-MB-231 cells have a global tendency toward promoter methylation, so it is possible that not only the ER promoter is hypermethylated in these cells, but that the DUSP5 promoter may be, as well.
Figure 5-6. DUSP5 expression is repressed by ERK1/2 phosphorylation and increases in response to HDAC and MEK inhibitors. Figures from “Signal-dependent repression of DUSP5 by class I HDACs controls nuclear ERK activity and cardiomyocyte hypertrophy” [181]. A. Increased DUSP5 mRNA expression in the presence of phenylephrine (PE) by class I HDAC inhibition with MGCD. B. Reduced DUSP5 mRNA expression in response to increased phosphor-ERK1/2 following PE treatment. C. Reduced DUSP5 mRNA expression by PE can be rescued by treatment with trichostatin A (TSA) or MGCD. D. DUSP5 mRNA repression by PE can be reversed through treatment with ERK1/2 MAPK inhibitors, but not by a p38 MAPK inhibitor or a jnk inhibitor.
The phosphorylation-resistant form of ERK2 in the heart was shown to be preserved in HDAC inhibitor-treated cells. Additionally, expression of an auto-phosphorylation and nuclear accumulation of ERK2 in cardiomyocytes. Unlike MEK1 overexpression, which stimulates DUSP5 expression, leading to dephosphorylation of nuclear ERK2 upon stimulation of GPCRs that couple to Gβγ subunits. Stress stimuli trigger the downstream kinase ERK and JNK phosphorylation, which is highly selective for dephosphorylation of ERK versus p38 and JNK in cardiomyocytes exposed to HDAC inhibitors.

**Figure 5-7.** Hypothesized feedback loop leading to increased DUSP5 expression and reduced ERK phosphorylation in response to class I HDAC inhibition. Figure from “Signal-dependent repression of DUSP5 by class I HDACs controls nuclear ERK activity and cardiomyocyte hypertrophy” [181].

**Figure 5-8.** DUSP5 expression is significantly associated with ER positivity in invasive ductal breast carcinoma. Oncomine data from the Hatzis breast cancer database showing DUSP5 mRNA expression in ER negative breast cancers versus ER positive breast cancers.
Figure 5-9. DUSP5 expression is significantly inversely associated with tumor grade and triple negativity in invasive ductal breast carcinoma. Oncomine data from the Hatzis breast cancer database showing DUSP5 mRNA expression in grades I, II, and III and triple negative versus not triple negative invasive breast carcinomas.
Figure 5-10. DUSP5 expression increases in response to class I HDAC inhibition and MEK inhibition in ER negative breast cancers lacking ER promoter methylation. Western blots of three ER negative cell lines lacking ER promoter methylation (SUM229, SUM149, and DT22) and one ER negative cell line with ER promoter methylation (MDA-MB-231). Cells were treated for 48 hours with CI994 (class I HDAC inhibitor) or AZD6244 (MEK inhibitor). Westerns were probed for vinculin (loading control) and DUSP5.

5.1.4. Effects of HDAC modulation on PR expression

When beginning our studies of epigenetic modulation of ER expression, we sought to utilize progesterone receptor mRNA expression as a reading for estrogen receptor activity following HDAC inhibition. Hyperactive MAPK signaling not only suppresses ER expression through transcriptional repression, but also through destabilization of mRNA and protein, therefore functional ER expression following HDAC inhibition is not expected to be significant. However, results from the preliminary small molecule screen using the lentiviral ERE-luciferase reporter
indicated that some level of functional ER is present following only HDAC inhibition. Luciferase expression in reporter-transduced MCF-7 cells mirrored that of PR expression in response to estradiol and faslodex (ICI182780).

A number of studies investigating the re-expression of ER in ER negative cell lines with promoter methylation by epigenetic means have utilized PR mRNA expression as a proxy for estrogen receptor activity [144, 147]. They have also noted that HDACs are involved in the repression of PR in response to partial anti-estrogens through their involvement in co-repressor complexes, such as the HDAC1/2 containing Sin3 and Mi2/NuRD complexes and the HDAC3-containing NCoR complex [189-191]. Sharma et al discovered occupancy of ER-responsive promoters by HDAC2 and 3 following re-expression of ER by DNMT and HDAC inhibitor combination treatment [144]. It is interesting that HDACs are recruited to ER-responsive promoters in these cells that are under the influence of HDAC inhibitors in order to induce ER re-expression. It may be that the HDACs retain the ability to be recruited by their respective co-repressor complexes even while their deacetylase enzymatic activity is inhibited. Also interesting is that prior to utilizing PR mRNA expression as a downstream indicator of ER activity, they did not investigate whether this PR expression was estrogen receptor-dependent.

Preliminary investigations of PR mRNA expression following HDAC inhibitor treatment led to the discovery that, in the absence of estradiol treatment as well as in the induced absence of ER by faslodex, PR expression remained high (figure 5-11).
Figure 5-11. Progesterone receptor mRNA expression increases in response to HDAC inhibitors in the presence or absence of estrogen receptor. RT-PCR for PR mRNA in SUM149 (upper) and DT22 (lower) cells following treatment with one of three HDAC inhibitors and ethanol vehicle, 10nM estradiol, or 100nM faslodex (ICI).
Reports have indicated that when ER positive breast cancer cells such as MCF-7 are treated with HDAC inhibitors, downregulation of PR mRNA is seen [192]. This modulation of PR expression was seen at the protein level, as well, with decreased PR protein expression following HDAC inhibitor treatment with either valproic acid or vorinostat (SAHA) and this effect could be recreated by siRNA silencing of HDAC2. Simultaneous tamoxifen and HDAC inhibitor treatment of these cells further reduced their PR expression led to significantly increased apoptosis. It is odd that tamoxifen and HDAC inhibitor treatment combined could further reduce PR expression, as a mechanism of tamoxifen action is recruitment of HDAC-containing co-repressor complexes with ER to the PR promoter. The cooperative reduction in PR expression with this combination in ER positive cells indicates that an additional mechanism must be responsible for PR repression in response to partial ER agonists.

Bicaku et al also studied the effects of HDAC inhibitors and the combination of HDAC inhibitors and tamoxifen on PR expression in MDA-MB-231 cells [192]. They noted no increases in ER or PR mRNA or protein in response to either of the treatments. Results from our studies contrast with these findings, as we have seen large increases in PR mRNA expression in MDA-MB-231 cells in response to a class I HDAC inhibitor. However, further PR mRNA induction was not induced by estradiol treatment, indicating that this PR expression was not due to transcriptional activation by ER (figure 5-12).
Figure 5-12. PR mRNA expression increases in MDA-MB-231 cells following class I HDAC inhibition, but does not respond to estradiol treatment. RT-PCR for PR mRNA expression in MDA-MB-231 cells following 48 hour treatment with CI-994 (10uM) or DMSO (vehicle control) with or without estradiol (10nM E2) treatment.

5.1.5. ER mRNA expression in response to combined HDAC and MEK inhibition

HDAC inhibitors have been evaluated for the treatment of solid and hematologic malignancies both as a single agent and in combination with other therapeutics [193]. Due to the possible ERK-phosphatase, class I HDAC, MAPK feedback loop predicted by the results of our studies, we determined that it would be prudent to investigate the effects of combined HDAC and MEK inhibition on our ER negative cell lines lacking ER promoter methylation.

Our lab has studied receptor tyrosine kinase signaling in ER negative breast cancers for some time, and have discovered the repressive action of
hyperactive MAPK signaling on ER mRNA and protein expression. This signaling pathway results in increased expression of c-myc and cyclin D1 and reduced activity of cell cycle checkpoint proteins p21 and p27 [194]. These alterations result in cell cycle progression and survival, therefore the inhibition of this pathway is effective in treating a variety of cancers.

Histone deacetylase inhibitors affect similar components involved in cell cycle progression, including increasing expression of tumor suppressors p21 and p27. They are also reported to induce the degradation of c-myc and reduce expression of cyclin D1 [195-197]. As such, the combination of tyrosine kinase pathway inhibitors and HDAC inhibitors has been previously evaluated.

Erlotinib and gefitinib are small-molecule inhibitors of EGF signaling that have been approved for the treatment of lung/pancreatic and lung cancers, respectively [193]. Gefitinib-resistant small-cell lung cancers have been pretreated with HDAC inhibitors and the expression of e-cadherin and ERB-3, both indicators of gefitinib sensitivity, were induced [198]. Additionally, the combination of HDAC inhibition and gefitinib treatment induced growth inhibition and apoptosis in gefitinib-resistant cancer cells lines [199]. Phase I and II trials are currently underway combining gefitinib/erlotinib and HDAC inhibitors for the treatment of head and neck, and lung cancers.

Sorafenib, a multikinase inhibitor that blocks a number of tyrosine receptor kinases as well as Raf kinases, has shown promise in preclinical trials of combination treatment with HDAC inhibitors [200, 201]. Antiproliferative, anti-angiogenic, and pro-apoptotic effects of combined treatment have been seen.
This therapeutic combination is currently being evaluated in a number of Phase I clinical studies.

Because the full transcriptional effect of HDAC inhibitor treatment would not be apparent in the presence of ER mRNA destabilizing effects of hMAPK signaling, and because a potential synergistic feedback loop ties histone deacetylation to ERK signaling, we investigated the combined effects of MEK and HDAC inhibition. Since compound CI994 addition led to the greatest increase in ER mRNA expression in many of the cell lines tested, this HDAC inhibitor was combined with the MEK inhibitor AZD6244 in 48 hour treatments to determine the effects of combined MEK and class I HDAC inhibition of ER mRNA expression. While the HDAC inhibitor and MEK inhibitor alone led to significant increases in ER mRNA expression in the cell lines lacking ER promoter methylation (P<0.05; excluding MEK inhibitor treatment of DT22), the combination drug treatment led to significantly greater increases (p<0.01) (figure 5-13). Additionally, combined HDAC and MEK inhibition elicited greater ER protein expression than MEK inhibition alone (figure 5-14). ER mRNA expression did increase in response to MEK and HDAC inhibitor treatment, as well as the two combined, in the MDA-MB-231 cells. However, the response was far less pronounced than those of the cell lines lacking ER promoter methylation. These increases were also much smaller than those seen with the combination of a DNA methyltransferase inhibitor with an HDAC inhibitor in MDA-MB-231 cells [145].
Figure 5-13. Simultaneous pharmacologic inhibition of class I HDACs and MEK synergistically increased ER mRNA expression in ER negative breast cancer cell lines lacking ER promoter methylation. A. rtPCR for ERα mRNA in SUM229, SUM149, DT22, and MDA-MB-231 cells treated with ethanol vehicle, 10uM CI994 (HDACi), 10uM AZD6244 (MEKi), or combination CI994 and AZD6244 for 48 hrs.
Figure 5-14. Simultaneous pharmacologic inhibition of class I HDACs and MEK synergistically increases ER protein expression in ER negative breast cancer cell lines lacking ER promoter methylation. Western blots of SUM229, SUM149, DT22, and MDA-MB-231 with cell conditions listed in figure 5-11. Blots were probed for vinculin (loading control), phospho-ERK1/2, total ERK1/2, HDAC1, HDAC2, HDAC3, and acetylated histone H3 (acH3).
5.2. HISTONE HYPOACETYLATION IN RESPONSE TO HMAPK SIGNALING IN ER NEGATIVE BREAST CANCER

5.2.1. ChIP assays indicate increased acetylation of histone H3 following MEK inhibition

In order to link hMAPK signaling to decreased acetylation of histones at the ER promoter, we performed ChIP assays on an hMAPK cell line (SUM229) and an hMAPK primary tumor culture (DT22) following 8 hour treatment with AZD6244, a specific MEK inhibitor, or CI994, an HDAC inhibitor (Fig. 5-15). Western blots of these cell lines following 8 hour treatment with MEK inhibitor can be seen in figure 5-3. ER promoter acetylation of ER positive MCF-7 cells is shown as a reference for active ER transcription. DT22 and SUM229 cells exhibit only a fraction of the ER promoter-associated histone acetylation in MCF-7 cells; however, treatment with the MEK inhibitor or HDAC inhibitor significantly increases the acetylation (p<0.01). While we have shown that hMAPK signaling leads to increased phosphorylation of HDAC1, 2, and 3, and MEK inhibition leads to reduced expression of these HDACs, this data directly ties hyperactive MAPK signaling to histone hypoacetylation at the ER promoter providing a mechanism by which MAPK signaling transcriptionally represses ER.
Figure 5-15. Abrogation of hMAPK signaling in ER negative cell line and primary tumor culture lacking ER promoter methylation leads to increased histone H3 acetylation at the ER promoter. ChIP assays with (A) DT22 primary tumor cells and (B) SUM229 cells with antibodies against acetylated histone H3 or control IgG. MCF-7 serves as a positive control for an active ER promoter. Primers for the ESR1 promoter were used in RT-PCR.
5.2.2. Differential class I HDAC, ERK2, and Elk1 binding at the ER promoter in the presence or absence of HDAC and MEK inhibition

ChIP analysis of the ER promoter-associated histones revealed increased histone H3 acetylation following 8hour MEK inhibition or HDAC inhibition. Additionally, we have shown that the silencing of HDAC1, 2, and 3 leads to derepression of ER transcription and class I HDAC inhibitors are able to increase ER mRNA expression. We then investigated whether class I HDAC binding could be seen at the ER promoter with ChIP assays using antibodies for HDAC1, 2, and 3 (figure 5-16). We saw a large amount of HDAC1 and 3 binding at the ER promoter in SUM229 cells that decreased in response to 8hour treatment with class I HDAC inhibitor CI994. HDAC2 binding followed a similar pattern, though the amount of HDAC2 bound to the ER promoter was significantly less (figure 5-16). An important concept to consider is that it is not necessarily the presence or absence of HDAC binding at promoters that determines the local chromatin conformation and promoter activity, but the balance of histone deacetylases and histone acetyltransferases that determines transcriptional activity. As was mentioned previously, Malacuso et al discovered similar complexes bound to the ER promoters of MDA-MB231 cells and MCF-7 cells differing only in their inclusion of a DNA methyltransferase or a histone acetyltransferase. These complexes in both cell lines, whether ER positive or negative, contained HDAC1 [173]. This dynamic balance between histone acetylation and deacetylation determines transcriptional activity at promoters, and therefore rather than analyzing the presence or absence of histone deacetylases, a true indicator of promoter activity is overall histone acetylation at specific promoter regions.
Madak-Erdogan et al identified genome-wide ERK2 binding sites using ChIP-on-chip technology [202]. Five of these ERK2 binding sites were located within the ESR1 gene locus and Stossi et al discovered recruitment of ERK2 to three of these binding sites in response to activation of MAPK signaling in MCF-7 cells [203]. Because this activation of MAPK signaling also involved loss of ER mRNA expression, they then sought to determine whether this ER transcriptional repression was dependent on ERK2. Silencing of ERK2 by siRNA prevented the loss of ER in response to MAPK activation, as did silencing of c-Jun, which was found to co-localize with ERK2 at one of the ERK2 ER binding sites and be necessary for the recruitment of ERK2 to the ER gene. This ERK2 binding site at which c-Jun localized with ERK2 is located at -1,020 bp from the transcriptional start site to +38bp, which includes the ER promoter. One possible shortcoming of this method is that, through the siRNA silencing of ERK2, downstream non-ER promoter related MAPK targets may have been altered, as activated ERK1 and 2 mediate the effects of active MAPK signaling. Altering the ERK2 binding site in the ER promoter to prevent ERK2 binding, allowing activated ERK2 to modulate other downstream MAPK functions, would indicate whether ERK2 binding to this site in the ER promoter is necessary for the downregulation in an isolated fashion.
Due to these recent findings from the Katzenellenbogen laboratory, we analyzed whether ERK2 binding is occurring at the ER promoter of our cell lines exhibiting hyperactive MAPK signaling but lacking ER promoter methylation. We saw ERK2 binding at the ER promoter and, similar to the class I HDAC data, this binding was reduced by class I HDAC inhibition (figure 5-17).

Figure 5-16. HDAC1, 2, and 3 binding at the ESR1 promoter in SUM229 cells untreated or following HDAC inhibition. ChIP assays with antibodies against normal mouse IgG, HDAC1, HDAC2, or HDAC3 followed by RT-PCR for the ESR1 promoter. Results were normalized to input and are expressed as a percentage of said input.
Cells are able to control their sensitivity to stimuli through downregulation of receptors by their own ligands. The estrogen receptor is controlled in this manner by agonist binding leading to ubiquitination and proteasomal degradation of ER protein and repression of ER transcription. Repression of ER transcription following ligand-induced activation of ER involves the recruitment of co-repressor complex Sin3a to the proximal ER promoter [100]. This co-repressor complex consists of a Sin3a scaffold protein as well as HDA1, HDAC2, RbAp46/RbAp48 (stabilize the complex to nucleosomes) and SAP18/SAP30 (stabilize the interaction between Sin3a and HDACs). Sin3a does not contain a DNA binding domain but is targeted to promoter through interactions with DNA-binding or
adapter proteins [101, 102]. Ellison-Zelski et al discovered that activated ER binds its own promoter following ligand binding and recruits the co-repressor complex Sin3a to the proximal promoter leading to hypoacetylation, thus preventing RNA polymerase II binding and repressing ER transcription.

Downregulation of ER protein in response to MAPK signaling similarly occurs via ubiquitination and proteasomal degradation, though the events leading to this ubiquitination are not fully elucidated. It is possible that transcriptional repression of ER in response to MAPK signaling may be similar to that occurring in response to ligand activation of ER, as well. The Elk-1 transcription factor has been reported to associate physically with Sin3a [204]. The N-terminal transcriptional repression domain of Elk-1 can recruit Sin3a and this recruitment is enhanced in response to activation of ERK1/2 MAPK signaling. Analysis of the ER promoter reveals one Elk-1 binding site (below, highlighted GGAA), though Elk-1 binding at the ER promoter has not been reported.

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1 attgaatcac gggggcgggt cttctccggtg ctgttctcgt ggtaatgaat aagtctcacg
61 agatctgatg gttttataaa tgggagttct ctttcacgag ctctctgccg tgccgcctatg
121 taagaaggcgc ctttgccttt cctttgtctt ctgccatgat tgtgaggcct tcccagccat
181 gtggaactgt gagtccatta acaccttttc ctttataaat taccagctt ttgatatgct
241 tttattagca gtgtgaaaat ggactaatac atgagccaca ttgttacaga gtttctgaag
301 gtcaatgaaga gattccatgt cgtgggctct agctgggact caaagttcg caagagagtc
361 cagtgcacac accacgaagg gcccatcttg aactcctaag gcagggcaga gctgggcttt
421 atggagacat gtggtcttca g
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We wanted to determine whether ELK1 binding to the ER promoter is altered in response to either MEK inhibition or HDAC inhibition. When untreated, the ELK1 occupancy of the ER promoter is much higher than that of Mcf7 cells; however, treatment with either a MEK inhibitor or HDAC inhibitor significantly reduced the amount of ELK1 located at the ER promoter, inverse to the pattern
of histone acetylation at the ER promoter in response to these treatments (figure 5-18).

Figure 5-18. Elk1 binding at the ESR1 promoter in SUM229 and DT22 cells untreated or following HDAC inhibition or MEK inhibition. ChIP assays with antibodies against normal mouse IgG or Elk1 followed by RT-PCR for the ESR1 promoter. Results were normalized to input DNA samples and are expressed as a percentage of this input.

Of note, ERK2 is targeted to the Elk-1 transcription factor. This interaction occurs through a domain in Elk-1 that differs from the Elk-1 phosphoacceptor domain [205]. Binding of ERK2 and/or Elk-1 at the ER promoter could be recruiting HDAC-containing co-repressor complexes mediating the repression of ER transcription.

Evidence of reduced histone acetylation at the ER promoter in response to MAPK signaling implicates class I HDAC-containing co-repressor complexes in the repression of ER transcription. Further research is warranted to determine
which co-repressor complexes may be responsible for ER transcriptional repression and how they are being targeted to the ER promoter.

5.3. SUMMARY

While short term MEK inhibition leads to decreased phosphorylation of class I HDACs, MEK inhibition for longer periods of time (i.e. 48 hours) led to decreased total protein levels of HDAC1, 2, and 3. Long-term HDAC inhibition caused a reduction in ERK phosphorylation. A number of studies have noted this phenomenon and have attributed this HDAC inhibitor induced reduction of ERK signaling to a variety of mechanisms. Studies on cardiomyocyte hypertrophy have revealed the HDAC dependent repression of DUSP5, an ERK1/2 phosphatase whose expression is highly correlated with ER positivity in breast cancer microarray datasets. Following HDAC inhibition in cardiomyocytes, there was increased DUSP5 expression, which led to decreased ERK1/2 activation [181]. While the reduction in ERK1/2 phosphorylation we see with HDAC inhibition is not to the extent that would fully explain the ER mRNA increases, the reduction may be explained by increased MAPK phosphatase expression that we saw following HDAC inhibitor treatment. A feedback loop consisting of class I HDAC inhibition leading to the de-repression of DUSP5 and de-phosphorylation of ERK1/2, leading to further HDAC inhibition could be involved in ER negative breast cancers and could explain the synergism seen when combining a MAPK inhibitor with a class I HDAC inhibitor in restoring ER mRNA and protein expression.
ChIP assays to directly analyze histone acetylation at the ER promoter confirmed increased acetylation not only in response to the HDAC inhibitors, as expected, but in response to MEK inhibition, as well. These results directly link hMAPK signaling to the hypoacetylation of histones at the ER promoter and provide a mechanism by which hMAPK signaling is able to transcriptionally repress ER expression. Stossi, et al localized ERK2 to the ER promoter in response to macrophage-induced activation of hMAPK in MCF-7 cells [203]. It is likely that HDAC recruitment to the ER promoter occurs as a part of a complex and the recruitment of activated ERK2 and/or Elk1 to the ER promoter in response to hMAPK signaling may be involved in the complex formation and localization.

Our previous studies utilizing our hMAPK mRNA signature indicates that hyperactive MAPK signaling is present in the majority of ER negative breast cancers, and the ability to restore ER expression in several ER-negative breast cancer cell lines and primary culture models implicates hMAPK signaling as the driver of ER loss in a significant number of ER-negative breast cancers [109, 132, 138]. The role of class I HDACs in the hMAPK-mediated transcriptional repression of ER identified in this study identifies HDAC inhibition as a potential therapeutic in ER-negative breast cancer for restoring ER transcription. Additionally, as histone deacetylation is intimately involved in the repression of ER transcription associated with ER promoter hypermethylation, these data suggest a much wider role for HDAC mediated transcriptional repression in ER negative breast cancer and implicate deacetylation of ER promoter-associated
histones as a common mechanism for loss of ER mRNA in ER negative breast cancers with methylated or unmethylated ER promoters.
CHAPTER 6: DISCUSSION

Breast cancer statistics predict that 1 in 8 women will develop breast cancer in her lifetime. Clinically, breast cancers are classified by their expression of three biomarkers: estrogen receptor, progesterone receptor, and erbB2/HER2. Approximately 30-40% of breast cancers present as ER negative. ER negative breast cancers, as compared to ER positive, are more aggressive and invasive and are associated with shorter disease-free and overall survival in patients [1]. While tamoxifen and other anti-estrogen therapies have greatly improved the treatment options for breast cancer, many cancers are either de-novo resistant to these therapies or develop resistance over the course of treatment [206]. Anti-estrogen directed therapies are not effective in ER negative breast cancers and thus the mechanisms explaining lack of ER expression in ER negative breast cancers are of great importance. Currently, there are two main mechanisms established for the ER negative phenotype: hypermethylation of the ER promoter, a permanent repressive mechanism seen in approximately 25% of ER negative breast cancers, and dysregulation of growth factor signaling resulting in a dynamic and reversible repression of ER protein and/or mRNA expression, shown to play a role in a much larger fraction of ER negative breast cancers [107, 132].

Previously, studies have demonstrated that DNA methyltransferase and histone deacetylase inhibition are able to induce the re-expression of ER mRNA and protein in ER negative breast cancer cell lines exhibiting methylation of the ER promoter [107, 114, 118]. Though hypermethylation of the ER promoter is
cited as a main mechanism leading to ER negativity in breast cancer, it is important to note that this prevention of ER transcription is not sufficient to create an ER negative breast cancer. As is apparent from the use of ER antagonists and down-regulators in the treatment of ER positive breast cancers, the loss of ER in cells that depend on ER signaling for proliferation and survival leads to growth arrest and/or apoptosis of those cells. Pro-survival and proliferation signaling in cancer cells that have lost estrogen receptor expression is necessary for their survival and continued cancerous growth. Additionally, as ER negative breast cancers tend to be more aggressive and invasive than those expressing ER, the signaling pathways replacing ER for survival and growth tend to be robust. While hypermethylation of the ER promoter is certainly a mechanism of transcriptional repression of ER, it seems that it does not explain the cellular environment that leads to ER negativity in breast cancer. It is likely that the requirement of hyperactive growth factor signaling exists in these breast cancers, as well.

More recently, hyperactivation of tyrosine kinase induced signaling pathways has been shown to lead to ER repression. Overexpression of epidermal growth factor receptor (EGFR) occurs in approximately 50% of breast cancers while overexpression or amplification of Her2 is seen in approximately 20%. Importantly, EGFR and Her2 overexpression is inversely correlated to ER expression (Sainsbury, 1985). We previously demonstrated that hyperactivation of ERK1/2 MAPK (hMAPK) downstream of overexpressed EGFR or overexpression/amplification of Her2 represses ER protein and mRNA
expression [131, 138]. This hMAPK-mediated ER repression involves the repression of transcription, decreased mRNA stability, and decreased protein stability through proteasomal degradation. Stable transfection of constitutively active c-erbB-2 [126], ligand inducible EGFR [128], and constitutively active c-Raf1 [124] and MEK1 leading to hyperactivation of ERK1/2 MAPK signaling results in downregulation of ER expression that was reversible through the use of pharmacologic MEK inhibitors or dominant negative ERKs or siRNAs for ERK1/2 [131, 139]. Similarly, in a number of ER negative breast cancer cell lines and primary cultures developed from patient tumor samples showing hyperactive MAPK signaling, hMAPK inhibition resulted in the re-expression of ER and sensitization to anti-estrogen therapies [132].

Induced hyperactive MAPK signaling in ER positive cells was shown to decrease ER mRNA levels and ER promoter activity. We have also demonstrated a loss of DNase hypersensitivity sites in the 5' region of the ER promoter of (ca)Raf MCF-7s as compared to control MCF-7s. The loss of these hypersensitive sites indicates altered factor binding at the ER promoter. In an attempt to locate promoter sequences responsible for hMAPK-mediated repression of ER promoter activity, they utilized reporter promoter deletion constructs. They were not able to identify any repressor binding sites able to abrogate hMAPK mediated reductions in ER transcription in the promoter region of the (ca)Raf or (ca)erbB2 cells. These findings suggested that the DNase I hypersensitivity data was due to a shift in chromatin accessibility to transcription
factors and led us to suspect chromatin conformational changes were responsible for the hMAPK mediated transcriptional repression of ER.

We performed an epigenetic small molecule screen of the ER negative hMAPK SUM149 cell line, which lacks ER promoter methylation. The small molecule library contained 60 epigenetic compounds consisting of HDAC inhibitors/stimulators, histone lysine deacetylase inhibitors, HIF1α inhibitors, histone demethylates, histone acetyltransferase inhibitors, and sirtuin inhibitors/activators. The screen identified a number of HDAC inhibitors as possessing the ability to re-express ER. Of the 14 compounds leading to greater than 50% increase in ERE-luciferase expression over the SUM149 cells not receiving compound, 12 were HDAC inhibitors, indicating that this is the predominant class of epigenetic compound inhibitor able to produce this effect. In preliminary studies, we identified three HDAC inhibitors that consistently led to the largest increases in ER mRNA expression in our ER negative cell lines lacking ER promoter methylation. These were SAHA, CI994, and M344.

SAHA, clinically marketed as Vorinostat, was the first FDA approved HDAC inhibitor and was approved for the treatment of cutaneous T-cell lymphoma. It targets class I and II HDACs and has shown promise in the treatment of a variety of malignancies [160]. CI994, also known as Tacedinaline, is a synthetic benzamide derivative that was initially developed as an anticonvulsant and later found to act as an HDAC inhibitor [207]. CI994 inhibits HDAC1, 2, and 3 at micromolar concentrations and has been the most effective of the HDAC inhibitors tested in leading to ER mRNA re-expression. M344,
Ondansetron, is a benzamide analog of the hydroyxamic acid HDAC inhibitor trichostatin A. It has shown promise in a variety of malignancies, as well, and similarly targets HDAC1, 2, and 3 at micromolar concentrations [168]. Because these inhibitors targeting only HDAC1, 2, and 3 were equally effective as the global HDAC inhibitors in inducing ER expression in the ER negative cells lacking ER promoter methylation, we focused our study on the individual and combined effects of perturbing these HDACs in our ER negative cell lines and models and, whether these HDACs might be activated in response to hMAPK signaling.

Studies have shown that ERKs are able to phosphorylate, and therefore activate, HDACs. ERK1 and 2 have been linked to the phosphorylation of HDACs 4 and 6 in C2C12 myoblast cells and 293T cells, respectively [179, 183]. We saw that MEK inhibition in breast cancer cells with hMAPK leads to both a reduction in the phosphorylation of HDACs 1, 2, and 3 and a reduction in overall protein expression of these HDACs at later time points. To determine whether ERK1/2 could phosphorylate the class I HDACs directly, we analyzed their amino acid sequences to identify possible ERK1/2 phosphorylation sites. ERK1 and 2 are proline-directed kinases and phosphorylate serine or threonine residues that are neighboring a proline residue, with the optimal consensus sequence being proline-X-serine/threonine-proline. While a number of partial consensus sites, meaning serine or threonine neighbored by one proline residue, exist in HDAC1, 2, and 3, none of these sites are phosphorylation sites shown to mediate activation in these HDACs. It is possible that ERKs phosphorylate these sites
and they have not yet been identified as activating phosphorylation sites in HDACs 1, 2, and 3. Another possibility is that ERK1/2 activation modulates HDAC1, 2, and 3 phosphorylation through a downstream mechanism.

When we silenced HDAC1, 2, and 3 alone and in combinations of two and three with siRNA we saw significantly increased ER mRNA expression (p<0.05) in cell lines and tumor samples lacking ER promoter methylation. We did not see significant increases in ER mRNA expression in the MDA-MB-231 cells, which have a methylated ER promoter, following silencing of HDAC1, 2, and 3. As several groups have discovered previously, we saw that HDAC1 and 2 compensate for one another in the event that expression of one of them is lost. While HDAC2 overexpression is significantly associated with ER negativity, high HDAC1 expression is associated with ER positivity. However, due to their compensatory abilities, in order to abrogate the repression of ER transcription by HDAC2, it is necessary that both be silenced.

Two studies have analyzed the protein expression of the class I HDACs, focusing on HDACs 1, 2, and 3, in breast cancer tissue microarrays to determine if their expression correlates with clinicopathological characteristics [174, 175]. While Krusche, et al found that HDAC1 and 3 were correlated with ER and PR positivity, B. Müller, et al observed that HDACs 2 and 3 were correlated with ER negativity and more aggressive tumor types [174, 175]. In agreement with Müller, Oncomine clinical mRNA expression data supports the inverse correlation between HDAC2 and ER expression. However, the clinical datasets do not support the findings of HDAC1 overexpression associating with ER positivity.
Rather, there are no significant differences seen in HDAC1 mRNA expression between ER positive and ER negative invasive breast carcinomas. Additionally, in agreement with Claudia et al, HDAC3 expression was significantly associated with ER positivity, though the fold change values for HDAC3 mRNA expression were very small. Using RPPA data from the TGCA dataset through cBioPortal, we also saw that ER and PR protein expression are significantly lower in breast cancers overexpressing HDAC2 mRNA by 1.5-fold or higher.

We saw that long-term (48hour) HDAC inhibition caused a reduction in ERK phosphorylation, while short term (30 minute, 1hour, 8hour) HDAC inhibition did not show this effect. Other laboratories have seen this reduction in ERK phosphorylation in response to long-term HDAC inhibition and mechanisms have been proposed to account for it. A study investigating cardiomyocyte hypertrophy discovered the class I HDAC dependent repression of DUSP5, a dual-specificity phosphatase known to affect ERK1/2, whose expression is highly correlated with ER positivity in breast cancer microarray datasets. Following class I HDAC inhibition in cardiomyocytes, DUSP5 mRNA expression increased, which led to decreased ERK1/2 activation [181]. Additionally, DUSP5 mRNA expression was increased following MEK inhibition, suggesting a feedback loop between ERK1/2 activation, class I HDAC activity, and DUSP5 expression. We analyzed DUSP5 protein expression following 48hour class I HDAC inhibition or MEK inhibition in SUM229, SUM149, DT22, and MDA-MB-231 cells. We saw increased DUSP5 expression in response to both HDAC inhibition and MEK inhibition in the ER negative cell lines lacking ER promoter methylation. Increased DUSP5
expression in response to HDAC and MEK inhibition was not visible in the MDA-MB-231 cells, nor was reduced ERK1/2 phosphorylation following long-term HDAC inhibition. This may be due to hypermethylation at the DUSP5 promoter, as MDA-MB-231 cells are said to exhibit a ‘methylator phenotype, characterized by overexpression of DNMT3b and increased CpG island methylation in the promoters of a subset of genes [208]. While the reduction in ERK1/2 phosphorylation we see with HDAC inhibition is not to the extent that would fully explain the ER mRNA increases, as we hypothesize that this effect is due to class I HDAC activity directly at the ER promoter, the reduction may be explained by increased MAPK phosphatase expression following HDAC inhibitor treatment (figure 6-1).

Figure 6-1. Feedback loop involving class I HDACs, ERK1/2, and DUSP5. We hypothesize that class I HDAC inhibition leading to increased DUSP5 expression leads to reduced ERK1/2 phosphorylation/activation, leading to further decreased class I HDAC activity. This reduced HDAC activity further increases both DUSP5 and ER expression, leading to further reduced ERK1/2 phosphorylation.
Growth factor

Receptor tyrosine kinase

Grb2, Sos, Ras, Raf, MEK, ERK

DUSP5

ER

HDAC#

Class I HDACi
Increased mRNA expression of ER in the ER negative cell lines lacking promoter methylation may be elicited through treatment of the cells with either class I HDAC inhibition/knockdown or MAPK inhibition. Because MAPK represses ER via additional mechanisms and due to the potential HDAC inhibition of MAPK through increased expression of DUSP5, we were interested in the combined effects of these treatments on ER expression. This combination CI994 and AZD6244 led to a greater increase in ER mRNA expression than was seen with pharmacologic inhibition of either alone. ER protein expression was also greatly increased by the combination of HDAC and MEK inhibitor. ER protein expression is not seen with treatment of these ER negative cell lines lacking promoter methylation with HDAC inhibitors alone. hMAPK signaling induces proteasomal degradation of ER protein, and this down-regulation mechanism is not affected by HDAC inhibition. While this mechanism of ER protein downregulation by hMAPK is abrogated by MEK inhibition, the addition of the HDAC inhibitor further increases ER mRNA synthesis, leading to greater protein expression. These results indicate that these treatments may have a synergistic effect on relieving the transcriptional repression of ER.

In addition to ER transcriptional control, it appears as though histone deacetylases play a role in the repression of progesterone receptor transcription, as well. Because PR is an ER target gene, we analyzed PR expression in response to HDAC inhibition in order to use PR mRNA expression as an indicator of ER activation. In an ER positive cell line in the absence of HDAC inhibitors, PR expression is significantly induced by estradiol treatment. This
induction is blocked by an ER down-regulator such as faslodex. In the presence of HDAC inhibitors, PR expression is greatly increased and is not induced further by estradiol treatment. PR expression is similarly not reduced by treatment with an ER down-regulator, indicating that this expression is not estrogen-dependent. HDAC inhibition is able to induce PR mRNA expression in the absence of estrogen receptor, indicating that histone deacetylases act to repress PR transcription in the absence of activated ER.

Through ChIP assays, we have demonstrated increased acetylation at the ER promoter in response to both HDAC inhibition and MEK inhibition. This data links hyperactive MAPK signaling to the hypoacetylation of histones at the ER promoter providing a mechanism by which hMAPK signaling transcriptionally represses ER. We also discovered class I HDAC binding, as well as ERK2 and Elk1 binding at the ER promoter of our ER negative cell lines lacking ER promoter methylation. We believe that class I HDACs are being targeted to the ER promoter as members of large co-repressor complexes in response to hMAPK signaling. This may be occurring through interactions with Elk-1, a transcription factor that is activated by ERK and that has been shown to associate with the HDAC-containing co-repressor complex Sin3a [209]. The ER promoter contains an Elk-1 binding site making this interaction possible and our observations that HDAC inhibition and MEK inhibition lead to reduced Elk-1 binding at the ER promoter support this hypothesis.

In summary, we hypothesized that epigenetic mechanisms play a role in the hMAPK induced transcriptional repression of ER. In order to identify these
epigenetic mechanisms in a comprehensive approach, a plate-array based epigenetic compound screen of an ER negative breast cancer cell line both lacking ER promoter methylation and exhibiting hMAPK dependent ER repression was used to identify those compounds that could abrogate repression of ER. Multiple compounds, all histone deacetylase inhibitors, were identified and have been validated for their efficacy in other ER-negative breast cancer cell lines and primary tumor cultures lacking ER promoter methylation. We have demonstrated the ability of HDAC inhibition or silencing to cause re-expression of ER mRNA in ER negative breast cancer cell lines lacking ER promoter methylation, establishing HDAC mediated transcriptional repression as a common mechanism between breast cancers with methylated or unmethylated ER promoter CpG islands. Additionally, we have identified HDAC-mediated transcriptional repression of ER as the downstream effector of hMAPK ER repression by showing that hMAPK-dependent hypoacetylation of ER promoter-associated histones contributes to the repression of ER transcription by hMAPK signaling.

We have illustrated that hyperactive MAPK signaling is present in the majority of ER negative breast cancers through studies using our hMAPK mRNA signature [138]. Additionally, we have been able to restore ER expression in a number of ER negative breast cancer cell lines and primary culture models [132]. These findings indicate that hMAPK signaling drives the loss of ER expression in a significant number of ER negative breast cancers. In this study, we have identified class I HDACs as mediating hMAPK induced transcriptional repression of ER through deacetylation of histones at the ER promoter. We uncovered a
feedback mechanism through DUSP5, and ERK1/2 phosphatase, leading to the synergistic activity of HDAC and MEK inhibitors in increasing ER mRNA and protein expression (figure 6-2). While a multitude of research has been done concerning ER negative breast cancers with methylated ER promoters, linking this promoter methylation with histone deacetylation and combining DNMT and HDAC inhibitors to restore ER expression, this study suggests a role of HDAC mediated transcriptional repression of ER in cells with unmethylated ER promoters, as well. We hope that this research will serve to further the understanding of the generation of the ER negative breast cancer phenotype and help to shape future therapeutic innovations in the treatment of these cancers.

Figure 6-2. ER promoter-associated histone hypoacetylation in response to hyperactive MAPK signaling. Figure depicting our theory of HDAC activation downstream of hMAPK signaling leading to the hypoacetylation of ER promoter-associated histones that may be reversed through the use of class I HDAC inhibitors or RNAi knockdown of HDACs 1, 2, and 3. This mechanism is involved in transcriptional repression of those ER negative tumors with hypermethylated ER promoters through the interaction of DNA methyltransferases with histone deacetylases leading to more stable repression of ER transcription. Additionally, class I HDAC inhibition leads to expression of DUSP5, an ERK1/2 phosphatase that, when expressed, reduces ERK1/2 signaling. This results in further reduction of class I HDAC activity, resulting in a feedback loop propagating histone acetylation and ER expression.
Growth factor

Receptor tyrosine kinase

Additional modulators of ER expression (i.e. BRCA1)

DNMT

HDAC#

DUSP5

ERK

MEK

Raf

Sos

Groz

Ras

HDAC#

HDAC#

HDAC#

HDAC#

ER expression

Class I HDACi

Growth factor

Receptor tyrosine kinase

Additional modulators of ER expression (i.e. BRCA1)

DNMT

HDAC#

DUSP5

ERK

MEK

Raf

Sos

Groz

Ras

HDAC#

HDAC#

HDAC#

HDAC#

ER expression

Class I HDACi
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