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Polyomavirus Enhancer Activator 3 (PEA3), a Member of the Ets Family of Transcription Factors, Is a Transcriptional Activator of Notch-1 and Notch-4 in Breast Cancer: An Opportunity for Novel Combinational Therapy

Anthony George Clementz
Loyola University Chicago

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POLYOMAVIRUS ENHANCER ACTIVATOR 3 (PEA3),
A MEMBER OF THE ETS FAMILY OF TRANSCRIPTION FACTORS,
IS A TRANSCRIPTIONAL ACTIVATOR OF NOTCH-1 AND NOTCH-4
IN BREAST CANCER: AN OPPORTUNITY
FOR NOVEL COMBINATIONAL THERAPY

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

ANTHONY G. CLEMENTZ

CHICAGO, ILLINOIS

DECEMBER 2010
ACKNOWLEDGEMENTS

I am grateful to Dr. Clodia Osipo for her continual support, guidance, and excellent mentorship. She has taught me how to be a strong scientist and person. When I entered the laboratory, one of the first things she said to me was, “I am going to teach you to be a good scientist, and above all else, as a young researcher, I will teach you patience.” Not only have I learned patience, but the importance of integrity, communication, and dedication. I must say that Dr. Osipo has gone above and beyond in her mentorship and is herself an amazing scientist. I am very honored to be her first Ph.D. graduate student.

I would like to thank my committee members, Dr. Mary Druse-Manteuffel, Dr. Richard Schultz, Dr. Maurizio Bocchetta, and Dr. Paola Rizzo, for their support and direction during my doctoral research studies. I would like to recognize Dr. Paola Rizzo, a dear friend that has guided me not only in science, but also in many areas of my life; I am ever grateful for our long talks and many laughs. I also would like to thank Dr. Osipo’s lab personnel, former master’s student Parul Patel, Kinnari Pandya, Allison Rogowski, and Kathleen Meeke, who are all excellent scientists, great friends, and made my experience within the lab fun and unforgettable.

I would like to thank the Department of Cell Biology, Neurobiology, and Anatomy: Division of Molecular and Cellular Biochemistry for educating and assisting
me throughout their program. I would also like to thank Loyola University, the many faculty, and friends both at Loyola and outside Loyola. I have met many friends and colleagues that have made my experience here memorable.

Most importantly, I must recognize and thank my dearest parents, Peter and Toni Clementz, and family, Gina, Peter, Mia, and Andria, who stuck with me through it all, listened to my stories, and continues to give endless support and love. I have been blessed to have learned from the greatest people and to them I am truly grateful. I attribute my gifts to four amazing people in my life and I have carried with me a part of each of them: among so many other things, my father has taught me determination, caring, and problem solving; my mother, respect, openness, and consideration. To my incomparable grandfather, who taught me strength, passion, art, and always saw my potential before I could even see it in myself; and my grandmother, for her wisdom, prayer, and hope.

As I evolve into a young scientist, I find that the more I study concerning the vast molecular infrastructure of the human body and life in general, I find myself humbled by the intelligent design innate in us. It is within all of us—the beauty and blessings of life.
To my Father and Mother,
And for Dorothy and Sebastian
Considering the wonderful frame of the human body, this infinitely complicated engine, in which, to the due performance of the several functions and offices of life, so many strings and springs, so many receptacles and channels are necessary, and all to be in their right frame and order; and in which, besides the infinite, imperceptible and secret ways of mortality, there are so many sluices and flood-gates to let death in, and life out, it is next to a miracle we survived the day we were born.

J. Puckle, 1798

I awoke to the reality that I did not choose science; it chose me.

A.G.Clementz, 2010
Breast cancer continues to be the second leading cause of cancer-related deaths among women. It is a disease that transcends gender boundaries, arising in both women and men. Currently, according to the statistics acquired by the National Institute of Health, there are approximately 200,000 female and 2,000 male new breast cancer cases in the United States of America. Roughly 41,000 females and 450 males will parish this year from the disease. However, with the advancement of current research, these numbers are substantially lower than the past, and the percentage of patients reaching their five year disease free survival is increasing. The advent of new surgical and oncological techniques enables patients to receive a lumpectomy, radiation treatment, targeted chemotherapeutics, and reconstruction or breast-conserving surgery, which not only leads to increased survival rate, but also aims to regain physically and psychologically the patient’s former quality of life. Nevertheless, breast cancer is an aggressive and devastating illness. With continued support from global governments, commercial industries, and personal conquests, the prospect for a cure is on the horizon. Eradication of breast cancer is no longer a hopeful wish, but an emerging and tangible promise.
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<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>A-FOS</td>
<td>Dominant negative c-FOS</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APBI</td>
<td>Accelerated partial breast irradiation</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic leucine zipper domain</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>CoA</td>
<td>Co-activator</td>
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<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CoR</td>
<td>Co-repressor</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>CR</td>
<td>Cysteine rich region</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle number at threshold</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCIS</td>
<td>Breast ductal carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EGF-like</td>
<td>Epidermal growth factor like repeats</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FL</td>
<td>Notch full length</td>
</tr>
<tr>
<td>GSI</td>
<td>Gamma-secretase inhibitor</td>
</tr>
<tr>
<td>HC</td>
<td>Heavy chain IgG isotype control</td>
</tr>
<tr>
<td>HER2</td>
<td>Epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>IC</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IMEM</td>
<td>Iscove’s Minimal Essential Media</td>
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<td>Long chain IgG isotype control</td>
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<td>LNR</td>
<td>Lin12 repeats</td>
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<td>MAML-1</td>
<td>Mastermind-like 1</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activating pathway kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
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<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>Mut</td>
<td>Mutation</td>
</tr>
<tr>
<td>NCR</td>
<td>Cytokine response element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
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<tr>
<td>N&lt;sup&gt;EC&lt;/sup&gt;</td>
<td>Notch extracellular domain</td>
</tr>
<tr>
<td>N&lt;sup&gt;IC&lt;/sup&gt;</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>N&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Notch transmembrane domain</td>
</tr>
<tr>
<td>O-fut</td>
<td>O-fucosyl transferase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP-ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEA3</td>
<td>Polyomavirus Enhancer Activator 3</td>
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<td>PEA3&lt;i&gt;&lt;i&gt;i&lt;/i&gt;&lt;/i&gt;</td>
<td>PEA3 siRNA</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline glutamic acid serine threonine</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PR</td>
<td>Progestrone receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
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<td>RT-PCR</td>
<td>Realtime polymerase chain reaction</td>
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<tr>
<td>SCRBi</td>
<td>Scrambled control random siRNA</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SUMO</td>
<td>Sumoylation</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-alpha converting enzyme</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAM-67</td>
<td>Dominant negative c-JUN</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffer saline tween</td>
</tr>
<tr>
<td>TM</td>
<td>Notch transmembrane portion</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>Wt</td>
<td>Wildtype</td>
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ABSTRACT

Women diagnosed with triple-negative breast cancer have the worst overall prognosis and frequently present with metastatic tumors. To date, there are no targeted therapies available to combat this aggressive form of breast cancer due to the lack of expression of well-known targets such as ERα, PR, or HER2/neu. Therefore, there is an immediate need to identify novel targets that are responsible for the proliferation, survival, and invasive phenotype. Notch-1 and Notch-4, both potent breast oncogenes, are overexpressed in triple-negative breast cancers-associated with the poorest overall survival. PEA3 (polyomavirus enhancer activator 3), a member of the Ets family of transcription factors, is overexpressed in triple-negative breast cancer and also correlates with aggressive behavior and poor overall survival. Here, we provide new evidence for transcriptional regulation of Notch in triple negative breast cancer and other subtypes of breast cancer. Our results showed that PEA3 is a transcriptional activator of both Notch-1 and Notch-4 genes using a PEA3 siRNA and measuring both transcripts and proteins in MDA-MB-231 cells where PEA3 levels are endogenously high. In SKBr3 and BT474 breast cancer cells where PEA3 levels are low, exogenous overexpression of PEA3 significantly increased Notch-4 transcripts. Chromatin immunoprecipitation confirmed enrichment of PEA3 within the promoter regions of both Notch-1 and Notch-4. Notch-1
recruitment appears to be AP-1 independent, whereas PEA3 recruitment on the Notch-4 promoter is dependent upon c-JUN. Furthermore, results showed that either c-Jun or Fra-1 are required for Notch-4 transcription while c-FOS was a repressor. Importantly, the combined inhibition of Notch signaling via a gamma-secretase inhibitor (GSI) and knockdown of PEA3 (via siRNA) significantly growth arrested breast cancer cells in G1 and decreased both anchorage dependent and independent growth in vitro. In correlation, a significant decrease in cell viability and tumorigenicity, as well as an increase in apoptotic cells were observed when a GSI was combined with PEA3 siRNA. Interestingly, a combined inhibition of Notch signaling and PEA3 showed no significant changes in invasion and migration. Taken together, results from this study suggest for the first time that Notch-1 and Notch-4 are novel transcriptional targets of PEA3 in breast cancer cells. PEA3-mediated Notch-1 transcription is AP-1 independent while Notch-4 transcription requires both PEA3 and AP-1 most probably composed of the c-Jun:Fra-1 complex. Moreover, both PEA3 and Notch signaling are essential for proliferation and survival of MDA-MB-231 breast cancer cells. Thus, dual targeting of both PEA3 and Notch pathways might provide a new therapeutic strategy for triple-negative breast cancer as well as additional therapeutic targeting in other breast cancer subtypes.
CHAPTER I
AN INTRODUCTION TO BREAST CANCER

Introduction

Breast cancers are thought to originate from a small number of cancer cells that are capable of self renewal and pluripotency. These breast cancer “stem cells” are capable of initiating and sustaining breast tumor growth. The cancer-initiating cells or cancer stem cells were originally identified in hematological malignancies but is now being recognized in breast and several other solid tumors (Subramaniam et al., 2010). The normal breast anatomy contains lobular glands that produce milk, the luminal ducts that carry the milk to the nipple, and the surrounding environment consisting of adipose, connective, and lymphatic tissue (Figure 1). Transformed stem cells lose their internal proliferation checkpoints and develop into a benign palpable mass or neoplastic tumor. When tumor cells further transform into migratory cells or under epithelial-to-mesenchymal transition (EMT), they become invasive or infiltrating breast cancers distinguishing them from benign or breast cancer in situ. The breast undergoes multiple remodeling events similar to EMT throughout a woman’s lifetime and most notably during pregnancy. Multiple steps during development and pregnancy could be targets of mutation and subsequent transformation where cancer may develop. Consequently,
Figure 1: Anatomy of the Breast.
breast cancer assumes diverse intrinsic gene patterns leading to the formation, identification, and classification of particular molecular subtypes (Micalizzi et al., 2010)

**Categorization and Features of Breast Cancers**

Breast cancer is a heterogeneous disease. Molecular and pathological portraits of breast cancer provide insight into the vast genetic and phenotypic varieties that may present in the clinic. A hierarchical division among breast cancer using a both pathological and genetic analysis of expression have been formulated (Perou et al., 2000). They subdivided breast cancer into four minimal subtypes: luminal, HER2, normal breast-like, and basal-like. Soon after, further investigations divided the luminal subtype into luminal A and luminal B based on gene profiling and clinical outcomes (Sorlie et al., 2001). The classification of tumors stemming from the lumen or the myoepithelial (basal) layer was determined by immunohistochemistry on sections of patient samples using antibodies to keratin 5,8,17, and 18 (Perou et al., 2000). Those tumors that showed positive keratin 8 and 18 staining were considered of luminal origin and those that were positive for keratin 5 and 17 were derived from the basal-epithelial layer. Additionally, whether or not there was presence of gene expression patterns of the estrogen receptor and its corresponding hormone responsive genes, aided in the categorization of luminal versus basal-like cancers, respectively. Further classifications are continuing to emerge such as the molecular apocrine and claudin-low breast cancer subtypes. Although segregated into different categories, sometimes the classification of breast cancer subtypes share common gene expression and tend to overlap. However, four classic subtypes have
withstood the test of time, and are commonly referenced: luminal A, luminal B, HER2, and basal-like (including triple negative) (Table 1) (Kao et al., 2009).

1. Luminal A Breast Cancers

The luminal A subtype makes up approximately 60% of breast cancers and originates from the cells that nest within the lumen of the breast that have undergone oncogenic transformation. They are classically hormone receptor positive, that is estrogen receptor alpha positive (ERα+) and progesterone receptor positive (PR+) (Sorlie et al., 2001). They are negative for the overexpression or gene amplification of HER2, a member of the EGFR family, and tend to express low levels of genes responsible for proliferation. They lack expression of basal markers such as cytokeratin 5/6, but usually express GATA3 (Chou et al., 2010; Usary et al., 2004), a 13% rate of TP53 mutations (Langerod et al., 2007; Sorlie et al., 2001), and have a distinct characteristic gene expression profile (Perou et al., 2000). Luminal A tumors usually present with a low histological grade and have overall good prognostics when detected early. Patients with luminal A breast cancer are six-times less likely to die from the disease when compared to the aggressive basal-like subtype (Langerod et al., 2007). This overall good survival may be due in part to the availability of targeted therapy directed at ERα. Common hormonal treatments given to these patients to treat early and advanced breast cancer include tamoxifen, a selective estrogen receptor modulator (SERM), often given to pre-menopausal women, and/or letrozole, an aromatase inhibitor (AI), often a better choice for post-menopausal women (Swaby and Jordan, 2008). Luminal A breast cancer tends
to have a 29% rate of relapse 15 years after initial diagnosis and have the longest survival rates when compared to the other subtypes (Kennecke et al., 2010; Peppercorn et al., 2008). However, intrinsic and acquired resistance to adjuvant anti-hormonal therapy is still a major problem showing a 63% rate of recurrence (Peppercorn et al., 2008).

2. Luminal B Breast Cancers

Like luminal A, the luminal B subtype makes up 55% of breast cancers and originates from luminal cells nested in the breast. However, the luminal B gene profile differs from the luminal A, thus creating its own subcategory in breast cancer. They are classically hormone receptor positive (ERα+/PR+), however, a key aspect of luminal B tumors is the presence of ErbB-2/HER2/neu (HER2+) (Sorlie et al., 2001). These tumors are more aggressive in nature than luminal A tumors and often co-express EGFR and/or cyclin E1 (Sorlie et al., 2003). They tend to have high expression of proliferation-associated genes and high histological grade. They too lack expression of basal markers such as cytokeratin 5/6, but express GATA3 (Usary et al., 2004), and have a 40%-70% rate of TP53 mutation status (Langerod et al., 2007; Peppercorn et al., 2008). Much of current research is devoted to attacking this aggressive form of luminal B breast cancer. If presented in the clinic as a non-metastatic lesion, these patients have overall good survival due to combinatory treatment strategies involving pharmacological drugs targeted at the estrogen receptor (tamoxifen or AI), and the humanized monoclonal antibody directed against HER2 (trastuzumab) or a small molecule tyrosine kinase inhibitor against the EGFR/HER2 receptor (lapatinib) (Amar et al., 2008; Bedard et al.,
2009; Dean-Colomb and Esteva, 2008). Compared to luminal A, HER2+, or basal-like subtypes, 50% of patients with luminal B breast cancer have a five year disease-free survival (Sorlie et al., 2001). However, intrinsic or acquired resistance to anti-hormonal or anti-HER2 therapy remains problematic as evidenced by a 81% rate of recurrence (Peppercorn et al., 2008).

3. HER2+ Breast Cancers

Approximately 15-25% of breast cancers have acquired an overexpression or gene amplification for HER2 on chromosome 17q12 (Rouzier et al., 2005), whose transcripts can be elevated by two-fold to greater than twenty-fold (Slamon et al., 1989). Interestingly, the HER2+ subtype is hormone receptor negative showing no positive expression of the estrogen or progesterone receptors, and low levels of their hormone responsive genes (Sorlie et al., 2001). HER2+ breast cancers tend to have aggressive clinical behavior acquiring 4.2 fold increase in expression of proliferation-associated genes when compared to luminal A tumors (Peppercorn et al., 2008). They also have high histological grade and harbor a 71% rate of TP53 mutations (Peppercorn et al., 2008; Weigelt et al., 2009). Although they exhibit luminal features, they are associated with only a 30% rate of disease-free survival at 5 years (Cheang et al., 2008; Sorlie et al., 2001). HER2+ tumors are associated with early relapse, high risk of recurrence, and more likely to involve metastasis to the axillary lymph nodes before diagnosis (Sorlie et al., 2001). However, current anti-HER2 targeted therapy, such as the monoclonal antibody, trastuzumab, or the EGFR/HER2 tyrosine kinase inhibitor, lapatinib, has
reduced the risk of relapse and dramatically improved survival outcomes (Romond et al., 2005). In the adjuvant setting, the overall response rate of 26% to lapatinib treatment was increased to 90% when lapatinib was combined with chemotherapeutic agents in HER2+ breast cancers (Amar et al., 2008; Bedard et al., 2009; Dean-Colomb and Esteva, 2008). Although responsive to chemotherapy and regimented targeted therapy, 10-15% of women acquire resistance, particularly those that present with metastatic tumors (Amar et al., 2008; Romond et al., 2005).

4. Basal-like, Triple Negative Breast Cancers

The basal-like subtype makes up approximately 15-20% of breast cancer cases. They are defined as those tumors which lack ER, PR, and HER2 expression (Sorlie et al., 2001). They are prevalent among pre-menopausal, African American women: 20.8% in African American women versus 10.4% other races including those harboring a BRCA1 mutation (Morris et al., 2007; Peppercorn et al., 2008). Basal-like tumors are very aggressive in nature, have high rates of metastasis (Nielsen et al., 2004), and lead to poor overall survival (van de Rijn et al., 2002). They are considered basal-like because the tumor expresses genes usually associated with the normal breast basal/myoepithelial cells (Jones et al., 2004), such as cytokeratin 5 and 17 (Perou et al., 2000). These tumors are highly proliferative, exhibit elevated expression of proliferation-associated genes, have a high histological grade, up to 85% tend to harbor TP53 mutations (Sorlie et al., 2001; Weigelt et al., 2009), and approximately 60% overexpress EGFR (Nielsen et al., 2004; Reis-Filho et al., 2006). Pathology of basal-like tumors demonstrates high central
necrotic zones, typical and atypical medullary features, and metaplastic areas (Fulford et al., 2006; Livasy et al., 2006). It is important to note, that not all basal-like tumors are triple negative. In fact, a small percentage (<5%) of basal-like tumors contain ER, PR, or HER2 gene expression, implying that the idea of “triple-negative breast cancer” may not be synonymous with basal-like (Reis-Filho and Tutt, 2008). Reis-Filho made it quite clear the importance of this separation in the clinic:

Patients with triple negative cancers expressing a basal phenotype had a significantly shorter disease-free survival than those with triple-negative cancers lacking the expression of basal markers. Therefore, caution should be exercised not to equate a triple negative phenotype with a basal-like profile. Triple-negative cancer is not a synonym for basal-like cancer (Reis-Filho and Tutt, 2008).

This is a clear testament of the diverse signaling pathways present within the cell, the intrinsic differences between breast cancers, and the need to better comprehend and target aberrant factors controlling this transformation.

The interest in basal-like, triple-negative breast cancer is in part due its highly aggressive nature and the fact that currently there is no known targeted therapy. Cytotoxic chemotherapeutics such as taxenes (62% initial response rate), epidermal growth factor receptor (EGFR) inhibitors (49% initial response rate), vascular endothelial growth factor (VEGF) inhibitors (63% initial response rate), and poly-ADP-ribose polymerase (PARP) inhibitors (48% initial response rate) are currently under investigation for the treatment of triple-negative breast cancers (Bartsch et al., 2010).
Although the percent response rates appear high, unfortunately the risk of resistance and recurrence is also high usually developing between the first and third years following therapy (Dent et al., 2007). The majority of patients never make their five year survival mark. The standard of care is a combination of cytotoxic drugs: cyclophosphamide, doxorubicin, and fluorouracil (Hortobagyi, 1998) or recently shown to be more effective in triple negative breast cancer was the combination of cyclophosphamide, methotrexate, and fluorouracil (Colleoni et al., 2010). Their rate of survival is shorter than those who present with luminal type tumors (Sorlie et al., 2001). Thus, there is an immediate need for the elucidation of novel targets to treat these patients and increase their survival.

5. Normal-like Breast Cancers

Normal-like breast cancer is still in its infancy regarding lucid categorization. It is known to be ER positive or negative, PR status undetermined (most likely negative), and HER2 negative. It does show signs of basal markers, leading some to believe it stems from the branch of the basal-like subtype. The grade, presence of TP53 mutations, and relative levels of genes responsible for proliferation are low. Interestingly, Normal-like breast tumors have been shown to consistently cluster with fibroadenoma and normal breast samples. These Normal-like breast tumors contain genes normally associated with adipose tissue (Peppercorn et al., 2008).
6. Claudin-low Breast Cancers

Claudin-low breast cancer acquires its name because of the reduced expression of genes involved in tight junctions and cell-cell adhesion. It could be considered a branch of the triple negative subtype in that it lacks expression of ER, PR, or HER2+. The histological grade and relative levels of genes responsible for proliferation are high. The TP53 status is unknown. However, what differentiates this type from the triple negative is that the transcriptomic features of the tumors are suggestive of ‘cancer stem cell-like’ (Hennessy et al., 2009). Investigations into this subtype may prove to be advantageous and interesting to the field of cancer stem cell research.

7. Molecular Apocrine Breast Cancers

Molecular apocrine breast cancer has recently surfaced as a category, which is believed to be close to the HER2 positive, ER negative, and PR negative subtype. What distinguishes it from the HER2+ subtype and all other ER-negative tumors is the presence of an active androgen receptor (AR) and its subsequent target genes (Farmer et al., 2005). However, the relative levels of genes responsible for proliferation are high. With these gene signatures, apocrine tumors represent 8–14% of breast tumors. The knowledge of elevated AR may provide a novel strategy for targeted therapy in this type of breast cancer.
Risk Factors for Breast Cancer

Age, race, family history, breast density, early menarche, age of first pregnancy, and late menopause can all contribute to the risk of breast cancer (Peppercorn et al., 2008). These are factors over which there is limited personal control. However, certain hormonal therapies such as hormone replacement therapy, obesity, alcohol consumption, inactivity, diet, smoking, and radiation exposure are other risk factors that can be monitored and/or avoided. Similarly, increase instances in ER positive over ER negative breast cancers have been associated with an increase in alcohol consumption (Suzuki et al., 2005). Long-term aspirin use has been associated with decreased development of ER positive tumors, but increased ER negative tumors (Marshall et al., 2005). Poor diets, even ones primarily rich in vegetables, and inactivity has been associated with the development of ER negative breast cancers (Fung et al., 2006). Along with a healthy balanced diet, folate (Vitamin B9) has been linked to a decreased risk of ER negative breast cancer (Zhang et al., 2005). Even though correlations have been observed, it is important to understand that there are multiple factors that are required to develop breast cancer and that none of these alone can be solely attributed to breast cancer development. Nevertheless, there is no doubt that overall psychological and physiological well being helps to provide a better defense against breast cancer development.

Treatment Strategies for Breast Cancer

Breast cancer is a diverse and aggressive disease. When presented at time of diagnosis, each patient is unique and their breast cancer varies according to their grade,
type, and response to treatment. With such individualization, the ultimate decision of proper treatment lies with the patient, their doctors, and their families. Many times, multiple treatment strategies are administered to help eradicate the disease. Currently, the most common strategies to treat breast cancer are surgery, radiation therapy, systemic therapy, and a low-fat/low-alcohol diet.

1. Surgery

When a patient presents in the clinic, a biopsy is taken, and surgery usually follows after diagnosis. Surgery can be minimal such as a lumpectomy, or as extensive as radical mastectomy (Maughan et al., 2010). A lumpectomy is the first choice of surgery for early stage breast cancers, where the surgeon will remove the tumor and a layer of lining stroma. After surgery, there is usually a five to seven week follow-up with radiation therapy. A second surgical choice is simple or total mastectomy, where there is removal of the entire breast. Later stage breast cancers may have infiltrated to the sentinel and/or axillary lymph nodes. A biopsy of the sentinel and then possibly the axillary lymph nodes is taken to determine the extent of metastasis. A modified radical mastectomy may be administered in which the total breast is removed as well as the lymph nodes. An extremely aggressive breast cancer that has infiltrated to the lymph nodes and chest wall requires a total radical mastectomy, where the breast, lymph nodes, and chest wall muscle are all removed (Fisher et al., 2002a; Fisher et al., 2002b; Veronesi et al., 2002). Normally, surgery is not a sole means of treatment. As the grade and stage
of the disease increases, the more combinatorial strategies are taken (e.g. surgery with chemotherapy and radiation).

2. Radiation Therapy

Radiation therapy is either given before surgery to help reduce the tumor burden, or after surgery to insure the annihilation of remaining cancer cells. There are two methods of administering radiation therapy: external or internal. In external radiation, a biophysical machine directs a beam of a radioactive isotope towards the affected breast and/or tumor. The external radiation is administered across an average five to seven weeks and is effective at eliminating and/or reducing tumorigenic cells. In internal radiation, the radioactive isotope is dispensed directly into the tumor through thin capillary wires or needle injection. This method is also termed brachytherapy and is increasingly becoming more effective. Some patients undergo both forms of radiation treatment, but the type and stage of the cancer may favor one method over the other. Currently, accelerated partial breast irradiation (APBI) is a method being developed to reduce doses, area, and duration of treatment (Dirbas, 2009). Although reduced tumor burden has been observed, radiation is rarely used as a sole form of treatment. Its effectiveness relies on both surgery and systemic therapy.

3. Systemic Therapy

Systemic therapy is a means to treat breast cancer through oral administration or injection, which consequently circulates through the entire body. Treatment may be
given before surgery (neoadjuvant), after surgery (adjuvant), or when surgery is not an option. Currently, hormone therapy, targeted biological therapy, and cytotoxic chemotherapy are the most common forms of systemic therapy.

**A. Hormone Therapy**

The estrogen receptor has emerged as a critical hormonal target to modulate in ER positive breast cancers and has been shown to promote tumor growth. Tamoxifen, a selective estrogen receptor modulator (SERM) and partial agonist, is a leading triphenyl-ethylene compound and medicinally modified moiety that binds and competitively inhibits the estrogen receptor, dampens the estrogenic signaling, and consequently reduces the estrogenic responsive effects (Figure 2) (Jordan et al., 1981). Tamoxifen is given orally to both pre- and post-menopausal women for a period of five years (Swaby and Jordan, 2008). It has been demonstrated that following treatment, there is an approximately 33% reduction in death rate and 41% reduction in recurrence (Coates et al., 2007). Tamoxifen has undesirable thromboembolism events and increased endometrial cancer risk as major side effects (Hind et al., 2007). Another anti-estrogen is Fulvestrant (Faslodex), a complete antagonist and competitive inhibitor of the estrogen receptor which is given as an intramuscular injection (Diagram 2) (Buzdar, 2008). It binds to the estrogen receptor and destabilizes the three dimensional structure thus facilitating E3 ubiquitin ligase/proteasome-mediated receptor degradation (Berry et al., 2008; Howell, 2006). Consequently, estrogenic signaling and tumor growth is significantly reduced. Patients taking Faslodex have reported gastrointestinal side effects.
Figure 2: Chemical Structures of Common Estrogenic and Aromatase Inhibitors
Aromatase Inhibitors (AIs) are a class of drugs that inhibit the aromatase enzyme that converts either androstenedione into 17β-estradiol, or directly testosterone into 17β-estradiol. Current drugs include letrozole (Femara), anastrozole (Arimidex), and exemestane (Aromasin) (Figure 2). They are effective in post-menopausal women where small amounts of estrogen are produced in the adipose tissue surrounding the tumor. Current research has shown that AIs alone or interspersed with tamoxifen treatments is more advantageous than tamoxifen alone (Buzdar et al., 2006). AIs are relatively more expensive and possess undesirable side-effects such as osteopenia and bone pain (Hind et al., 2007).

B. Targeted Biological Therapy

Since approximately 30% of breast cancers contain a gene amplification or overexpression of the HER2 receptor linked to tumor survival, HER2 has become a critical target to inhibit. In 2006, trastuzumab (Herceptin) was approved by the FDA to treat patients with HER2 positive breast cancer (O'Mahony and Bishop, 2006). Trastuzumab is a humanized, monoclonal antibody that binds to the extracellular portion of the HER2 receptor (Carter et al., 1992). HER2 dimerization is disrupted and its ability to promote survival is dampened. Studies have shown that treatment with trastuzumab in combination with cytotoxic chemotherapy such as a taxane have reduced patient death rate by 33% and risk of recurrence by 52% (Romond et al., 2005). Lapatinib, a small molecule tyrosine kinase inhibitor targeted against EGFR and HER2, is also used to target the signaling cascade from the EGFR family of receptors (McArthur, 2009).
Another current form of targeted biological therapy is bevacizumab (Avastin), which is a humanized monoclonal antibody against the vascular endothelial growth factor (VEGF) (Sachdev and Jahanzeb, 2008). VEGF is responsible for angiogenesis, and since a tumor needs an enriched blood supply, a VEGF inhibitor prevents tumor vascularization and angiogenesis. Avastin was approved by the FDA in 2008 as an added chemotherapeutic strategy for advanced breast cancer (Spalding, 2008). Other small molecules or antibodies are being developed, tested, and currently are in clinical trials. Targeted biological therapy shows much promise particularly when combined with surgery and cytotoxic chemotherapy.

C. Cytotoxic Chemotherapy

Some breast cancers require a more general systemic treatment. Cytotoxic chemotherapy is designed to target rapidly dividing cells, which is a hallmark of a highly proliferative tumor. Since cytotoxic chemotherapy target all rapidly dividing cells, several non-cancerous cells may unfortunately be targets as well. Rapidly dividing benign cells are found in the hair shaft, intestines, and in the bone marrow, which are why many patients experience side effects such as hair loss, gastrointestinal disturbances, and anemia (Chan and Giaccia, 2008). Some examples of cytotoxic drugs include taxanes (paclitaxel, Taxol), cyclophosphamide, fluorouracil, and cisplatin (Mouridsen, 1990; Rowinsky et al., 1992; Sledge, 1992). Taken together, systemic therapy when combined with other targeted drugs, surgery, and radiation have been shown to be more advantageous than with one form of treatment (Hortobagyi, 1998; Mauri et al., 2008). As
medicines are being developed and studied, future research needs to understand and elucidate novel aspects of breast cancer biology.

**Conclusions: Breast Cancer Now and the Future**

Breast cancer continues to be one of the major causes of cancer-related death in women. Presently, there are four classic subtypes of breast cancer: luminal A, luminal B, HER2/neu, and basal-like, but more novel categorizations are being developed as the systems biology and gene arrays uncover new patterns of expression. Currently, tamoxifen still remains a leading chemotherapeutic choice for hormone positive breast cancers. In addition, the advent of novel aromatase inhibitors and more potent medicinally modified estrogenic inhibitors are becoming an additional choice for treatment. However, approximately half of the women treated will develop resistance to the anti-estrogens, driving current research to develop targeted combinational strategies. Today, trastuzumab (Herceptin) and lapatinib, are chemotherapeutic strategies to treat patients with HER2+ breast cancer. Unfortunately, the triple negative basal-like subtype still manifests high mortality, high recurrence, and no known targeted treatment to date. Therefore, there is an immediate need to identify novel targets that are responsible for the proliferation and invasive phenotype for these cancers so as to increase overall patient survival. Taken together, the future of breast cancer lies within the very biochemical infrastructure of the transformed cells, their stem-likeness, and their microenvironment, which still remains relatively elusive. As each compromised cell responds to its extrinsic signaling cascades and its downstream aberrant intrinsic transcriptional effects,
progressive research will uncover and exploit the molecular mechanisms and novel targets that drive its progression, aggression, and survival.
<table>
<thead>
<tr>
<th>SUBTYPE</th>
<th>EXAMPLE CELL LINE</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>TP53 (MUT)</th>
<th>GRADE</th>
<th>PROLIFERATION GENE PROFILE</th>
<th>CLINICAL FEATURES</th>
<th>TREATMENT STRATEGIES</th>
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<td>LUMINAL A</td>
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<td>Lower risk of reoccurrence</td>
<td>Letrozole Exemestane AI(s)</td>
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<td>LOW</td>
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<td>LOW</td>
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<td>Less responsive to chemotherapy</td>
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<td>LUMINAL B</td>
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<td>+</td>
<td>+</td>
<td>MODERATE</td>
<td>MODERATE</td>
<td>HIGH</td>
<td>Endocrine therapy responsive</td>
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<td>Moderate response to chemotherapy</td>
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<td>MODERATE</td>
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<td>HIGH</td>
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<td>HIGH</td>
<td>Likely involvement of axillary lymph nodes upon diagnosis</td>
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<td>High risk of reoccurrence</td>
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<td>HIGH</td>
<td>HIGH</td>
<td>HIGH</td>
<td>Responsive to chemotherapy</td>
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<td>BASAL-LIKE</td>
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<td>–</td>
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<td>HIGH</td>
<td>Association with BRCA-1 mutations</td>
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<td>No known targeted treatment</td>
<td>Cyotoxic chemotherapy</td>
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</table>

*Abbreviations: ER, estrogen receptor; PR, progesterone receptor; MUT, mutations. References: (Peppercorn et al. 2008, Kao 2009)*

**Table 1: Properties of the Classic Subtypes of Breast Cancer.**
CHAPTER II
NOTCH, PEA3, AND AP-1: A LITERATURE REVIEW

The molecular profile of breast cancer involves a series of interconnected pathways that when perturbed, disrupts normal cellular homeostasis and drives the cell towards transformation. The cell discovers new ways of utilizing signaling cascades and transcriptional events to become hyper-sensitive through amplification or enhanced activity. In addition, elimination of genes, often termed “tumor suppressors,” is exploited so the cell can acquire resistance to apoptosis, immortality, self-sufficiency, and migratory capabilities. Oncogenic pathways that increase proliferation and prevent apoptosis have emerged as potential targets for the treatment of breast cancer. For example, Notch signaling, PEA3 transcriptional effects, and AP-1 dynamic partnership all have been associated with aggressive breast cancers, particularly the triple negative subtype, and are pathways the cancer cell has manipulated to drive its survival and progression.

**Notch Signaling**

In the early twentieth century, the discovery of “Notch” and its name came after an investigation of genetic gene mutants in Drosophila. Upon exploration of the mutations,
one particular mutant resulted in a “notch” of missing tissue at the tip of the fly wing blades (Morgan, 1917). This identification eventually led to the discovery of the Notch pathway. As years passed, Notch signaling became very interesting to scientists both in development –implicated in insulin-secreting pancreatic beta-cells (Apelqvist et al., 1999), the inner ear hair cell development (Lanford et al., 1999), and intestinal crypt and goblet cells (van Es et al., 2005) –and eventually cancer. Notch has emerged as a potent oncogene and is aberrantly expressed in breast cancer (Stylianou et al., 2006). Elevated levels of Notch-1 and one of its ligands, Jagged-1, confer poor prognosis and overall diminished survival (Dickson et al., 2007; Reedijk et al., 2005). Currently, Notch is becoming an advantageous pathway to explore and exploit in neoplastic cells as well as potential cancer stem cells having been implicated in proliferation of tumor initiating or mammary cancer stem cells (Harrison et al., 2010). Currently, Notch is becoming an advantageous pathway to explore and exploit in neoplastic cells as well as potential cancer stem cells having been implicated in proliferation of tumor initiating or mammary cancer stem cells (Harrison et al., 2010).

1. An Overview of Notch Signaling

Notch signaling has emerged as a target for the treatment of breast cancer. In the mammalian system, there are four Notch receptors (Notch-1,2,3,4) which are all type-I transmembrane proteins (Blaumueller and Artavanis-Tsakonas, 1997) and five known ligands (Delta-like 1,2,4, and Jagged 1,2) (Dunwoodie et al., 1997; Lindsell et al., 1995; Shawber et al., 1996). Cell-to-cell contact activates Notch signaling, which subsequently
enables the pathway to modulate genes involved in cell fate, proliferation and differentiation (Callahan and Raafat, 2001). Notch is processed in the trans-Golgi apparatus, where it undergoes the first of three proteolytic cleavages (Figure 3) responsible for transcriptional activation. The first cleavage (S1) takes place in the trans-Golgi by furin-like convertase converting the single polypeptide into the mature heterodimeric Notch receptor held together by di-cations (Ca\(^{2+}\)). The receptor is trafficked to the cellular membrane where it awaits physical contact with its ligand (Kopan and Ilagan, 2009). When ligand binds through EGF-like repeats and electrostatic interactions, the extracellular portion of Notch is co-endocytosed with ligand into the neighboring, ligand-expressing cell thus exposing a second cleavage site (S2) on the transmembrane portion of the Notch (\(N^{TM}\)) which is then cleaved by TNF-alpha converting enzyme (TACE), a disintegrin and metalloproteinase (ADAM10/17) (Brou et al., 2000). Consequently, a third cleavage site (S3) within the transmembrane domain of Notch is now susceptible to proteolysis by the membrane associated aspartyl proteinase, the \(\gamma\)-secretase complex (presenilin, nicastrin, APH1, PEN2) (Kopan and Ilagan, 2004), liberating the intracellular portion of Notch (\(N^{IC}\)) (Saxena et al., 2001). Notch (\(N^{IC}\)) translocates to the nucleus and binds to CBF-1/Serrate/Lag-1, a constitutive repressor on target genes that binds the core sequence CGTGGGAA and other related sequences. \(N^{IC}\) then displaces co-repressors (SMRT, N-coR, SKIP, and histone deacetylases) (Hsieh et al., 1999), and recruits co-activators such as p300, histone acetyl transferases, and mastermind-like 1 (MAML1-3) (Wu et al., 2000). Notch is known to activate many genes including, the pro-differentiation transcription factors, HES and HEY family of
RECEPTORS:  
Notch-1  
Notch-2  
Notch-3  
Notch-4  

LIGANDS:  
Jagged-1  
Jagged-2  
Delta-like 1  
Delta-like 2  
Delta-like 4  

Figure 3: The Notch Signaling Pathway.

Abbreviations- a disintegrin and metalloproteases, ADAM; Notch intracellular protein, NIC; co-activators, CoA; co-repressors, CoR; mastermind-like 1, MAML-1.
basic helix-loop-helix transcription factors, genes implicated in angiogenesis (Fischer et al., 2004) and in epithelial-to-mesenchymal transition (Iso et al., 2003; Maier and Gessler, 2000; Zavadil et al., 2004), cyclin D1 (Ronchini and Capobianco, 2001), c-Myc (Weng et al., 2006), NF-κB (Cheng et al., 2001), and the IGF-1R (Eliasz et al., 2010).

2. Notch Receptors

A. Structural Analysis of Notch Receptors

In the mammalian system, there are four Notch receptors (Notch-1,2,3,4) (Blaumueller and Artavanis-Tsakonas, 1997). The structure of the receptor is conserved, but emerging evidence demonstrates distinct properties among the different receptors (Figure 4). The region of Notch that extends into the intercellular space (N EC) consists of a series of epidermal growth factor-like (EGF-like) repeats. These EGF-like repeats make up the ligand binding surface. The number of EGF-like repeats varies among the receptors: Notch-1 having the most and Notch-4 the least. The EGF-like repeats contain sites for O-linked glycosylation by the O-fucosyl transferase (O-fut) enzyme (Haines and Irvine, 2003). Glycosylation plays a vital role in the function, folding, and ligand binding preference of Notch (Sasamura et al., 2003; Shi and Stanley, 2003). Juxtaposed to the cellular membrane, there are three Lin12 Notch repeats (LNR) that help stabilize and direct appropriate ligand binding.

The next portion of Notch is the transmembrane region (N TM), which transverses the lipid bilayer and contains the cleavage site for the γ-secretase complex. The intracellular portion (N IC) contains a RAM domain (a domain involved in protein
Figure 4: The Four Mammalian Notch Receptors, Their Ligands, and Their Domains.

Abbreviations- epidermal growth factor repeats, EGF; Lin12, LNR; ankyrin repeats, ANK; nuclear localization signal, NLS; cytokine response element, NCR; transactivation domain, TAD; proline glutamic acid serine threonine, PEST; Notch extracellular region, \( N^{EC} \); Notch intracellular region, \( N^{IC} \); cysteine rich region, CR.
interactions), ankyrin repeats (site of protein-protein interactions), two nuclear localization sequence (NLS; Notch-1 contains three), and a C-terminal PEST domain (a signal region for protein degradation). Notch-1 and Notch-2 contain transactivation domains (TAD) and cytokine response elements (NCR), where as Notch-3 only contains a NCR region (Kopan and Ilagan, 2009). All these elements together are vital to the successful activity of Notch signaling.

B. Notch Receptors and Breast Cancer

The Notch receptors have been linked to many types of cancers and are found to play a vital role in the development and progression of mammary tumorigensis (Miele et al., 2006). For example, elevated expression of Notch-1 and its ligand Jagged-1 in ERalpha negative, basal-like, triple negative, or HER2 positive cells are associated with poorest overall patient survival (Dickson et al., 2007; Reedijk et al., 2005).

Using a xenograft MDA-MB-231 model expressing active Notch-2 (N2IC), there was overall decreased tumor take and increased apoptosis (O'Neill et al., 2007). In agreement in endothelial cells, expression Notch-2 (N2IC) stimulated pro-apoptotic genes such as caspases 3 and 7, as well as PARP cleavage (Quillard et al., 2009). These studies imply that unlike Notch-1, Notch-3, or Notch-4, Notch-2 has a pro-apoptotic and possibly tumor suppressor function. This brings to question whether pan-pharmacological Notch inhibitors should be used, or should more specific targeting be explored.

Notch-3 has also been implicated in mammary tumor formation as well as mammary gland development. Similar to studies performed using Notch-1, MMTV-
driven Notch-3 (N3\textsuperscript{IC}) transgenic mice showed similar exacerbation of mammary tumorigenesis (Hu et al., 2006). In HER2 negative breast cancers, downregulation of Notch-3 resulted in decreased proliferation and increased apoptosis even more than Notch-1 (Yamaguchi et al., 2008). Similar to Notch-4, Notch-3 promotes self-renewal and expansion of mammary gland stem and progenitor cells (Sansone et al., 2007). Thus, Notch-3 like Notch-1 and Notch-4 plays a critical role in the transformation and progression of breast cancer.

The Notch-4 gene contains a site of integration (int-3) for retroviral insertion such as mouse mammary tumor virus (MMTV) (Uyttendaele et al., 1996). This site occurs downstream of the Lin12 portion, yet upstream of the transmembrane portion of Notch (see Figure 4). Loss of the extracellular portion from Lin12 to the N-terminus results in a truncated C-terminus form of Notch-4 that is constitutively active. Notch-1 has similar integration sites and is found to also be constitutively active (Dievart et al., 1999). In a separate study, active intracellular Notch-1 or Notch-4 (N1\textsuperscript{IC} and N4\textsuperscript{IC}) was demonstrated to form spontaneous mammary tumors in mice (Callahan and Raafat, 2001). Recently, Notch-4 has been implicated in survival and proliferation of tumor initiating or mammary cancer stem cells (Harrison et al., 2010). Aberrant Notch signaling has become evident not only in tumor initiation, but also progression playing a role in undifferentiated stem-like and more terminally differentiated neoplastic cells.
3. Notch Ligands

In the mammalian system, there are five known ligands (Delta-like 1,2,4, and Jagged 1,2) (Dunwoodie et al., 1997; Lindsell et al., 1995; Shawber et al., 1996). The ligands are divided into two groups depending upon whether or not they contain a cysteine rich region (Delta-class or Jagged, respectively) (see Figure 4). In common, both classes have a DSL domain and a series of EGF-like repeats, which are essential for Notch receptor binding. Notch receptor binding to its ligand on an adjacent cell is critical for proteolysis and signaling events. However in Drosophila, cleaved and/or soluble ligands have been found to activate or downregulate the signaling cascade independent of cell to cell contact depending upon context (Klueg et al., 1998; Sun and Artavanis-Tsakonas, 1997). Interestingly during hematopoiesis, these “decoy” ligands provide an exciting new strategy to modulate the Notch pathway, which could be exploited in other biological systems (Masuya et al., 2002). High Jagged-1 expression has been correlated with the poorest overall patient survival in ERalpha negative breast cancer subtypes including the basal-like, triple negative, and HER2 positive tumors (Dickson et al., 2007; Reedijk et al., 2005).

4. Inhibitors of Notch Signaling

With the understanding that ligand engagement is critical for Notch activation, receptor decoys could be a means to interrupt the ligand-receptor mediated activation (Nickoloff et al., 2002). Disruption of the O-linked glycosylation required for mature receptor formation could also be used to inhibit the activity of Notch. Specific Notch
inhibition could be achieved by using siRNA, antisense, or monoclonal antibodies directed at specific receptors (Miele et al., 2006). Other methods could be mastermind decoys, RAS inhibitors, or even transcription factor-stapled peptides that disrupt the co-activation complex in the nucleus (Moellering et al., 2009).

Enzymatic inhibition has shown promise in dampening Notch signaling. Anyone of the three enzymatic cleavage steps that process Notch (furin-like convertase [S1], ADAM10/17 [S2], or γ-secretase [S1]) (see Figure 3 for more detail) may be used as a means to attenuate signaling. Of great interest is the final cleavage step required for the liberation of the active, intracellular form of Notch (N\text{IC}) to activate Notch signaling (Kopan and Ilagan, 2004; Kopan and Ilagan, 2009). Its inhibition can be exploited through emerging pharmacological drugs identified as γ-secretase inhibitors (GSIs), which inhibit signaling from all four receptors. These can be either peptide-mimics or small molecule inhibitors. One such GSI is the compound developed by Merck Pharmaceuticals known as MRK-003 (Figure 5) and used in this investigation (Lewis et al., 2007). This compound is a small medicinally modified chemical that binds to the ATP-dependent binding pocket of presenilin, the catalytic subunit of the γ-secretase complex. Since this is an experimental drug designed by Merck Pharmaceuticals, the rates, pharmacokinetics, and pharmacodynamics are confidential information secured by the company. Other GSI compounds include LY 411,575, a reversible competitive inhibitor, Calbiochem, an irreversible covalent suicide inhibitor (see Figure 5 for chemical structures), and Z-Leu-Leu-Nle-CHO, a tripeptide inhibitor (Curry et al., 2005). Recent studies demonstrate that GSI treatment in combination with tamoxifen inhibits
Figure 5: Chemical Structures of Common GSI(s).
ER+ breast tumor growth in athymic, nude mice (Rizzo et al., 2008) providing evidence of novel therapeutic approaches to enhancing current treatments. Certain GSI(s) are currently in clinical trials for the treatment of breast cancer.

5. Regulation of Notch Signaling

A. Post-transcriptional Regulation of Notch

Notch signaling is regulated post-transcriptionally by several different methods. Fringe glycosylation of the extracellular portion of Notch determines the relative affinity for contact with its ligands (Okajima and Irvine, 2002). Phosphorylation events on Notch also aid its signaling sustainability such as phosphorylation by GSK-3beta (Foltz et al., 2002). Ubiquitination by E3 ligases such as Neuralized and Mindbomb are important for endocytosis of Notch ligands and subsequent activation events (Le Borgne et al., 2005; Wang and Struhl, 2005). Alpha-adaptin mediated endocytosis by negative regulator Numb also plays a critical role in the regulation of Notch signaling (Berdnik et al., 2002). Numb is shown to interact with the intracellular Notch domain and recruit the Nedd4 family of E3-ubiquitin ligases. This leads to the ubiquitination and degradation of Notch IC (McGill et al., 2009; McGill and McGlade, 2003). Other post-transcriptional events are currently being investigated and could be manipulated as a means to dampen Notch signaling in cancer. However, Notch is already processed and reaching maturity by this time. A better option could be to understand the transcriptional regulation of Notch and terminate its transcription prior to protein synthesis.


B. Transcriptional Regulation of Notch

Notch begins its life in the nucleus as a gene awaiting transcription. The factors that are transcriptional regulators of the Notch receptors are still widely unknown. It was shown in human keratinocytes that p53 regulates the Notch-1 receptor (Alimirah et al., 2007). In vascular endothelial cells, AP-1 was shown to be important in the regulation of Notch-4 transcription (Wu et al., 2005). Demonstrated by the same group, AP-1 was shown to stabilize the glucocorticoid receptor on the Notch-4 promoter in vascular endothelial cells and aid in its expression (Wu and Bresnick, 2007). During T-cell development, NKAP was shown to be a Notch transcriptional repressor (Pajerowski et al., 2009). During hair shaft differentiation, both Msx2 and Foxn1 were demonstrated to be responsible for Notch-1 expression (Cai et al., 2009). However, more detailed transcriptional regulators are left widely understudied, particularly in breast cancer. Thus, novel regulators of the Notch pathway will prove to be significant in the fight against breast cancer.

Promoter analysis of Notch genes reveals evidence of p53, AP-1, SP-1, Ets, as well as several other consensus sites that may all play a role in the transcriptional regulation of Notch which is most likely cell context-dependent. In breast cancer, particularly the basal-like/triple negative breast cancer, used in this investigation, p53 mutations are known to exist, which raises the question of what else is regulating these transcripts. Evidence of Ets consensus sequences is exciting, due to the fact that PEA3, a well known member of the Ets family of transcription factors, is overexpressed in
aggressive breast cancers. Therefore, could the transcription factor PEA3 be responsible for the regulation of Notch in breast cancer?

**Polyomavirus Enhancer Activator 3 (PEA3)**

**1. Ets Transcription Factors**

Ets proteins are a family of transcription factors of which there are over 30 known members. The members are categorized into 13 groups: Ets, TEL, YAN, SPI, ERG, PEA3, ELF, DETS4, ELK, GABP, ER71, ERF, and ESE. These transcription factors share a common DNA Ets domain which binds through its winged helix-turn-helix motif (Graves and Petersen, 1998; Laudet et al., 1999) to the canonical 11 base pair DNA sequences with the core being GGAA/T on target genes (Sharrock, 2001). The affinity of binding relies on the proximal sequences surrounding the Ets binding site. The domain is important for protein-protein co-regulatory interactions. Although there is marked similarities among the Ets domain, differences in a single amino acid within the C-terminal portion of the Ets domain can lead to altered DNA specificity and interactions with other transcription factors (Fitzsimmons et al., 1996; Shore et al., 1996). Activation and/or repression domains are often present in these factors, which aid their transcriptional control based on context (Oikawa and Yamada, 2003). This further individualizes and separates the Ets transcription factors into unique groups.
2. Polyomavirus Enhancer Activator 3 (PEA3)

Polyomavirus enhancer activator 3, commonly known as PEA3, E1AF, or ETV4, is a member of the Ets family of transcription factors and located on chromosome 17q21 (Oikawa and Yamada, 2003). Other family members with PEA3 are ER81 and Erm. PEA3 is important in embryogenesis and is rarely expressed at elevated levels during adulthood. Interestingly, PEA3 levels re-emerge during remodeling events and cancer progression. Approximately 76% of human breast tumors contain elevated levels of PEA3; the highest found in the triple negative breast cancers conferring overall poor prognosis (Trimble et al., 1993). Interestingly, mRNA levels of PEA3 demonstrated marked over-expression, yet endogenous PEA3 protein is rarely detectable (Baert et al., 1997). This may be impart do to the rapid turnover rate and protein stability (Baert et al., 1997). Several studies associate PEA3 in the regulation of enzymes involved in tissue remodeling and invasion such as matrix metalloproteases (MMP-1, MMP-3, MMP-7, MMP-9) (Benbow and Brinckerhoff, 1997), urokinase-type plasminogen activator (uPA) (Evans et al., 2001), cyclooxygenase (COX-2) (Subbaramaiah et al., 2002), HER2/ErbB-2 (Matsui et al., 2006), and IL-8 (Davidson et al., 2003) implicating it as a potential target for therapeutics in a diseased state. However, little concurring evidence of a mechanistic role of PEA3 in breast cancer has left the field widely controversial.

A. The Structure of PEA3

The gene is made up of 13 exons spanning approximately 15 kilobases on chromosome 17q21 (Coutte et al., 1999). The 62 kD protein contains an Ets DNA
binding domain with a tryptophan repeat and a conserved trans-activation domain flanked by negative regulatory domains (Figure 6) (Bojovic and Hassell, 2001). It also contains an acidic region and glutamine-rich region, which have activation capabilities (Higashino et al., 1993). The C-terminus domain of PEA3 has been found to be important to regulate its stability (Takahashi et al., 2005).

B. Activity and Regulation of PEA3

Activation of PEA3 is through phosphorylation of serine and threonine residues by the mitogen activation protein kinase pathway (MAPK: Ras, Raf-1, MEK, ERK-1, and ERK-2) (Wasylyk et al., 1998). The protein is relatively unstable exhibiting a turnover half-life of about 30 minutes via the ubiquitin-proteasome pathway (Takahashi et al., 2005). Recently, PEA3 was suggested to be negatively regulated in part by sumoylation (Bojovic and Hassell, 2008; Takahashi et al., 2005). SUMO-E3 ligase PIASy has been implicated directly in sumoylating PEA3 (Nishida et al., 2007). Sumoylation sites have been identified as lysines 96, 222, and 256 (Bojovic and Hassell, 2008), and have been demonstrated to either transiently enhance the activity of PEA3 or mark it for degradation by the ubiquitin-proteasome pathway (Guo and Sharrocks, 2009). Therefore, there is a direct connection and interplay between both sumoylation and ubiquitinylation pathways.

3. PEA3 and Breast Cancer

PEA3 is overexpressed in 76% of breast cancers. The highest expression is observed in invasive breast cancers particularly the triple negative subtype (Trimble et al.,
Figure 6: Protein Structure of Polyomavirus Enhancer Activator 3 (PEA3).

Abbreviations- phosphorylation motif, P; transactivation domain, TAD; threonine, T; serine, S; lysine, K; sumoylation moiety, SUMO.
1993), which is associated with overall poor prognosis, and is also highly expressed in HER2 positive tumors (Benz et al., 1997). PEA3 is present during embryogenesis and expressed within the epithelial buds of the mammary gland (Chotteau-Lelievre et al., 1997; Chotteau-Lelievre et al., 2003). Later studies confirmed the presence of PEA3 in undifferentiated epithelial cap cells of the terminal end buds (Shepherd and Hassell, 2001). In a mouse model of MMTV-neu-induced mammary primary adenocarcinomas, overexpression of PEA3 resulted in enhanced metastatic lesions (Trimble et al., 1993). Conversely, expression of a dominant negative form of PEA3 resulted in a delay of tumor onset and progression in a MMTV-neu mouse model (Shepherd et al., 2001). PEA3 also enhances transcriptional activity of important genes involved in breast cancer metastasis including, but not limited to cyclooxygenase-2 (Subbaramaiah et al., 2002), MUC4 (Perez et al., 2003), and osteopontin (El-Tanani et al., 2004). Taken together, PEA3 has been correlated with tumor initiation, progression, and invasiveness and appears to play a vital role in breast cancer.

Interestingly, PEA3 has been implicated recently in multipotent progenitor regulation of lineage-specific differentiation (Kurpios et al., 2009) implying a potential function in mammary stem cells. This is similar to the recent study mentioned previously regarding Notch-4 in survival of tumor initiating cells/cancer stem cells (Harrison et al., 2010). This may provide an exciting correlation between PEA3 and Notch-4 expression.

Conflicting studies also have been shown regarding the role of PEA3 in regulation of HER2 and its clinical correlations. Originally, it was identified that the HER2 promoter contained a conserved Ets consensus site that when mutated dampened HER2
transcription (Scott et al., 1994). Shortly after, PEA3 was shown to be upregulated in 93% of HER2/Neu-overexpressing human breast tumors and correlated with breast cancer initiation and progression (Benz et al., 1997). Again in 2001, PEA3 was linked to the positive expression of HER2 helping confirm the potential of PEA3 as a therapeutic target (Shepherd et al., 2001).

However, other studies demonstrated that PEA3 repressed HER2 expression and that PEA3 does not positively correlate with HER2 expression in breast cancer (Xia et al., 2006; Xing et al., 2000). Although the field presents a “gray area” regarding the role of PEA3 in HER2 regulation, this controversy actually provokes the notion of dynamic molecular variability. The role of PEA3 on other genes in different systems implies the importance of cellular context. In fact, further studies demonstrated that PEA3 and c-JUN independently had slight roles in the regulation of HER2. However, their coordinate expression along with histone acetyltransferase p300 stimulated transcription of the HER2 gene by 20-fold in the MCF-7 breast cancer cell line (Matsui et al., 2006). This stresses the importance of transcription factors such as c-JUN working in tandem with PEA3 to appropriately regulate PEA3 target genes. Moreover, PEA3 directly interacts with p300 (Liu et al., 2004) and USF-1 (Firlej et al., 2005), and many other co-regulatory factors to enhance gene transcription. Therefore could PEA3 be responsible with other factors such as AP-1 for the regulation of Notch transcription in breast cancer?
**Activating Protein 1 (AP-1)**

A common player in transcriptional control and implicated in various cellular reactions is the homo- or heterodimeric transcription factor complex AP-1. The dimeric complex classically consists of the FOS (c-FOS, FosB, Fra-1, Fra-2) and JUN (c-JUN, JunB, and JunD) families (Curran and Franza, 1988). Other dimeric complexes can also include ATF and MAF protein families. c-JUN:c-JUN homodimers can form, but are less stable than c-JUN:c-FOS heterodimers (Smeal et al., 1989). Homodimers of c-FOS have yet to be identified (Figure 7). AP-1 contains a leucine zipper motif and binds through its basic DNA binding domain (DBD) to a canonical DNA binding element: TGAg/cTCA. This is known as the TPA-responsive element (TRE) named after its enhanced DNA binding to this consensus site upon treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter. AP-1 is activated through phosphorylation by the mitogen activating pathway (MAPK) or the stress pathway (JNK) (Chen et al., 1996). It is negatively regulated by the ubiquitin-proteasome degradation pathway.

AP-1 cooperates with other proteins including, but not limited to, NFκB, CBP/p300, Rb, and PEA3 (Hesselbrock et al., 2005; Matthews et al., 2007). AP-1 members generally favor a dynamic “switch of partners” to mediate targeted effects as opposed to a “static predetermined partner” model. Expression profiles and partnerships of the different members vary among cell types, even in different breast cancers (Table 2). The functional role of AP-1 is to promote proliferation, differentiation, inflammation,
Figure 7: AP-1 Members and Dynamic Partnerships.

Table 2: Relative Levels of AP-1 Family Members in Breast Cancers.

<table>
<thead>
<tr>
<th>AP-1 Members</th>
<th>Reference Normal</th>
<th>Transformed HBL-100</th>
<th>Invasive MDA-MB-231</th>
<th>Non-invasive T47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-FOS</td>
<td>----</td>
<td>High</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>FosB</td>
<td>Low</td>
<td>----</td>
<td>----</td>
<td>High</td>
</tr>
<tr>
<td>Fra-1</td>
<td>----</td>
<td>Medium</td>
<td>High</td>
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</tr>
<tr>
<td>Fra-2</td>
<td>----</td>
<td>Low</td>
<td>Low</td>
<td>----</td>
</tr>
<tr>
<td>c-Jun</td>
<td>----</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>JunB</td>
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<td>junD</td>
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AP-1 expression pattern was determined in separate cell types: HBL-100 cells, transformed mammary epithelial cells derived from a normal lactating breast, MDA-MB-231 cells which are highly invasive, and T47D cells which are weakly or non-invasive as compared to normal breast tissue as a base reference (Bamberger et al. 1999).
and/or apoptosis. The perturbed expression and activity of AP-1 varies among cancers, and is positively linked to tumor promotion and progression.

1. The c-JUN Family

A. c-JUN

c-JUN was originally identified and characterized as the AP-1 founding member. The protein is approximately 330 amino acids in length and harbors many domains important for its function (Hattori et al., 1988). c-JUN contains a N-terminal transactivation domain (TAD), a domain consisting of a stretch of basic residues considered the DNA binding domain (DBD), and the C-terminal leucine zipper domain (ZIP) (Figure 8). The basic DNA binding region allows for appropriate DNA binding by interaction with the negatively charged DNA backbone. The C-terminal leucine zipper motif aids in protein-protein interactions and dimerization between AP-1 partners. Activation of c-JUN occurs through phosphorylation events by MAPK or JNK within the transactivation domain at serines 63 and 73, and threonines 91 and 93 (Smeal et al., 1991). Phosphorylation events are important for the stability and interaction with other proteins. This transactivation domain has been completely removed to create a dominant negative form of c-JUN and consequently the AP-1 complex itself, known as TAM-67 (Figure 8). This was originally created by Brown et al. in 1993 (Brown et al., 1993). TAM-67 is a transdominant inhibitor of AP-1, which can bind DNA but quenches AP-1 complexes by competing for wildtype c-JUN and c-FOS members (Brown et al., 1994; Brown et al., 1996) thus dampening AP-1 activity. Like PEA3, ubiquitinylation directs c-JUN
Figure 8: Protein Structure of c-JUN and its Dominant Negative Form, TAM-67.

Abbreviations- serine, S; threonine, T; transactivation domain, TAD; DNA binding domain, DBD; basic leucine zipper domain, bZIP.
proteasome-mediated degradation. Interestingly, c-JUN knockout mice are embryonic lethal showing effects on liver, heart, and hepatoblasts (Eferl and Wagner, 2003).

The critical member of the AP-1 family, c-JUN is associated with cancer acting as a major transcription factor. It is overexpressed in triple negative breast cancer cells, MDA-MB-231 cells (Bamberger et al., 1999). c-JUN is associated with proliferation showing marked proliferation alterations in c-JUN deficient fibroblasts (Wisdom et al., 1999). Also, c-JUN cooperates with p300 and PEA3 to promote HER2 mediated transcription in MCF-7 cells, luminal A HER2 expressing breast cancer cells (Matsui et al., 2006). Correlation studies determined that c-JUN is involved in both proliferation, hyperphosphorylated Rb, angiogenesis, and vascular endothelial growth factor (VEGF), showing expression on 38% of invasive breast cancers in 103 breast cancer cases (Vleugel et al., 2006). Interestingly, high expression of c-JUN has been associated with insensitivity to chemotherapy and drug resistance in MCF-7 breast cancer cells (Daschner et al., 1999). Other associations regarding cellular proliferation have shown that c-JUN expression and activity upregulates cyclin D1, a critical mediator of cell cycle progression from G1-S phase (Albanese et al., 1995; Bakiri et al., 2000; Herber et al., 1994) and downregulates p16, an important cell cycle G1/S checkpoint inhibitory protein (Passegue and Wagner, 2000). In contrast, another member of the JUN family, JunB, downregulates cyclin D1 (Bakiri et al., 2000) and upregulates p16 (Passegue and Wagner, 2000), which limits cell proliferation and provides an interesting and distinctive role among each AP-1 member.
B. JunB

JunB has a similar structure to c-JUN, but lacks the important serines 63 and 73 phosphorylation sites on (Kallunki et al., 1996), which may be responsible for the interesting and opposite role JunB has on cellular transformation. Many studies have reported an antagonism between c-JUN and JunB (Bakiri et al., 2000; Passegue and Wagner, 2000); their expression tend to be inversely proportional (Shaulian and Karin, 2002). Its expression in MDA-MB-231 cells is very low (Bamberger et al., 1999). JunB is less active than c-JUN. c-JUN and c-FOS heterodimers are much more potent activators of gene transcription than c-JUN and JunB dimers (Deng and Karin, 1993). This opposite effect is illustrated in overexpression studies in which c-JUN expression alone can transform immortalized rat fibroblasts, but JunB expression has no transformation capability (Schutte et al., 1989). This interesting opposition only stresses the notion that cellular context and dynamic partnership between AP-1 members and their interacting protein partners determines the appropriate cellular response.

C. JunD

JunD has a similar structure as the other JUN family members, but unlike JunB, JunD contains the serines 63 and 73 phosphorylation sites as c-JUN. The functional role of JunD is still being discerned showing both positive and negative effects on cell proliferation and apoptosis (Pfarr et al., 1994; Weitzman et al., 2000). JunD null mice do not form spontaneous tumors, yet inhibition of JunD suppresses neoplastic growth indicative of a tumor promoting effect (Agarwal et al., 1999; Thepot et al., 2000). In a
study investigating 53 mammary carcinomas and three cell lines, relative expression of 
JunD varied greatly with no distinct pattern (Bamberger et al., 1999). It is expressed in 
MDA-MB-231 cells (Bamberger et al., 1999), but its functional role is relatively 
unknown. To further demonstrate the spurious nature of JunD, studies have shown that 
when the interaction with JunD and menin, a known tumor suppressor, is abolished, JunD 
changes its activity from a tumor suppressive function to one that is tumor promoting 
(Agarwal et al., 2003). However, in Ras transformed fibroblasts, JunD protects cells 
from oxidative stress and reduces angiogenesis (Gerald et al., 2004). This further 
demonstrates the necessity of comprehending appropriate cellular context and not 
limiting AP-1 members to a specific function, namely tumor suppressive or tumor 
promoting.

2. The c-FOS Family

A. c-FOS

c-FOS was originally identified and characterized when the viral protein bound to 
the same consensus as c-JUN (Rauscher et al., 1988). It contains 381 amino acids 
consisting of a basic DNA binding domain (DBD), a transactivation domain (TAD), and 
a leucine zipper domain (ZIP) (Figure 9). Similarly to c-JUN, c-FOS has been exploited 
to create a dominant negative AP-1 known as A-FOS. A-FOS is mutant form of c-FOS 
in which the DNA binding domain has been mutated (Figure 9) (Olive et al., 1997). The 
dominant negative mutant incorporates an acidic extension made up of many negatively 
charged glutamate amino acids (Olive et al., 1997). This charge reversal
Figure 9: Protein Structure of c-FOS and its Dominant Negative Form, A-FOS.

Abbreviations- serine, S; threonine, T; leucine, L; transactivation domain, TAD; DNA binding domain, DBD; basic leucine zipper domain, bZIP.
destroys the intermolecular charge attraction between the negative DNA backbone and the DNA binding domain of A-FOS, thus dampening AP-1 activity (Ransone et al., 1990). c-FOS heterodimerizes with c-JUN to form a stable dimeric transcription factor capable of regulating genes important in cell proliferation, differentiation, and apoptosis (Shaulian and Karin, 2001). Homodimers of c-FOS do not exist, but additional binding partners of c-FOS are MAF proteins (Kataoka et al., 1996), CBP (Bannister and Kouzarides, 1995), and GATA-4 (McBride et al., 2003). Similar to c-JUN, c-FOS is phosphorylated on threonine 232 by MAP kinases (Deng and Karin, 1994) and by ERKs on threonines 325, 331, and 374 (Monje et al., 2003) to enhance its activity. c-FOS relies on dimerization for its activity, since it is highly unstable and rapidly degraded by the ubiquitin-mediated proteasome degradation (Bossis et al., 2003).

In cancer, c-FOS upregulates genes responsible for angiogenesis such as VEGF (Marconcini et al., 1999) and MMP-1 and MMP-3 (Hu et al., 1994). It has also been shown that c-FOS upregulates DNA 5-methylcytosine transferase responsible for methylating and silencing tumor suppressor genes (Bakin and Curran, 1999). Alternatively, c-FOS upregulates FAS ligand (FASL) to induce apoptosis through the extrinsic death-receptor pathway in T-lymphocytes (Kasibhatla et al., 1998). It is highly expressed in triple negative breast cancer, MDA-MB-231 cells (Bamberger et al., 1999). Even though the majority of the literature associates active c-FOS with cancer progression, the specific role of c-FOS depends on cell type and cellular context. With this in mind, c-FOS becomes a more dynamic partner relying greatly on other cellular signals.
**B. Fra-1**

Elevated levels of AP-1, especially Fra-1, are commonly found in the most aggressive estrogen receptor negative mammary tumors when compared to less metastatic estrogen receptor positive mammary tumors (Zajchowski et al., 2001). Exogeneous overexpression of Fra-1 in the non-invasive MCF-7 breast cancer cells disturbs cell morphology and increases the invasive potential of the cells. Conversely, if endogenous Fra-1 is inhibited using RNA interference in MDA-MB-231 cells, overall motility and aggressiveness are diminished (Belguise et al., 2005). In a cDNA array of breast cancer subtypes, increased expression of both Fra-1 and c-JUN partners was correlated to overall breast tumor aggressiveness (Zajchowski et al., 2001). Similarly, Fra-1 expression in epithelioid adenocarcinoma cells demonstrated a positive effect on motility and invasiveness (Kustikova et al., 1998). In MDA-MB-231 cells, motility was not affected by Fra-1, but its invasiveness potential was highly enhanced (Milde-Langosch et al., 2004). Recently, Baan et al. demonstrated that invasive breast cancer cells including MDA-MD-231 favored the c-JUN:Fra-1 heterodimer, compared to less invasive breast cancer cells, which favored the c-JUN:c-FOS heterodimer (Baan et al., 2010). Fra-1 is known to regulate genes involved in proliferation (p19-ARF) (Ameyar-Zazoua et al., 2005), invasion (MMP-1,9,) (Milde-Langosch et al., 2004), and angiogenesis (IL-8) (Freund et al., 2004). Taken together, AP-1 complexes involving Fra-1 most probably are critical for mammary tumorigenesis and invasion.
C. Fra-2

Most focus on AP-1 has encompassed c-JUN, c-FOS, and Fra-1 leaving the other members like Fra-2 in the shadows. Nevertheless, Fra-2 does play an interesting role in development and cancer. Fra-2 is involved in cellular differentiation during development. The expression pattern of Fra-2 is observed in the epithelia and central nervous system during mouse development (Carrasco and Bravo, 1995). Its expression in triple negative breast cancer cells MDA-MB-231 is relatively low (Bamberger et al., 1999). Fra-2 overexpression in mouse mammary adenocarinoma cells (CSML0) had no affect on motility, but in a fibroblastoid cell line, its overexpression effected motility (Tkach et al., 2003). In MDA-MB-231 cells, Fra-2 expression had a mild positive effect on cellular invasion (Milde-Langosch et al., 2004). Fra-2 tends to be a modular protein partner not only relying on cellular context, but also the AP-1 core complex.

D. FosB

FosB is the last member of the FOS family and possesses an opposite function than the other members. An inverse correlation between FosB and Fra-1 has been observed. In tumor tissues with high Fra-1 expression, FosB is low, but in tissues where FosB is elevated, low expression of Fra-1 is observed. Also, association of FosB with well-differentiated and estrogen positive breast cancer has been observed (Bamberger et al., 1999). The expression of FosB is relatively undetectable in poorly-differentiated steroid receptor negative clinical breast cancer tissues, but highly expressed in normal ductal mammary epithelial cells (Milde-Langosch et al., 2004). Opposite of the effects
demonstrated by expression of c-FOS, Fra-1, and Fra-2, FosB has an inhibitory effect on cell proliferation in correlation studies using breast cancer samples (Milde-Langosch et al., 2000). Although FosB has an inverse role than its siblings, the AP-1 partner choice and directed cellular signals could tip the balance and potentially change its function role.

3. AP-1 and PEA3

AP-1 interacts with partners on the promoter of many genes which is dependent on cellular context to determine function. Similarly, PEA3 exists in a dynamic equilibrium relying on partners to determine its function (Kurpios et al., 2003). Interestingly, PEA3 has been shown to partner with AP-1 on several genes such as MMP-1, MMP-3, MMP-7, MMP-9 (Benbow and Brinckerhoff, 1997), urokinase-type plasminogen activator (uPA) (Evans et al., 2001), cyclooxygenase (COX-2) (Subbaramaiah et al., 2002), HER2/ErbB-2 (Matsui et al., 2006), IL-8 (Davidson et al., 2003), and mammaglobin (Hesselbrock et al., 2005). AP-1 and PEA3 DNA binding elements are generally within relative close proximity to each other and generally to the transcriptional start site (Benbow and Brinckerhoff, 1997; Davidson et al., 2003; Evans et al., 2001; Hesselbrock et al., 2005; Matsui et al., 2006; Subbaramaiah et al., 2002). Taken together, AP-1 and PEA3 are most probably dynamic transcriptional partners that tend to share a common gene regulatory unit.
A Systems Approach: Linking Notch, PEA3, & AP-1

Biological systems are made up of interconnecting networks of cellular communication. These pathways involve a plethora of proteins that when stretched beyond homeostasis produces a diseased state. Notch signaling, PEA3, and AP-1 are shown to be atypically regulated in breast carcinomas. Studies have shown that elevated levels and/or misdirected signaling of these proteins lead to abnormal cell proliferation, increased aggressive phenotypes, and overall reduced patient survival (Angel and Karin, 1991; Coutte et al., 1999; Kopan and Ilagan, 2009). In our investigations, initial promoter scanning indicated proximal AP-1 and PEA3 sites on both the Notch-1 and Notch-4 genes. With vast research independently correlating Notch, PEA3, and AP-1 with breast cancer, we provide, to our knowledge, for the first time a link between these signaling pathways, with aim at exploiting and manipulating their function to create innovative strategies for better targeted treatment of triple-negative breast cancer. Women diagnosed with triple-negative breast cancer have the worst overall prognosis, frequently present with metastatic tumors, and have few targeted therapy options to date. And therefore, there is an immediate need to identify and understand the novel targets that are responsible for the proliferation, survival, and invasive phenotype of breast cancer.
CHAPTER III
MATERIALS AND METHODS

Cell Culture

MDA-MB-231, MCF-7, SKBr3 and BT474 breast cancer cells were purchased from American Type Culture Collection. All cell lines were supplemented with 100 μM nonessential amino acid and 1% L-glutamine (2 mM). SKBr3 cells [supplemented with 10% fetal bovine serum (FBS)] and MDA-MB-231 cells (5% FBS) were maintained in Iscove’s Minimal Essential Media (IMEM). MCF-7 cells (10% FBS) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12). BT474 cells (10% FBS) were maintained in Dulbecco's Modified Eagle Medium (DMEM). All cells were maintained at 37°C with 95% O2 and 5% CO2.

Drugs and Chemicals

Gamma-secretase inhibitor (MRK-003) was kindly provided by Merck Oncology International, Inc. (see Diagram 5 for chemical structure). MRK-003 was dissolved in the solvent dimethylsulfoxide (DMSO) and stored at -80°C. Lactacystin (L6785) was purchased from Sigma Aldrich (St. Louis, MO, USA), dissolved in water, and stored at -20°C until use.
**Expression Vectors**

pcDNA3.1 and pcDNA3.1-PEA3 expression vectors were kindly provided by Dr. Mein-Chie Hung (The University of Texas, MD Anderson Cancer Center, Houston, TX, USA). Control CMV-500, CMV-500-AFOS, Control pHMB, and pHMB-TAM67 vectors were kindly provided by Dr. Richard Schultz (Loyola University Medical Center, Maywood, IL, USA).

**RNA Interference and Reagents**

All small interfering RNA (siRNA) reagents were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Transfection reagents were Lipofectamine 2000 and Lipofectamine RNAiMAX purchased from Invitrogen (Carlsbad, CA, USA), and Fugene 6 was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA). Protocols were performed according to the manufacturer’s instructions. Co-transfections of DNA plasmid and siRNA were performed with Lipofectamine 2000. The siRNA sequences are shown in Table 3.

**Antibodies**

Notch-1(C-20): (sc-6014-R), Notch-4(H-225): (sc-5594), PEA3 (16): sc-113, c-FOS(H-125): (sc-7202), c-JUN(G-4): sc-74543, FRA-1(R-20): (sc-605) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Beta-actin (AC-15): (A5441) was purchased from Sigma Aldrich (St. Louis, MO, USA) and used as the internal
### Table 3: Sequences of siRNA(s).

<table>
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<td>Notch-1</td>
<td>sc-36095</td>
<td>5'-CACCAGUUUGAGUUGUCAA-A3'</td>
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<tr>
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<td></td>
<td>5'-CCAUUUACCACAUUGA-A3'</td>
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<tr>
<td>PEA3</td>
<td>sc-36205</td>
<td>5'-CCAGACAAUUGCCAUCAA-A3'</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>5'-CCAUUCGAUGAAACAGA-A3'</td>
</tr>
<tr>
<td>c-JUN</td>
<td>sc-29223</td>
<td>5'-GUAGAGGACUGUUUAGUA-A3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-CCAGGAUAAUUCAAAGA-A3'</td>
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<td>5'-GAGGGCCUUUGCUUAUGA-A3'</td>
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<tr>
<td></td>
<td></td>
<td>5'-GCAUUCUGAUUAGAAUCU-A3'</td>
</tr>
<tr>
<td>c-FOS</td>
<td>sc-29221</td>
<td>5'-CAAGCGACAGUUGUAUCU-A3'</td>
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<td></td>
<td></td>
<td>5'-CCGAUCACGUUUGAGAA-A3'</td>
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</table>

### Table 4: Sequences of PCR Primers.

<table>
<thead>
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<th>TARGETS</th>
<th>FORWARD PRIMERS</th>
<th>REVERSE PRIMERS</th>
</tr>
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<tbody>
<tr>
<td>Notch-1</td>
<td>5'-GTCAACGCCGTAGTGATGACC-3'</td>
<td>5'-TTGTAGGCCCCGTTCTTCAG-3'</td>
</tr>
<tr>
<td>Notch-2</td>
<td>5'-TCCACTTCTACCTACTTAGTTGA-3'</td>
<td>5'-TTGTTTCAGAGAAAACATA-3'</td>
</tr>
<tr>
<td>Notch-3</td>
<td>5'-GGGAAGAAAAGCCAATAGGCG-3'</td>
<td>5'-GGAGGGAAGCAACCTAGCAC-3'</td>
</tr>
<tr>
<td>Notch-4</td>
<td>5'-AACCTCCCTCCCAAGAATGCTG-3'</td>
<td>5'-CTCCATCAGAAGGAAGTT-3'</td>
</tr>
<tr>
<td>Hes-5</td>
<td>5'-ATGGCCCCCAGCCTTGTCG-3'</td>
<td>5'-CTGACAGCCATCTCCAGATG-3'</td>
</tr>
<tr>
<td>Hey-1</td>
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<td>5'-TTGTGAGATGCGAAACCAG-3'</td>
</tr>
<tr>
<td>cIAP2</td>
<td>5'-GGACATGCGTGTGCGGAACTG-3'</td>
<td>5'-CCTTTAATCTTCTTACTAGATCTACACCTT-3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>5'-ATGAACCAGTTATGACCTTGAT-3'</td>
<td>5'-CTGTTGAGCTGTCATTACAATA-3'</td>
</tr>
<tr>
<td>PEA3</td>
<td>5'-AGGAGACGTTGCTGCTGA-3'</td>
<td>5'-GGGGCTGTGGAAAGCTAGT-3'</td>
</tr>
<tr>
<td>COX-2</td>
<td>5'-CAGGATACAGCTCCACAGCA-3'</td>
<td>5'-ATCAGCGCTCTTCCATTGACC-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-TCGTGGTTCCACTCGGTGTT-3'</td>
<td>5'-GGCAGGGCTCTCAAGATGA-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-CAGCGGAAGGAAACCTCCTCACT-3'</td>
<td>5'-TCAGCCCTCTTTCATAAAACCTTCTCC-3'</td>
</tr>
</tbody>
</table>
control. Secondary antibodies include donkey anti-mouse IgG-HRP (sc-2314) and donkey anti-rabbit IgG-HRP (sc-2313) (Santa Cruz, CA, USA).

**Real-Time RT-PCR**

MDA-MB-231 (4x10⁵), MCF-7 (4x10⁵), SKBr3 (5x10⁵), and BT474 (5x10⁵) cells were plated in a 6-well tissue culture treated dish. Twenty-four hours later, the cells were transfected with appropriate reagents (7 μL of lipofectamine 2000 for plasmids or 5 μL of lipofectamine RNAiMAX for siRNA and 5 μL of siRNA reagents (16 nM) and/or 3 μg of pcDNA3.1-PEA3) and maintained at 37°C 95% O₂ and 5% CO₂. Forty-eight hours later, cells were harvested. Total RNA was extracted from the cells using the RNA extraction kit: RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). Total RNA recovered was then quantified using the NanoDrop Spectrophotometer (Thermo Scientific). The cDNA was reverse transcribed (RT) from 1 μg total RNA extracted in a total 100 μL volume containing 1X RT buffer, 5.5 mM MgCl₂, 500 μM dNTPs, 2.5 μM random hexamers, 0.4 U/μL RNase inhibitors, and 1.25 U/μL RT enzyme (MultiScribe™ Reverse Transcriptase Kit, Applied Biosystems, Foster City, CA, USA). The parameters were as follows: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, and held at 25°C until use. Analysis of mRNA relative fold copy number adjusted to hypoxanthine-guanine phosphoribosyl transferase (HPRT), an endogenous control, was carried out by real-time, quantitative PCR using iTaq™ SYBR® Green Supermix with ROX (BioRad, Hercules, CA, USA). In a 96-optical PCR plate, 2.5 μL of cDNA (10 ng/μL) was added to 22.5 μL of mastermix (2X Syber green Universal Master Mix, and 1 μM forward/reverse primers).
The quantitative PCR parameters were as follows: the initial denature temperature was 95°C for 10 minutes; PCR cycling for 40 cycles was carried out at 95°C for 10 seconds, and 60°C for 45 seconds. A melt curve was added after completion of the 40 cycles set by the StepOnePlus thermocycler manufacturer (Applied Biosystems). Cycle number at threshold (Ct) values were subsequently determined relative to the value for the quantified cDNA that was amplified. Analysis of mRNA copy number adjusted to hypoxanthine-guanine phosphoribosyl transferase (HPRT), an endogenous control, was used to calculate relative fold induction or decrease compared to a control sample. The PCR primers that were used for detection of specific transcripts are shown in Table 4.

**Western Blot Analysis**

MDA-MB-231 (4x10^5) cells were plated in a 6-well tissue culture treated dish. Twenty-four hours later, the cells were transfected with appropriate reagents (7 μL of lipofectamine 2000 with plasmid addition or 5 μL of lipofectamine RNAiMAX with siRNA addition and 5 μL of siRNA (16 nM) reagents and/or 3 μg of pcDNA3.1-PEA3) and maintained at 37°C with 95% O₂ and 5% CO₂. Forty-eight hours later, cells were harvested. The cells were lysed in radioimmunoprecipitation assay buffer (RIPA, pH 8.0 containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin and 1 mg/mL leupeptin). Lysates were sonicated six times for three seconds each and incubated on ice for 30 minutes. Lysates were then centrifuged at 10,000 g for
5 minutes at 4°C. The supernatant was isolated for further testing. Protein within the supernatant was measured using the BCA protein concentration assay (Pierce Chemicals, Rockford, IL), incubated for 30 minutes at 37°C with 95% O₂ and 5% CO₂, and measured using a 96 plate UV/Visible spectrophotometer (PolarStar Omega, BMG Labtech, Durham, NC). Samples were treated with an equal volume of sample buffer containing NuPAGE 1X LDS Sample buffer, 1X NuPAGE reducing agent or 1 mM DTT. Samples were heated at 70°C for 10 minutes. Total denatured proteins in lysates were added to wells of a 4-12% NuPAGE® Bis-Tris Gels in 3-(N-morpholino) propanesulfonic acid running buffer (MOPS). The gel was run at 175V for 1.5 hours and separated proteins were transferred to PVDF membranes at 38V for 2 hours in Transfer Buffer (25 mM Tris, 192 mM glycine, 10% methanol). The membranes were then blocked in 5% non-fat milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 0.2%NP-40 at pH 8.0) for one hour at room temperature while constantly shaking. Primary antibodies were added (1:200) overnight in 5% non-fat milk in TBST at 4°C. Blots were washed three times in TBST at intervals of 10 minutes at room temperature while constantly shaking. Secondary antibodies conjugated to horseradish peroxidase were added (1:2000) to 5% non-fat milk in TBST for one hour at room temperature while constantly shaking. Blots were then subjected to a second wash cycle: washed three times in TBST at intervals of 10 minutes at room temperature while constantly shaking. Substrate for protein detection was SuperSignal® West Dura (Thermo Scientific, Rockford, IL, USA) added in equal parts, incubated for 5 minutes at room temperature while rocking, and visualized by the FUGIFILM™ Las-300 imager.
To re-probe western blots, the membranes washed three times in TBST at intervals of 10 minutes at room temperature while vigorously shaking. Restore Plus Western Blot Stripping buffer (Thermo Scientific) was added to cover the membrane and incubated for 1 hour at 47°C. The blots were then rocked at room temperature for 30 minutes followed by three quick rinses with deionized water. The membranes were then rocked at room temperature in deionized water for 5 minutes, and then in TBST for 5 minutes. Membranes were then stripped and Western blot analysis was repeated as described above.

**Luciferase Constructs and Assay**

Notch-4 luciferase constructs were generously provided by Dr. Emery Bresnick (University of Wisconsin at Madison, Madison, WI, USA) and previously described (Wu et al., 2005). Notch-1 luciferase constructs were generously provided by Dr. Paolo Dotto (Massachusetts General Hospital: Cutaneous Biology Research Center, Charlestown, MA, USA). The AP-1 luciferase construct was generously provided by Dr. Richard Schultz (Loyola University Medical Center, Maywood, IL, USA). MDA-MB-231 (1x10^5) cells were plated in a 6-well tissue culture treated dish. Twenty-four hours later, the cells were co-transfected in each well with appropriate reagents: 7 μL of lipofectamine 2000 with plasmid addition 4 μg of Firefly luciferase (AP-1 or Notch-4 wildtype or mutant reporters) and 0.4 μg pTL-TK (Renilla luciferase and internal control), and/or 5 μL (16 nM) of PEA3 siRNA reagent. The cells were incubated at 37°C with 95% O₂ and 5% CO₂. Forty-eight hours later media was removed from the cells, they were washed with
ice cold PBS, and harvested in 1X passive lysis (300 μL) for 30 minutes while rocking at room temperature. The plates were then frozen at -80°C for 1 hour to over night. The plates were then removed from the freezer and rocked at room temperature for 15 minutes. Ten microliters of each sample was added to a white coated luminescent 96-well plate and subjected to the manufactured dual-luciferase assay (Promega, Madison, WI, USA). Luminescence was measured using the Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). The range of luminescence was between 350-650 nm; Firefly luciferase read at 560 nm and pTL-TK at 480 nm. The graphs represent the ratio of Firefly luciferase to Renilla luciferase (560 nm / 480 nm).

**Chromatin Immunoprecipitation (ChIP)**

MDA-MB-231 cells (3x10^6) were plated in 150 cm² petri dishes for 24 hours. The cells were transfected with 3 μg of pcDNA3.1, 3 μg of pcDNA3.1-PEA3, and 3 μg of pcDNA3.1-PEA3 and 5 μL of 10 μM solution of PEA3 siRNA for 48 hours at 37°C with 95% O₂ and 5% CO₂. The total volume in the wells was 3 mL. Media was aspirated and the cells washed once with ice-cold PBS. The cells were cross-linked with 1% formaldehyde in fresh media and incubated at 37°C with 95% O₂ and 5% CO₂ for 10 minutes. A final concentration of 0.125 M glycine in the 150 cm² petri dishes was added while rocking at room temperature for 5 minutes to stop the reaction. The media was aspirated while on ice, and washed twice with ice-cold PBS. Cells were then scraped, centrifuged at 2000 rpm at 4°C, and lysed in 500 μL SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing a protease inhibitor cocktail tablet (Roche
Diagnostics, Indianapolis, IN). The samples were allowed to incubate on ice for 10 minutes. Following incubation, the lysates were sonicated using the Branson Sonifier 250 at output 4.5, duty cycle 50, and pulsed 10 times. The lysates were then diluted in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, pH 8.1) to achieve a 1 to 10 dilution ratio and separated into two tubes: 1) IgG mouse isotype control, and 2) specific PEA3 antibody. Lysates were pre-cleared with 30 μL of protein G-plus beads (sc-2002, Santa Cruz) for 1 hour at 4°C with agitation. Supernatants were isolated and 4 μg of PEA3 (sc-113X) antibody or mouse IgG isotype control were added and incubated at 4°C overnight with agitation. Fifty microliters of protein G-plus beads (sc-2002, Santa Cruz) were added to the immune complexes for 2 hours while gently rocking. Immune complexes/beads were washed in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.1), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.1), LiCl buffer (1% sodium deoxycholate, 1% NP-40, 0.25 M LiCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The protein/antibody complexes from beads were eluted in freshly prepared elution buffer (1% SDS, 0.1 M sodium bicarbonate, pH 8.0). Protein/DNA was reversed crosslinked by heating at 65°C overnight while gentle rocking. The protein was degraded using a proteinase solution (0.5 M EDTA, 1 M Tris-HCl, 10 mg/mL proteinase K, pH 8.0) and incubated at 52°C for 1 hour. DNA was isolated using the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). 20 μL of total lysate was set as an input control. Enrichment at promoter sites was detected quantitative PCR using iTaq™
SYBR® Green Supermix with ROX (BioRad, Hercules, CA, USA). Ct values were normalized to the IgG control. Quantitative real-time PCR was carried out as described under the Real-Time PCR method section. The following primers flanking specific promoter regions used in detection are shown in Table 5. A schematic representation of the Notch-1 and Notch-4 promoters is shown in Diagram 10.

**Co-Immunoprecipitation (Co-IP)**

MDA-MB-231 cells (3x10^6) were plated in 150 cm^2 petri dishes for 24 hours. The cells were transfected with 3 μg of pcDNA3.1, 3 μg of pcDNA3.1-PEA3, and 3 μg of pcDNA3.1-PEA3 and 5 μL of 10 uM solution of PEA3 siRNA for 48 hours at 37°C with 95% O_2 and 5% CO_2. The total volume in the wells was 3 mL. Media was aspirated and the cells were washed once with ice-cold PBS. The cells were cross-linked with 1% formaldehyde in fresh media and incubated at 37°C with 95% O_2 and 5% CO_2 for 10 minutes. A final concentration of 0.125 M glycine in the 150 cm^2 petri dishes was added while rocking at room temperature for 5 minutes to stop reaction. The media was aspirated while on ice, and washed twice with ice-cold PBS. Cells were then scraped, centrifuged at 2000 rpm at 4°C, and lysed in 500 μL SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing a protease inhibitor cocktail tablet. The samples were allowed to incubate on ice for 10 minutes. Following incubation, the lysates were sonicated using the Branson Sonifier 250 at output 4.5, duty cycle 50, and pulsed 10 times. The lysate concentration was ascertained using the protein BCA protein assay (Pierce Chemicals, Rockford, IL), incubated for 30 minutes at 37°C with 95% O_2
Table 5: Chromatin Immunoprecipitation Primers.

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<th>ChIP Primers</th>
<th>#</th>
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<td>2 x PEA3 (+Ctrl)</td>
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<td>E</td>
<td>5'-CTCCTTGCGCCTTAACCAGG-3'</td>
<td>5'-CCAGAAAGCAAAACGGGTC-3'</td>
</tr>
<tr>
<td>AP-1 (A)</td>
<td>B</td>
<td>5'-GGTGCCTGTGTTGAAAGCTCT-3'</td>
<td>5'-TCTTCAAGGCCCCCTGCA-3'</td>
</tr>
<tr>
<td>AP-1 (B)</td>
<td>C</td>
<td>5'-TCCGCAACCAGGCTCTG-3'</td>
<td>5'-ATGGGCTGCACTGCGG-3'</td>
</tr>
<tr>
<td><strong>B) Notch-4 Promoter</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>D</td>
<td>5'-AGTTGGGACAGAGGAGTG-3'</td>
<td>5'-TGGGTCTGACCAGCTGAC-3'</td>
</tr>
<tr>
<td>CBF-1 (+Ctrl)</td>
<td>A</td>
<td>5'-GTTGACCCCCGTGCAATGAT-3'</td>
<td>5'-GCTCAGGCTATTAGAGCATTG-3'</td>
</tr>
<tr>
<td>AP-1 (A)</td>
<td>B</td>
<td>5'-AGCTGCCACTGACACCTTCT-3'</td>
<td>5'-CAGGGAATGCCAGTCAAGAAT-3'</td>
</tr>
<tr>
<td>AP-1 (B)</td>
<td>C</td>
<td>5'-ACTTCCCCAGGGTTGTC-3'</td>
<td>5'-CTTCCTCTCGGCTCCTGCT-3'</td>
</tr>
</tbody>
</table>

Figure 10: Schematic Representation of Notch-1 and Notch-4 Promoters Labeled to Designate Areas of Primer Design.
and 5% CO₂, and measured using a 96 plate UV/Visible spectrophotometer. The lysates were equally diluted in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, pH 8.1) to achieve a 1 to 10 dilution ratio and separated into two tubes: 1) IgG mouse isotype control, and 2) specific PEA3 antibody. Lysates were pre-cleared with 30 μL of protein G-plus beads (sc-2002, Santa Cruz) for 1 hour at 4°C with agitation. Precleared lysates were incubated with 3 μg of PEA3 (sc-113X) or mouse IgG overnight. Thirty microliters of protein G-plus beads (sc-2002, Santa Cruz) were added to the immune complexes for 2 hours while gently rocking. Immune complexes/beads were washed three times in phosphate buffered saline (PBS). The pellet was resuspended in Laemmli sample buffer supplemented with beta-mercaptoethanol (BioRad, Hercules, CA, USA) and heated for 5 minutes at 95°C while vigorously shaking. Western blot technique was used for detection of co-immunoprecipitation proteins as described in the Western Blot section of these methods. Antibodies used for detection were: PEA3 (16), c-FOS (H-125), c-JUN (G-4), and FRA-1 (R-20) from Santa Cruz Biotechnologies, CA.

**Cell Cycle Analysis**

MDA-MB-231 cells (1x10⁵) were seeded in a 6-well plate. After 24 hours, the cells were treated/transfected with either DMSO + scrambled siRNA, DMSO + PEA3 siRNA, MRK-003 (10 μM) + scrambled siRNA, or MRK-003 (10 μM) + PEA3 siRNA. After 24 and 48 hours post-treatment/transfection at 37°C with 95% O₂ and 5% CO₂, the cells were trypsinized, and the media was isolated. Cell and media were centrifuged at
4°C, 500 rpm for 5 minutes. The cell pellet was resuspended in 2 mL ice-cold PBS. From the 2 mL single cell suspension, 500 μL of solution was placed into FACS tubes. Cells were pelleted at 1200 rpm (4°C) for 5 minutes. The supernatant was removed by inverting and decanting and washed by adding 2 mL of ice cold 5% bovine calf serum (BCS) in PBS. Cells were again pelleted at 1200 rpm (4°C) for 5 minutes and fixed: cells were resuspend cells in 100 μL ice cold 5% BCS and 600 μL of ice cold 100% EtOH was slowly added while gently mixing. The tube was incubated on ice for 30 minutes and then washed by adding 2 mL of ice cold 5% BCS. Cells were then centrifuged at 1200 rpm (4°C) for 5 minutes and resuspended in 500 μL of 10 μg/mL RNase A. They were incubated at 37°C for 15 minutes and then allowed to incubate at room temperature for 5 minutes. To the 500 μL volume, 500 μL of 100 μg/mL Propidium Iodide (P4170, Sigma Aldrich) was added, gently mixed, and incubated at room temperature for at least one hour in a dark area void of light. The samples were measured by flow cytometry (FACS Canto, BD Biosciences).

**Annexin-V Apoptosis Assay**

MDA-MB-231 cells (1.5x10⁵) were seeded in a 6-well plate for 24 hours. Cells were treated and transfected with either DMSO + scrambled siRNA, DMSO + PEA3 siRNA, MRK-003 (10 μM) + scrambled siRNA, and MRK-003 (10 μM) + PEA3 siRNA. After 24 and 48 hours post-treatment/transfection at 37°C with 95% O₂ and 5% CO₂, cells were trypsinized, washed twice with ice-cold PBS, and resuspended in a 1X Annexin-V binding buffer (0.1 M HEPES/NaOH, 140 mM NaCl, 25 mM CaCl₂, pH 7.4).
From that suspension, 100 μL of the solution was placed into a FACS compatible tube. To the tube, 5 μL of 1 μg/mL FITC-Annexin-V stain (556420, BD Biosciences) and 5 μL of 50 μg/mL propidium iodide (P4170, Sigma Aldrich) were added, incubated for 15 minutes at room temperature in the dark. Then, 400 μL of 1X Annexin-V binding buffer was added back and immediately within the hour the samples were measured by flow cytometry (FACS Canto, BD Biosciences) using an Annexin-V specific antibody. Control tubes were used for compensation and background normalization: 1) a tube containing cell suspension in Annexin-V binding buffer alone, 2) a tube containing cell suspension in Annexin-V binding buffer with FITC-conjugated Annexin-V antibody, 3) a tube containing cell suspension in Annexin-V binding buffer with Propidium Iodide, and 4) a tube containing cell suspension in Annexin-V binding buffer, FITC-conjugated Annexin-V antibody, and Propidium Iodide.

**Cell Viability Assay**

MDA-MB-231 cells (1x10^5) were seeded in a 6-well plate. After 24 hours, the cell were treated and transfected with either DMSO + scrambled siRNA, DMSO + PEA3 siRNA, MRK-003 (10 μM) + scrambled siRNA, and MRK-003 (10 μM) + PEA3 siRNA. After 24 and 48 hours post-treatment and transfection at 37°C with 95% O₂ and 5% CO₂, the cells were trypsinized, and the media was collected. Cell and media were centrifuged at 4°C, 500 rpm for 5 minutes. The cell pellet was resuspended in 2 mL ice-cold PBS. From the 2 mL single cell suspension, 500 μL of solution was placed into 1.5 mL Eppendorf tube. 500 μL of Trypan Blue was added, mixed gently, and incubated at room
temperature for 20 minutes. Then, 20 μL of the solution was place on a heamocytometer. Stained cells were counted at 40X magnification under a standard light microscope (Leica DMIL, Leica Microsystems), normalized, and graphed as percent viability as compared to control.

**Colony Formation Assay**

A 1.75% solution of Nobel agar was autoclaved, allowed slightly to cool, and added in equal parts [1:1] to IMEM supplemented with 100 μM nonessential amino acid, 1% L-glutamine (2 mM), and 5% FBS (making a final agar solution of 0.8%). To a 6-well plate, 1 mL of the agar/media solution was added and allowed to completely solidify. Once cooled, 2 mL of a 1% methylcellulose (M0512, Sigma Aldrich)/IMEM solution (supplemented with 5% FBS, 1% nonessential amino acids, and 1% L-glutamine) was added on top of the agar. The methylcellulose/IMEM solution was made as follows: A 2% methylcellulose solution in serum-free media was made by stirring vigorously for 2-3 hours at room temperature and transferred/stirred at 4°C overnight. Then, 5%FBS, 1% NEAA, and 1% L-glutamine was added back to the solution, stirred at room temperature for 30 minutes, and transferred/stirred at 4°C for 1-2 hours depending upon incorporation rate. Once incorporated completely, the solution was centrifuged at 5500 rpm for 30 minutes at 4°C. The solution was then warmed, to accommodate the transfected/treated cells to 37°C. MDA-MB-231 (1x10³ cells/ml) were treated/transfected with either DMSO + scrambled siRNA, DMSO + PEA3 siRNA, Mrk-003 (5 μM) + scrambled siRNA, and Mrk-003 (5 μM) + PEA3 siRNA for 24 hours at 37°C and 5% CO₂. Forty-
eight hours later, the transfected/treated cells were trypsinized, counted, and added directly to the methylcellulose solution and 2 mL of 10,000 cells/well were added atop of the formerly solidified agar coated plates. The assay was left untouched for 14 days at 37°C and 5% CO₂. After 14 days, 20 μL of crystal violet stock (0.2% crystal violet and 2% ethanol diluted in deionized water) was added to each well and gently swirled to make a final stain concentration around 0.002%. The plate was then incubated for 1 hour in a 37°C, 5% CO₂ incubator. Colonies were photographed (4X magnification) and counted under a standard light microscope. Nine fields were counted per well and averaged.

**Statistical Analysis**

The statistics of these experiments are represented by means plus or minus standard deviations on graphs and numbers in parenthesis that denote standard deviation. The statistical significance of three or more independent studies in these experiments was determined by a two-tailed, unpaired Student’s 𝑡-test for two comparisons. Statistical significance of pairwise comparisons of more than two experiments was discerned using a one-way ANOVA.
CHAPTER IV
RESULTS

Even with the advent of modern technologies, breast cancer still remains an area of extensive research with hopes of uncovering novel strategies to help treat patients. Previous investigations have determined that overexpression of Notch-1 and Notch-4 play a critical role in breast tumorigenesis and PEA3 overexpression is associated with aggressive breast cancers particularly the triple-negative subtype. Preliminary research via promoter scanning using NCBI Entrez Gene bank identified on the Notch-1 and Notch-4 promoters several putative Ets binding sites, some independent with no known consensus sequences nearby, or interestingly some Ets sites adjacent to AP-1 consensus sites. Notch-2 and Notch-3 also have putative Ets binding sites, but preliminary real-time PCR and Western blot data directed the focus on Notch-1 and Notch-4 for these studies. Knowing the role of both these aberrant pathways in breast cancer, this investigation aims at determining a link between the Notch pathway and PEA3 signaling. The following hypothesis was proposed:

*PEA3 is a transcriptional activator of Notch-1 and Notch-4 in MDA-MB-231 triple-negative breast cancer cells. Dual inhibition of PEA3 and Notch signaling will result in a reduction in cell proliferation and survival implicating better therapeutic targeting.*
The following aims were formulated to address the hypothesis in MDA-MB-231 cells:

**Specific Aim 1.** To determine whether PEA3 is an upstream mediator of the Notch signaling pathway.

**Specific Aim 2.** To elucidate whether PEA3 acts a direct positive regulator of Notch-1 and Notch-4 transcription.

**Specific Aim 3.** To establish a biological significance by determining whether dual inhibition of Notch and PEA3 leads to reduced cell proliferation and tumorigenicity *in vitro*.

This research strategy provides novel evidence of a link between two pathways that are overexpressed in breast cancer. PEA3 is a transcriptional activator of both Notch-1 and Notch-4 mRNA in MDA-MB-231 cells. PEA3-mediated Notch-1 transcription is AP-1 independent while Notch-4 transcription requires both PEA3 and AP-1 (Figure 11). PEA3 and Notch signaling are essential for proliferation and survival in MDA-MB-231 cells. Furthermore, PEA3 is a transcriptional activator of both Notch-1 and Notch-4 mRNA in other breast cancer cells. Thus, the hypothesis remains that dual targeting of both PEA3 and Notch pathways might provide a new therapeutic strategy for triple-negative breast cancer as well as possibly therapeutic targeting in other breast cancer subtypes where PEA3 regulates Notch-1 and Notch-4.
Our novel findings

Previously known

Therapeutic strategy

HYPOTHESIS

PEA3

siRNA

AP-1

c-JUN
FR-A-1

Notch-1

GSI

Notch-4

MMP-9

COX-2

IL-8

Migration

Malignancy

Proliferation

HES

HEY-1

Cyclin-D1

Our novel findings

Previously known

Therapeutic strategy

Figure 11: Hypothesis of the Investigation.

PEA3 is a novel transcriptional activator of Notch-1 and Notch-4 in basal-like breast cancer. Dual inhibition of PEA3 and Notch signaling will result in a reduction in cell proliferation, tumorigenicity, and malignancy implicating better therapeutic targeting and overall survival.
SPECIFIC AIM-1:

To Determine Whether PEA3 is an Upstream Mediator of the Notch Signaling Pathway.

MDA-MB-231 cells are a representative cell line of triple-negative breast cancer. MDA-MB-231 cells lack expression of estrogen receptor, progesterone receptor, or HER2 overexpression. These cells are the model system used for the subsequent studies.

The investigation began by determining the efficiency of PEA3 knockdown by RNA interference to determine its resulting effects in vitro. Acquiring SMART-POOL of three specific PEA3 siRNAs from Santa Cruz Biotechnologies (Santa Cruz, CA), siRNA transfection in a dose dependent manner was performed to evaluate the optimal concentration of PEA3 siRNA. Using lipofectamine as the transfecting vehicle, 5 μL of siRNA (16 nM) was transfected into MDA-MB-231 cells. After 48 hours, cells were harvested for RNA and protein. Real-time PCR of PEA3 transcripts indicated that 5 μL of siRNA (16 nM) was sufficient to reduce PEA3 transcripts by ~90% (Figure 12). Endogenous PEA3 is extremely difficult to detect; the protein is relatively unstable exhibiting a turnover rate of about 30 minutes via the ubiquitin-proteasome pathway on the C-terminus domain of PEA3 (Takahashi et al., 2005). It is rare to capture the endogenous PEA3 protein levels (Baert et al., 1997). A Western blot analysis of endogenous PEA3 protein levels demonstrated the corresponding effect of PEA3 siRNA
MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or 5 μL of PEA3 siRNA (PEA3i) corresponding to overall siRNA concentrations of 16nM. Relative mRNA levels of PEA3 were measured 48 hours later as a knockdown control using quantitative PCR. Transcripts were normalized to HPRT and means were plotted. Error bars represent standard deviation. Statistical significance (p<0.0001) of three experiments was determined by two-tailed, unpaired Student’s t-test.
**Figure 13: Endogenous PEA3 protein upon RNA Interference.**

MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or 5 μL of PEA3 siRNA (PEA3i) corresponding to overall siRNA concentrations of 16nM. Protein levels of PEA3 were detected by Western blot analysis after 48 hours as described in the materials and methods. 50 μg of total protein lysate in each lane was subjected to SDS-PAGE 4-12% gel. Beta-actin was detected as an endogenous control.
on its protein levels (reduced by 50% upon siRNA transfection as measured by densitometry) (Figure 13).

1. PEA3 Regulates Notch-1 and Notch-4 Transcripts.

To investigate the role of PEA3 on Notch expression, the effect of PEA3 RNA interference on the expression of all four Notch receptor (1,2,3,4) mRNAs was examined. Cells were transfected with either scrambled control siRNA or PEA3 siRNA for 48 hours. As measured by real-time PCR, Notch-1 and Notch-4 transcripts were decreased by 50% and 70%, respectively upon PEA3 knockdown. Notch-3 transcript levels were unchanged. Interestingly, Notch-2 levels showed a moderate but significant increase upon PEA3 knockdown, which upon further investigation may prove to be advantageous since it has been correlated with pro-apoptotic and breast tumor suppressive function [(O'Neill et al., 2007) and discussed in Chapter III] (Figure 14).

2. PEA3 Regulates Notch-1 Protein Expression.

To determine if the effect on Notch-1 and Notch-4 transcripts by PEA3 correlated with protein expression, we measured steady protein levels using Western blotting in cells transfected with either control (SCRBi) or PEA3 siRNA for 48 hours. The results showed a reduction of Notch-1 full length (FL) and transmembrane (TM) receptor protein levels by 40% and 50%, respectively (Figure 15) agreeing with its corresponding transcripts. In contrast to Notch-1, PEA3 siRNA had no noticeable effects on steady-state Notch-4 IC protein levels (Figure 5, lanes 1 and 2). However, it is known that the
Figure 14: Effects of PEA3 siRNA on Notch Transcripts.

MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or PEA3 siRNA (PEA3i) for 48 hours. Relative mRNA levels of Notch receptors (NOTCH-1,2,3,4) were measured by quantitative PCR. Transcripts were normalized to HPRT and means were plotted. Error bars represent standard deviation. Statistical significance of three or more experiments was determined by two-tailed, unpaired Student's t-test.
Figure 15: Protein Levels of Notch-1 Upon PEA3 RNA Interference.

MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or 5 μL (16nM) of PEA3 siRNA (PEA3i). Protein levels of Notch-1 were detected by Western blot analysis after 48 hours as described in the materials and methods. 10 μg of total protein lysate in each lane was subjected to SDS-PAGE 10% gel. Both transmembrane portion (TM) and full length portion (FL) of Notch-1 were detected by the antibody. Beta-actin was detected as an endogenous control. Graphs represent quantification of changes in full length and transmembrane length Notch upon transfection with either scrambled or PEA3 siRNA as measured by densitometry. Statistical significance of three or more experiments was determined by two-tailed, unpaired Student’s t-test.
**Figure 16: Protein Levels of Notch-4 Upon PEA3 RNA Interference.**

MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or 5 μL (16nM) of PEA3 siRNA (PEA3i). Protein levels of Notch-4 were detected by Western blot analysis after 48 hours as described in the materials and methods. Cells were treated with or without 7 μM lactacystin after 24 hours of transfection for an additional 18 hours. 50 μg of total protein lysate in each lane was subjected to SDS-PAGE 4-12% gel. Beta-actin was detected as an endogenous control. Western Blot is representative of more than three experiments.
Notch-4 receptor is sensitive to stress and has a rapid turnover rate with a half life of 1.25 hours which is mediated by the proteasome (Wu et al., 2001). Thus, to determine if protein stability is critical for regulation of Notch-4 protein by PEA3, a proteasome inhibitor (lacacystin) was used to treat the cells prior to transfection with control or PEA3 siRNA. The protein levels of Notch-4 IC were increased almost two fold (Figure 16, lane 3) with lacacystin treatment and decreased by almost 90% upon PEA3 knockdown (Figure 16, lane 4). This result suggests that Notch-4IC protein expression is critically regulated by protein stability and not necessarily at the transcriptional level. Since PEA3 siRNA demonstrated little effects on Notch-2 and Notch-3 transcripts, their corresponding protein levels by Western blot analysis were not pursued.

3. Classic Gene Targets of PEA3 and Notch are Decreased by PEA3 Knockdown.

To confirm the effect of PEA3 siRNA on the activity of PEA3, its classical downstream targets were measured by real-time PCR after transfection with control or PEA3 siRNA for 48 hours. PEA3 target genes, namely IL-8 and MMP-9, were significantly reduced by 80% and 60%, respectively (Figure 17). To address whether the Notch signaling pathway was affected by PEA3 RNA interference, a classic downstream target of the Notch, HEY-1, was also significantly reduced upon PEA3 knockdown by 40% (Figure 6). Taken together, these results suggest that PEA3 positively regulates Notch-1 and Notch-4 receptor transcripts, Notch-1 protein and, Notch signaling in MDA-MB-231 cells.
Figure 17: Transcript Levels of Classic PEA3 and Notch Targets Upon PEA3 siRNA.

MDA-MB-231 cells were transfected with either scrambled siRNA (SCRBi) or PEA3 siRNA (PEA3i) for 48 hours. Downstream targets of PEA3 (IL-8, MMP-9) and Notch (HEY-1) were measured by quantitative PCR. Transcripts were normalized to HPRT and means were plotted. Error bars represent standard deviation. Statistical significance of three or more experiments was determined by two-tailed, unpaired Student’s t-test.
SPECIFIC AIM-2:

To Elucidate Whether PEA3 Acts as a Direct Positive Regulator of Notch-1 and Notch-4 Transcription.

To determine the mechanism by which PEA3 regulates Notch-1 and Notch-4 transcription, the promoter regions of Notch-1 and Notch-4 were scanned using NCBI Entrez Gene bank and identified several putative Ets binding sites (Figure 18). Interestingly both promoters contained two putative AP-1 sites adjacent to Ets sites. A classic CBF-1 site was also previously identified in the Notch-4 promoter and used as a control. Primers were subsequently designed to include these identified regions. Primers flanking promoter regions containing no known consensus sites were used as negative controls (Figure 18). Notch-2 and Notch-3 also contain minimal putative Ets sites. The quantitative PCR results on these promoters indicated mild changes in transcript levels (Figure 14) and therefore were not pursued. However, future studies on these sites may prove to be significant and/or may be used as negative controls.

Emphasizing the notion that PEA3 is highly unstable, endogenous protein levels are difficult to detect (Baert et al., 1997). Consequently, exogenous expression of PEA3 was required to perform subsequent chromatin immunoprecipitations (ChIPs). Empty vector (pcDNA3.1) and PEA3 expression vector (pcDNA3.1-PEA3) were tested in MDA-MB-231 cells in a dose dependent manner to determine an optimal concentration. Upon transfection of pcDNA3.1-PEA3 at concentrations of 0.5 μg, 1.0 μg, 2.0 μg, and
Figure 18: Schematic Representation of Notch-1 and Notch-4 Promoters.
4.0 μg, transcript levels of PEA3 increased in a dose dependent manner (Figure 19). Moreover, a classic target gene of PEA3, COX-2, was measured and demonstrated a similar dose dependent increase (Figure 19). Discerning that the maximal effect was at a concentration of 4 μg, 4 μg concentration of pcDNA3.1-PEA3 and pcDNA3.1 was used in the subsequent ChIP analyses.

To determine if exogenous PEA3 expression was compatible with immunoprecipitation, 4 μg of pcDNA3.1 and pcDNA3.1-PEA3 vectors were transfected in MDA-MB-231 cells for 48 hours prior to being subjected to immunoprecipitation. Endogenous levels of PEA3 were not detected by immunoprecipitation, however exogenous expression successfully immunoprecipitated PEA3 protein (Figure 20).

1. PEA3 is Enriched on the Notch-1 and Notch-4 Promoters.

To address whether PEA3 is recruited to the Notch-1 and Notch-4 promoters, chromatin immunoprecipitation experiments were performed on lysates from MDA-MB-231 cells that were transfected with PEA3 expression plasmid (pcDNA3.1-PEA3). Comparable to the immunoprecipitation studies, ChIP analysis on endogenous levels of PEA3 was not measurable (Figure 21) most likely a result of PEA3 protein instability, which it is rapidly turned over and undetectable (Baert et al., 1997).

Five Ets sites on the Notch-1 promoter and four on the Notch-4 promoter were identified and pursued via chromatin immunoprecipitation of PEA3. As a result of ChIP analysis, PEA3 enrichment was found on both Notch-1 (Figure 22) and Notch-4 (Figure 23) promoter regions within 1.1 kilobase or -70 nucleotides, respectively, upstream of the
Figure 19: Exogenous Expression of PEA3 and Its Effects on Target Genes.

MDA-MB-231 cells were transfected with either vector control plasmid (pcDNA3.1) or PEA3 expression vector (pcDNA3.1-PEA3) in a dose dependent manner for 48 hours. Relative mRNA levels of PEA3 and a classic target gene (COX-2) were measured by quantitative PCR. Transcripts were normalized to HPRT and means were plotted.
Vectors: pcDNA3.1 pcDNA3.1-PEA3 I.P.

Conjugated Antibodies: IgG PEA3 IgG PEA3

Figure 20: Immunoprecipitation of Endogenous and Exogenous PEA3.

MDA-MB-231 cells were transfected with either pcDNA3.1 or pcDNA3.1-PEA3 vectors. Immunoprecipitation of PEA3 and its isotype IgG control were performed after 48 hours as described in the materials and methods. Total immunoprecipitated protein lysates were subjected to SDS-PAGE 4-12% gel. Heavy and light chain IgG were detected as an endogenous loading control.
Figure 21: Chromatin Immunoprecipitation of Endogenous and Exogenous PEA3 on the Notch-1 and Notch-4 Promoter.

MDA-MB-231 cells were transfected with either pcDNA3.1 (Vector alone) or PEA3-pcDNA3.1 overexpression plasmid. Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-1 and Notch-4 promoter. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Below graphs is a schematic representation of the Notch-1 and Notch-4 promoters containing the identified enriched Ets sites. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 22: Chromatin Immunoprecipitation of PEA3 on the Notch-1 Promoter.

MDA-MB-231 cells were transfected with PEA3 expression plasmid (pcDNA3.1). Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-1. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Below graphs is a schematic representation of the Notch-1 promoter containing multiple AP-1 consensus sites and Ets sites. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 23: Chromatin Immunoprecipitation of PEA3 on the Notch-4 Promoter.

MDA-MB-231 cells were transfected with PEA3 expression plasmid (pcDNA3.1). Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-4 promoter. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Below graphs is a schematic representation of the Notch-4 promoters containing multiple AP-1 consensus sites and Ets sites. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
transcriptional start site. The other identified sites via promoter scanning showed no measurable PEA3 enrichment (Figure 22 and Figure 23).

To address the specificity of the PEA3 antibody to detect recruitment of PEA3 to these promoter regions, a SMART-POOL of three specific PEA3 siRNAs was used. Western blot analysis was performed to determine the efficiency of PEA3 siRNA on exogenous PEA3 expression (Figure 24). As a result from ChIP analysis, PEA3 enrichment on the -1.1 kb site on the Notch-1 promoter and the -70 bp site on the Notch-4 promoter was abrogated upon specific PEA3 knockdown (Figure 25).

2. PEA3 and AP-1 Regulate the Notch-4 Promoter.

Previous studies in vascular endothelial cells performed by Wu et al. at the University of Wisconsin at Madison indicated the importance of AP-1 on the regulation of Notch-4 (Wu et al., 2005). To assess if the -70 nucleotide region upstream of the start site was indeed necessary in MDA-MB-231 cells for the regulation of the Notch-4 promoter by PEA3 as identified by ChIP and potentially AP-1, a wild type AP-1 or mutant AP-1-containing Notch-4 promoter luciferase reporter constructs were obtained from the Dr. Emery Bresnick’s laboratory and previously described (Wu et al., 2005). The wild type construct contained an intact AP-1 and Ets consensus sites at -70 nucleotides. The mutant AP-1 construct contained an ablated AP-1 consensus site using site-directed mutagenesis; the adjacent Ets binding site was left preserved (Wu et al., 2005) (Figure 26).
Figure 24: Western Blot Analysis of PEA3 Expression.

MDA-MB-231 cells were transfected with either pcDNA3.1 and scrambled siRNA (SCRBi), pcDNA3.1-PEA3, PEA3 siRNA, or a combination of pcDNA3.1-PEA3 and PEA3 siRNA (PEA3i). Protein levels of PEA3 were detected by Western blot analysis after 48 hours as described in the materials and methods. Total protein lysate in each lane was subjected to SDS-PAGE 4-12% gel. Beta-actin was detected as an endogenous control.
Figure 25: PEA3 Enrichment Specificity on the Notch-1 and Notch-4 Promoters as Measured by Chromatin Immunoprecipitation.

MDA-MB-231 cells were co-transfected with PEA3 overexpression plasmid (pcDNA3.1) and either scrambled siRNA (SCRBi) or PEA3 siRNA (PEA3i) for 48 hours. Lysates then were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-1 and Notch-4 promoter. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Below graphs is a schematic representation of the Notch-1 and Notch-4 promoters containing the identified enriched Ets sites. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 26: Wildtype and Mutant Notch-4 Promoter Luciferase Constructs

Abbreviations: AP-1 consensus site construct, wt; Site-directed AP-1 consensus site mutant construct, mut;
The wildtype or mutant Notch-4 promoter Luciferase constructs were co-
transfected with either scrambled or PEA3 siRNA in our *in vitro* triple-negative MDA-
MB-231 model system. PEA3 siRNA significantly decreased wild type AP-1-containing
or mutant AP-1-containing reporter activity compared to the scrambled control (Figure
27). These results suggest that either PEA3 or AP-1 is critical for the regulation of the -70
region within the Notch-4 promoter in MDA-MB-231 cells. The mutant ablated AP-1
Luciferase results suggest that PEA3 may play an independent role from AP-1. However,
the construct does contain an additional, but minimal Ets site upstream of the AP-1:Ets
site, which may account for the further decrease in Luciferase on the mutant AP-1
construct. As a control study, we observed no significant difference in AP-1 luciferase
reporter activity upon PEA3 knockdown indicating that PEA3 is not mediating effects on
the Notch-4 promoter through AP-1 regulation (Figure 28).

To address if PEA3 acts directly with AP-1 on Notch-1 and Notch-4 promoter
regions, since it is known to work in accordance with c-JUN on several other genes
(referenced in Chapter III), transfected lysates with either pHMB empty vector or pHMB-
TAM-67, a dominant negative form of c-JUN missing the transactivation domain (Figure
8) (Liu et al., 2002), were subjected to chromatin immunoprecipitation. PEA3
enrichment on the Notch-1 promoter was not affected by TAM-67 (Figure 29). However,
PEA3 recruitment to an Ets site adjacent to an AP-1 site approximately -70 nucleotides
upstream of the start site of the Notch-4 promoter was abrogated by expression of TAM-
67 (Figure 29) indicating that the transactivation domain of c-JUN is required for PEA3
recruitment. As a control, dual Firefly-Renilla luciferase assays using the AP-1 reporter
Figure 27: Luciferase Assay of Wildtype and AP-1 Mutant Notch-4 Promoter.

Above is a schematic representation of the Notch-4 luciferase construct indicating mutated AP-1 consensus site and Ets site. MDA-MB-231 cells were co-transfected with either wildtype AP-1 or mutated AP-1 Notch-4 luciferase and either scrambled or PEA3 siRNA. Firefly luciferase (pGL2) was measured normalized to Renilla as an internal transfection control. Means and standard deviation of three or more experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 28: PEA3 Does Not Regulate AP-1 Activity.

Above is a schematic representation of the AP-1 luciferase construct. MDA-MB-231 cells were co-transfected with an AP-1 luciferase and either scrambled or PEA3 siRNA. Firefly luciferase (pGL2) was measured normalized to Renilla as an internal transfection control. Means and standard deviation of three or more experiments were plotted.
Figure 29: Dominant Negative c-JUN Ablates PEA3 Recruitment on Notch-4, And has No Effect on PEA3 Recruitment on Notch-1.

MDA-MB-231 cells were co-transfected with PEA3 expression plasmid (pcDNA3.1) and with either pHMB-vector or TAM-67. Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-1 and 4 promoters, respectively. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
construct containing four tandem AP-1 consensus sites (Figure 28) were performed to determine the efficiency of TAM-67 on AP-1 activity. Upon co-transfection with TAM-67 and AP-1 luciferase construct, AP-1 activity was diminished by 50% (Figure 30).

To determine if the c-JUN protein specifically is required for PEA3 enrichment on the identified Notch-4 promoter region, transfected lysates with either scrambled or a smart-pool of specific c-JUN siRNAs were subjected to chromatin immunoprecipitation. In agreement, PEA3 enrichment on the Notch-4 promoter region was abrogated upon c-JUN knockdown (Figure 31, top) suggesting that c-JUN is required for PEA3 enrichment on the identified Notch-4 promoter region. As a control, c-JUN siRNA was transfected in MDA-MB-231 cells and subjected to quantitative real-time PCR. As a result, c-JUN siRNA reduced endogenous c-JUN transcripts by 45% (Figure 31, bottom). Chromatin immunoprecipitation of c-JUN on Notch-1 and Notch-4 promoters was performed, but resulted in inconclusive findings. Other investigators have completed successful ChIP(s) of c-JUN (Zhao et al., 2010), which in our system will require optimization and increased sensitivity.

To determine whether PEA3 is in a complex with c-JUN since c-JUN is required for PEA3 enrichment as observed by the previous chromatin immunoprecipitation experiments, PEA3 was immunoprecipitated and Western blotting was performed to detect PEA3 and c-JUN proteins. Co-immunoprecipitation demonstrated that PEA3 was in complex with c-JUN (Figure 32). Taken together, these results strongly indicate that PEA3 and c-JUN are required to regulate the Notch-4 promoter.
Figure 30: Dominant Negative c-JUN (TAM-67) Reduces AP-1 Activity.

Above is a schematic representation of the wildtype and dominant negative c-JUN construct. MDA-MB-231 cells were co-transfected with AP-1 luciferase and either phMB-vector or TAM-67. Firefly luciferase (pGL2) was measured normalized to Renilla as an internal transfection control. Means and standard deviation of three or more experiments were plotted.
Figure 31: c-JUN is Required for PEA3 Enrichment on the Notch-4 Promoter.

MDA-MB-231 cells were co-transfected with PEA3 expression plasmid (pcDNA3.1) and with either scrambled or c-JUN siRNA. Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-4 promoter. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Means and standard deviation of more than three experiments were plotted. Below: Quantitative PCR was used to measure the efficiency of c-JUN siRNA knockdown. Transcripts were normalized to HPRT and means were plotted. Error bars represent standard deviation. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 32: Co-Immunprecipitation of PEA3 in Complex with c-JUN.

MDA-MB-231 cells were transfected with PEA3 expression plasmid (pcDNA3.1). Lysates were subjected to chromatin immunoprecipitation and subsequent Western blot analysis using either PEA3 antibody or isotype control IgG. Following Western blot analysis, PEA3 and c-JUN proteins were detected by immunoblotting. Western blot are representative of three independent experiments.
Since c-JUN is known to heterodimerize with the FOS family and to understand if c-FOS is required for PEA3 enrichment on Notch-1 and Notch-4 promoter regions, transfected lysates with either CMV-500 empty vector or CMV-500-AFOS, a dominant negative form of c-FOS (Figure 9), were subjected to chromatin immunoprecipitation. PEA3 enrichment on either the Notch-1 or Notch-4 promoter was not affected by A-FOS (Figure 33). Although c-JUN and c-FOS are preferred partners, c-JUN homodimers indeed exist as well as other FOS family members, which may contribute to PEA3 promoter enrichment. The dominant-negative effect of A-FOS on AP-1 activity was confirmed by co-transfecting A-FOS and AP-1 luciferase construct and performing a dual Firefly-Renilla luciferase assays (Figure 34). The results demonstrated a 40% reduction in AP-1 activity upon A-FOS transfection (Figure 34). Taken together, the results indicate that AP-1, with emphasis on c-JUN, and PEA3 regulate the Notch-4 promoter in MDA-MB-231 cells.

3. PEA3 and AP-1 Dynamically Regulate Notch-4 Transcription.

AP-1 members c-JUN, Fra-1, and c-FOS are overexpressed in MDA-MB-231 cells (Bamberger et al., 1999). To address which member(s) of the AP-1 complex is responsible for the regulation of Notch-4 gene transcription with PEA3, MDA-MB-231 cells were transfected with PEA3, Fra-1, c-JUN, or c-FOS siRNA individually for 48 hours.

As measured by quantitative real-time PCR, Notch-4 transcripts were reduced upon individual knockdown of PEA3, c-JUN, or Fra-1 and that the siRNA combinations
Figure 33: Dominant Negative c-FOS (A-FOS) Has No Effect on PEA3 Recruitment on Notch-1 and Notch-4 Promoters.

MDA-MB-231 cells were co-transfected with PEA3 expression plasmid (pcDNA3.1) and with either CMV-500-vector or A-FOS. Lysates were subjected to chromatin immunoprecipitation using either PEA3 antibody or isotype control IgG on the Notch-1 and 4 promoters, respectively. PEA3 enrichment was measured by quantitative PCR and normalized to IgG control. Means and standard deviation of more than three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 34: Dominant Negative c-FOS (A-FOS) Reduces AP-1 Activity.

Above is a schematic representation of the wildtype and dominant negative c-FOS construct. MDA-MB-231 cells were co-transfected with AP-1 luciferase and either CMV-500-vector or A-FOS. Firefly luciferase (pGL2) was measured normalized to Renilla as an internal transfection control. Means and standard deviation of three or more experiments were plotted.
of either c-JUN:PEA3, Fra-1:PEA3, Fra-1:c-JUN, or PEA3:Fra-1:c-JUN had no additional effect (Figure 35). Interestingly, in the presence of c-FOS siRNA, Notch-4 transcripts were increased suggesting that c-FOS may act as repressor in our MDA-MB-231 breast cancer *in vitro* model. The combination of c-JUN and c-FOS siRNAs had no measurable effect on Notch-4 transcripts compared to control siRNA suggesting the role of c-FOS as a potential repressor of Notch-4 (Figure 35). The combination of c-FOS and c-JUN siRNAs had the tendency to restore the transcript levels of Notch-4 to basal levels. This result would suggest that c-FOS could be a transcriptional repressor of Notch-4 mRNA. A one-way ANOVA resulted in no statistical significance because of the pairwise multiple comparisons and minimal repeated measurements (n=3). However, there is a clear trend and a Power Analysis would help to determine how many independent studies need to be performed to achieve statistical significance.

As a control experiment, Notch-1 transcripts were measured by quantitative real-time PCR under the same conditions as mentioned above. As a result, Notch-1 transcripts were not affected by AP-1 knockdown using c-JUN, Fra-1, or c-FOS siRNA(s). However, PEA3 siRNA reduced Notch-1 transcript levels as seen previously by 50%, indicating that PEA3 acts most probably independently of AP-1 to regulate the Notch-1 promoter (Figure 36). Interestingly, c-FOS in combination with PEA3 siRNA did not reduce Notch-1 transcripts. This suggests that there is a trend that c-FOS may serve as a repressor upstream or downstream on the Notch-1 promoter. Again, statistical analysis using one-way ANOVA resulted in no significance because of the pairwise
Figure 35: PEA3 and AP-1 Dynamically Regulate Notch-4 Transcription.

MDA-MB-231 cells were transfected individually or with various combinations of PEA3, Fra-1, c-JUN, or c-FOS siRNA. Notch-4 transcripts were normalized to HPRT and measured by quantitative PCR. Means and standard deviation of three or more experiments were plotted. Statistical significance was determined by one-way ANOVA.
Figure 36: Notch-1 Transcripts Are Not Affected by AP-1 siRNA Interference.

MDA-MB-231 cells were transfected individually or with various combinations of PEA3, Fra-1, c-JUN, or c-FOS siRNA. Notch-1 transcripts were normalized to HPRT and measured by quantitative PCR. Means and standard deviation of three or more experiments were plotted. Statistical significance was determined by one-way ANOVA.
multiple comparisons and minimal repeated measurements (n=3). More independent experiments might be needed to achieve statistical significance.

It is also important to mention that none of the siRNA treatments had any significant effect on each other, confirming that siRNA effects are not interacting with gene regulation (*data not shown*). As a final control, quantitative real-time PCR and Western blot analysis were performed on PEA3, c-JUN, Fra-1, and c-FOS to determined their individual siRNA knockdown efficiency. After 48 hours, PEA3, c-JUN, Fra-1, and c-FOS transcripts and protein levels were reduced by approximately 90%, 40%, 80%, 60% after their specific siRNA transfections, respectively (Figure 37).

SPECIFIC AIM-3:

*To Establish a Biological Significance by Determining Whether Dual Inhibition of Notch and PEA3 Leads to Reduced Cell Proliferation and Tumorigenicity in vitro.*

Notch is vital for proliferation and cell fate determination (Miele et al., 2006) whereas PEA3 is critical for migration and invasion (Hida et al., 1997; Kaya et al., 1996; Miele et al., 2006). Both aberrant and unregulated activities can lead to malignancy and overall poor survival (Reedijk et al., 2005; Shepherd et al., 2001). To understand the biological significance and their effect in our breast cancer model, we explored dual
Figure 37: RNA interference Knockdown of PEA3 and AP-1 Members.

MDA-MB-231 cells were transfected individually PEA3, Fra-1, c-JUN, or c-FOS siRNA. Their transcripts were normalized to HPRT and measured by quantitative PCR to determine siRNA knockdown efficiency. Means and standard deviation of three or more experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
inhibition of PEA3 and Notch using a specific PEA3 siRNA and the pre-clinical γ-secretase inhibitor (GSI: MRK-003), respectively.

1. Dual inhibition of Notch and PEA3 Inhibits Cell Proliferation.

To determine the effects of dual Notch and PEA3 inhibition on cell proliferation, MDA-MB-231 cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or GSI (10 μM), or a combination for 48 hours. Independently, PEA3 knockdown and/or Notch inhibition all had effects on cell cycle (Figure 38). PEA3 knockdown or GSI treatment of MDA-MB-231 cells alone showed an increase in G1 arrest. A significant increase (p<0.05) in G1 arrest was determined in the combination group of Notch inhibition and PEA3 knockdown as compared to control (Figure 38). PEA3 knockdown and GSI treatment alone showed a reduction in S-phase, while the combination of Notch inhibition and PEA3 knockdown significantly reduced S-phase (p<0.05) as compared to control (Figure 38). PEA3 siRNA and GSI treatment alone demonstrated a reduction in G2/M cell cycle, but the combination showed a significant reduction (p<0.05) as compared to control in G2/M (Figure 38). These results indicate that both PEA3 and Notch, whether working together or independently at the various stages of the cell cycle, are critical for proliferation of MDA-MB-231 cells.

2. Dual Inhibition of Notch and PEA3 Induces Apoptosis.

To address whether dual inhibition using both PEA3 knockdown and Notch inhibition affected cell viability potentially through increased apoptosis, MDA-MD-231
Figure 38: Effects of Dual Notch and PEA3 on Cell Proliferation.

MDA-MB-231 cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or a gamma-secretase inhibitor (GSI: MRK-003, 10 μM), or a combination thereof for 48 hours. Cell cycle analysis was performed by flow cytometry. Mean percentage and standard deviation of cells in each experiment were plotted. Statistical significance was determined by one-way ANOVA.
cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or GSI (10 μM), or a combination, and subjected to Annexin-V staining, and subsequently measured by flow cytometry 24 hours later as an early marker of apoptosis. Independently, PEA3 knockdown showed no change in apoptosis (Figure 39). GSI treatment alone increased apoptosis by 30%. Importantly, the combination of PEA3 knockdown and GSI treatment significantly increased apoptosis by almost 40% as measured by Annexin-V positive stained cells (Figure 39).

3. Dual Inhibition of Notch and PEA3 Reduces Cell Viability.

To confirm the effects of treatment/transfection on cell viability, a Trypan blue exclusion assay was performed after MDA-MD-231 cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or GSI (10 μM), or a combination for 48 hours. PEA3 siRNA alone showed no change in cell viability, but GSI alone resulted in decreased viability (Figure 40). In agreement with previous experiments, the combination of PEA3 knockdown and GSI treatment significantly diminished percent cell viability by more than 50% (Figure 40). These results taken together with the cell cycle data indicate that both PEA3 and Notch activities together are essential for cell proliferation and survival in MDA-MB-231 breast cancer cells.

4. Notch-1 and Notch-4 Overexpression Rescues the Effect of Dual Notch and PEA3 Inhibition.
Figure 39: Dual Inhibition of Notch and PEA3 Increases Apoptosis

MDA-MB-231 cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or a gamma-secretase inhibitor (GSI: MRK-003, 10 μM), or a combination thereof for 24 hours. Annexin-V staining was performed and measured by flow cytometry. Mean percentage and standard deviation of cells in each experiment were plotted. Statistical significance was determined by one-way ANOVA.
Figure 40: Dual Inhibition of Notch and PEA3 Decrease Cell Viability.

MDA-MB-231 cells were transfected with scrambled or PEA3 siRNA alone, or treated with vehicle or a gamma-secretase inhibitor (GSI: MRK-003, 10 μM), or a combination thereof for 48 hours. Percent viable cells were measured by Trypan blue exclusion study using a standard light microscope (10X magnitude). Mean percentage and standard deviation of cells in each experiment were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
To address whether dual inhibition of PEA3 and Notch activities have effects on anchorage independent growth as a measure of cell tumorigenicity, a colony formation assay was performed with MDA-MB-231 cells that were transfected with either scrambled or PEA3 siRNA and treated with vehicle or a gamma-secretase inhibitor (GSI: MRK-003, 5 μM) independently or in combination. Forty-eight hours after transfection, cells were suspended in methylcellulose and allowed to grow into colonies over 14 days. Colonies were photographed (Figure 41) and counted under a 40X magnification using a standard light microscope, and quantified (Figure 42). As a result, there was a reduction in the number of colonies to almost 60% after PEA3 siRNA knockdown or by 40% when Notch was inhibited using a GSI in the presence of vector alone. Furthermore, the percent number of colonies was reduced to 40% upon the combination of PEA3 knockdown and GSI treatment (Figure 41, 42).

GSI inhibits cleavage of all four Notch receptors (Kopan and Ilagan, 2009). To confirm the hypothesis that the effects of PEA3 inhibition on cell growth and survival are mediated primarily through Notch-1 and/or Notch-4, in MDA-MB-231 cells expression plasmids for the Notch-1 or Notch-4 intracellular domain (N1IC or N4IC) (10 ng) were simultaneously co-transfected independently or in combination in all four treatment groups (i.e. vehicle control, PEA3 siRNA, GSI, or GSI plus PEA3 siRNA) (Figure 41, column 2, 3, 4, quantified Figure 42). When Notch-1IC, Notch-4IC, or both were expressed in the absence of PEA3 siRNA, GSI treatment, or their combination, there was no measurable difference in percent number of colonies compared to control (Figure 41, row 1: E, I, M compared to control A, quantified Figure 42). The addition of Notch-1IC
MDA-MB-231 cells were transfected with either scrambled or PEA3 siRNA and treated with vehicle or gamma-secretase inhibitor (GSI: MRK-003, 5 μM) independently or in combination for 48 hours. Simultaneously, cells were co-transfected with expression plasmids for Notch-1 and Notch-4 intracellular domain (IC) independently or in combination. Colony formation assay was preformed using methylcellulose and incubated for 14 days. Colonies were photographed using a standard light microscope (40X magnitude) representative of three or more experiments.
Figure 42: Quantification of Notch-1 and Notch-4 Rescue on Colony Formation.

MDA-MB-231 cells were transfected with either scrambled or PEA3 siRNA and treated with vehicle or gamma-secretase inhibitor (GSI: MRK-003, 5μM) independently or in combination for 48 hours. Simultaneously, cells were co-transfected with expression plasmids for Notch-1 and Notch-4 intracellular domain (IC) independently or in combination. Colony formation assay was performed using methyl-cellulose and incubated for 14 days. Nine fields were counted and quantified per well and means plotted. Statistical significance was determined by one-way ANOVA.
alone and partially rescued the effects mediated by PEA3 siRNA, GSI, and their combination on percent colony formation (Figure 42, top). Notch-4IC alone partially rescued the effects in anchorage independent growth in the presence of GSI treatment alone and in combination with PEA3 siRNA. Notch-4IC rescue effects on PEA3 siRNA transfection alone were minimal (Figure 42, middle). In the presence of dual inhibition of PEA3 and Notch, Notch-1IC expression was able to restore anchorage independent growth by 80% than Notch-4IC in which only 70% of colony number was restored (Figure 41, row 4: H and L compared to D, respectively, Figure 42). When both Notch-1IC and Notch-4IC were co-expressed in this combination treatment group, complete restoration of percent number of colonies was observed by 93% (Figure 41, row 4: P compared to D, Figure 42, bottom). This result suggests that PEA3 mediates its effect primarily through expression of Notch-1 and Notch-4. While Notch-1IC had a greater colony restoration effect than Notch-4IC, both receptors are required to fully restore both the phenotypic appearance and percent number of colonies. Taken together, the results suggest that transcription of Notch-1 and Notch-4 genes by PEA3 and the activation of Notch-1 and Notch-4 receptors (after gamma-secretase cleavage leading to the formation of active Notch-1IC and Notch-4IC) are both critical biologic events required for proliferation and survival of triple negative, MDA-MB-231 breast cancer cells in vitro.

5. PEA3 Regulates Notch-1 and Notch-4 mRNA Expression in other Subtypes of Breast Cancer.
Our investigations are the first to show that PEA3 is an activator of Notch-1 mRNA and protein expression and Notch-4 mRNA in the triple negative subtype, MDA-MB-231 breast cancer cells. To determine whether this is a common mechanism among the other subtypes of breast cancer cells, MDA-MB-231 (triple-negative), MCF-7 (Luminal A), BT474 (Luminal B), and SKBr3 (HER2+) cells were transfected with either scrambled or PEA3 siRNA. Normalized to MDA-MB-231 cells, MCF-7 cells had slightly less PEA3 transcript levels followed by BT474 cells and then SKBr3 cells (Figure 43). Notch-1 and Notch-4 transcript levels were compared with either scrambled or PEA3 siRNA among the cell lines. PEA3 regulation of Notch-1 mRNA was seen in MDA-MB-231, SKBr3, and MCF-7 cells but not BT474 cells (Figure 44, top). However, PEA3-mediated effects on Notch-4 transcripts were only observed in MDA-MB-231 (Figure 44, bottom). This could be in part due to differences in AP-1 activity (Figure 45) or that PEA3 basal transcript levels were very low in other cell types (Figure 43). Provoked by this notion, we asked if we could drive the expression of Notch-4 by increasing levels of PEA3 in breast cancer cells that express low endogenous PEA3 levels. To address this, BT474 and SKBr3 cells were transfected with either pcDNA3.1 empty vector or pcDNA3.1-PEA3 expression plasmid. Notch-1 transcripts remained unchanged when PEA3 was overexpressed (Figure 46). Notch-4 transcript levels were significantly increased by nine fold in BT474 and sixteen fold in SKBr3 breast cancer cells (Figure 46). As a control, quantitative PCR was performed on PEA3 transcript levels after transfection of pcDNA3.1 and pcDNA3.1-PEA3 to determine PEA3 overexpression efficiency in both BT474 and SKBr3 cells (Figure 46, bottom). Taken together, PEA3 is required for
Notch-1 transcription in at least three subtypes of breast cancer cells: MDA-MB-231, SKBr3, and MCF-7. However, PEA3 is an activator of Notch-4 transcription in MDA-MB-231 cells where endogenous PEA3 levels are high. Exogenous expression of PEA3 in BT474 and SKBr3 cells provides evidence that it is also a potential activator of Notch-4 expression in other breast cancer subtypes.

Taken together, results from this study demonstrate for the first time that PEA3 is a novel activator of Notch-1 and Notch-4 transcription in different subtypes of breast cancer cells and could prove to be an important therapeutic target (targeted by nanotechnology, directed siRNAs, or staple interface masking peptides) when combined with a Notch inhibitor such as a GSI, particularly in triple negative breast cancer.
Figure 43: Relative PEA3 levels and Knockdown Efficiency in Breast Cancer Subtypes.

MDA-MB-231, MCF-7, BT474, and SKBr3 cells were transfected with either scrambled or PEA3 siRNA. Normalized to MDA-MB-231 cells, the transcript levels of PEA3 were compared and measured by quantitative PCR. Means and standard deviation of three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
Figure 44: PEA3 Regulates Notch in Other Breast Cancer Subtypes.

MDA-MB-231, SKBr3, MCF-7, and BT474 cells were transfected with either scrambled or PEA3 siRNA. Normalized to MDA-MB-231 cells, the transcript levels of Notch-1 and Notch-4 were compared and measured by quantitative PCR. Means and standard deviation of three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s t test.
BT474, MDA-MB-231, MCF-7, and SKBr3 cells were transfected with an AP-1 luciferase reporter construct (pGL2). After 48 hours, 10uL of each sample was added to a white coated luminescent 96-well plate and subjected to the manufactured dual-luciferase assay. Luminescence was measured using the Veritas Microplate Luminometer. The range of luminescence was between 350-650nm; Firefly luciferase read at 560nm and pTL-TK at 480nm. Firefly luciferase was measured and normalized to Renilla as an internal transfection control. Means and standard deviation of three or more experiments were plotted.
Figure 46: PEA3 Regulates Notch-4 in BT474 and SKBr3 Breast Cancer Subtypes.

BT474 and SKBr3 cells were transfected with either empty vector or PEA3 expression plasmid (pcDNA3.1). Notch-1, Notch-4, and PEA3 mRNA levels were normalized to empty vector and measured by quantitative PCR. Means and standard deviation of three experiments were plotted. Statistical significance was determined by two-tailed unpaired Student’s *t* test.
PEA3 was originally identified as a member of the Ets family of transcription factors (Xin et al., 1992). Since then, it has been observed that PEA3 is expressed during breast development, is quickly lost upon maturation, and yet re-emerges in metastatic breast cancers (Benz et al., 1997; Chotteau-Lelievre et al., 2003). Similarly, Notch-1 has been implicated in breast cancer demonstrating elevated expression and activity in breast tumors (Reedijk et al., 2005). Little is known about the factors that influence Notch gene expression, and why its levels are elevated in breast cancer.

MDA-MB-231 cells are an \textit{in vitro} model of triple negative breast cancer and therefore lack expression of estrogen receptor $\alpha$, progesterone receptor, or HER2 overexpression (Sorlie et al., 2001). They also contain a p53 mutation (Weigelt et al., 2009). Triple negative breast cancers have no known FDA approved, targeted treatment strategies, and as of yet have limited direct targeted chemotherapeutics. Their standard of care is a combination of cytotoxic drugs: cyclophosphamide, doxorubicin, and fluorouracil (Hortobagyi, 1998) or recently shown to be more effective in triple-negative breast cancer was the combination of cyclophosphamide, methotrexate, and fluorouracil (Colleoni et al., 2010). Therefore, it is imperative to understand the molecular infrastructure of triple-negative breast cancers to uncover and provide novel targeted
methods to help these patients reach their five year and beyond disease-free survival rate.

**The Analysis**

The initial results indicating that PEA3 is a transcriptional activator of Notch-1 and Notch-4 in triple-negative MDA-MB-231 cells provides new insight into targeted therapeutic options for patients afflicted with this illness. Targeting PEA3 in combination with inhibition of Notch shows promise *in vitro* to decrease MDA-MB-231 cellular viability (Figure 40), reduce cell proliferation (Figure 38), and increase apoptosis (Figure 39). What is even more intriguing is the result that PEA3 inhibition slightly, but significantly increased the transcript levels of Notch-2 (Figure 14). Notch-2 recently has been implicated as a tumor suppressor (O’Neill et al., 2007; Quillard et al., 2009). Minimal putative PEA3 Ets sites, although less abundant, were identified on the Notch-2 promoter (*data not shown*). This could indicate that targeting PEA3 in MDA-MB-231 could be beneficial in two ways: abrogating Notch-1 and Notch-4 transcripts and stimulating expression of Notch-2, thus increasing the chances of a complete reduction in proliferation and survival of the cancerous cells. This notion stems from other investigations of PEA3, which demonstrate it to be both an activator and repressor of gene transcription (Benz et al., 1997; Scott et al., 1994; Xia et al., 2006; Xing et al., 2000).

It was demonstrated that PEA3 siRNA alone showed no effect on cell proliferation (Figure 38), apoptosis (Figure 39), and viability (Figure 40) *in vitro* indicating that it is not efficient enough to inhibit PEA3 alone. Firstly, endogenous levels
of Notch-4 protein were not changed after PEA3 siRNA but were altered in the presence of lactacystin showing that inhibition of proteasome-mediated protein degradation inhibited pathways that resulted in transcription becoming rate controlling. Secondly, Notch-2 transcripts, although modest, were significantly increased in the presence of PEA3 siRNA and Notch-3 transcripts unaffected. A gamma-secretase inhibitor inhibits the third proteolytic cleavage responsible for the formation of active intracellular Notch-1,2,3, and 4 (Weijzen et al., 2002). The results shown here suggest that PEA3 only activates Notch-1 and Notch-4 transcripts. Therefore, one could speculate that the Notch-3 receptor could compensate for the dampening of Notch-1 and Notch-4 mRNA, and consequently maintain cell proliferation and viability, which were observed in these studies.

Notch-4 transcripts were positively regulated by PEA3 (Figure 14), however steady-state Notch-4 protein levels were not. In the presence of lactacystin, a specific proteasome inhibitor, the transcriptional effects of PEA3 on Notch-4 were observed (Figure 14). A speculative explanation could be that the rate of protein degradation could be decreased upon knockdown of PEA3, which may be a result of PEA3 regulating components of the proteasome. Another hypothesis could be that post-transcriptional steps regulating Notch-4 protein levels must be important since the endogenous Notch-4 protein levels do not correlate with Notch-4 mRNA levels. Examples of post-transcriptional steps include translation regulation, vesicular transport of Notch-4 protein through the endoplasmic reticulum and Golgi apparatus, ligand activation and gamma-secretase cleavage regulating plasma membrane Notch-4 protein levels, and proteasome
degradation of ubiquitinated Notch protein or other proteins involved in Notch-4 turnover. However, several experiments are necessary to answer these speculations. Nevertheless, the results suggest that Notch-4 is primarily regulated by the proteasome, but when compromised, the transcriptional effects of PEA3 on Notch-4 protein levels are observable.

To date, PEA3 has been implicated in regulating various genes in a complex with additional transcription factors (Firlej et al., 2005; Hesselbrock et al., 2005; Liu et al., 2004; Matsui et al., 2006). It was observed that AP-1 and PEA3 regulate Notch-4 transcription (Figures 27, 29, and 32). The results of this investigation suggest the importance of c-JUN, Fra-1, c-FOS, and PEA3 on the regulation of Notch-4 transcription. Interestingly, Fra-1 and c-FOS also regulate the Notch-4 promoter indicating that they either activate or dampen the transcription of Notch-4, respectively (Figure 35). This provides a unique future direction of understanding the partner preference on the Notch-4 promoter and the homeostatic balance needed for appropriate expression of Notch-4 in response to cellular stimuli. Recently, Baan et al. demonstrated that invasive breast cancer cells including MDA-MB-231 favored the c-JUN:Fra-1 heterodimer, compared to less invasive breast cancer cells, which favored the c-JUN:c-FOS heterodimer (Baan et al., 2010). Preference towards the c-JUN:Fra-1 AP-1 dimer may be responsible for Notch-4 mRNA modulation and subsequent tumor aggressiveness of MDA-MB-231 triple negative breast cancer cells. The data suggests that PEA3 expression could tip the balance and aid in this partner choice.
The partner choice of the AP-1 complex can be a heterodimer of JUN and FOS families or a homodimer of the JUN family (Eferl and Wagner, 2003). It was interesting to find that c-JUN was required for PEA3 recruitment to the Notch-4 promoter and that c-FOS was not as shown by the dominant negative ChIP studies (Figure 29 and Figure 33). Dominant negative c-JUN and c-FOS experiments may answer distinct questions. c-JUN is highly expressed in triple-negative MDA-MB-231 cells (Bamberger et al., 1999). TAM-67 only reduced AP-1 function by 50% (Figure 29). However, JUN:FOS is the preferred partnership, with c-JUN acting as an important member capable of homo- and hetero-dimerization among family members leading to subsequent AP-1 signaling effects (Eferl and Wagner, 2003). Thus upon transfection, TAM-67 has the capability to partner with JUN and FOS families limiting the cellular pool of potent gene regulators (Brown et al., 1994; Brown et al., 1996). A-FOS on the other hand can only partner the JUN family and not the FOS family (Olive et al., 1997). Therefore, important FOS family members are potentially left available to still regulate the Notch-4 promoter, namely Fra-1.

Since dynamic transcription factor partnering is plausible (Beckett, 2004), other putative regulatory factors must be critical for the regulation of Notch genes, because PEA3 is not solely responsible for the regulation of Notch-1 and Notch-4 transcription. This is evident by the observation that PEA3 RNA interference decreased Notch-1 and Notch-4 transcripts by 50% and 70% (Figure 14). If PEA3 was the sole transcription factor necessary, the transcripts would have been completely eliminated upon PEA3 knockdown. The results of this investigation demonstrate that PEA3 is one of many factors involved in the regulation of Notch transcription. A very dynamic interplay by
many additional factors may be responsible for the appropriate regulation of Notch-1 and Notch-4 transcripts. Moreover, key areas flanking the start site of the Notch-1 and Notch-4 gene including downstream intronic and extronic regions, unidentified cryptic sites, or regulatory transcriptional hubs within the gene (areas of three dimensional gene locus looping as seen in the CFTR gene (Ott et al., 2009)) could be putative sites of regulation. Endogenous genes are highly coiled, compact, and require chromatin remodeling events (Zhao et al., 2009). Therefore, PEA3 in addition with other putative factors potentially carrying histone acetyltransferases (Liu et al., 2004; Matsui et al., 2006) activity may be required to regulate Notch gene transcription.

These putative factors are yet to be identified especially on the Notch-1 promoter where the investigations indicate that PEA3 works independently of AP-1 (Figure 36). However, PEA3 is known in the literature to work in tandem with other transcription factors at variable genomic positions to regulate its target genes (Firlej et al., 2005; Liu et al., 2004). Therefore, it is highly unlikely that PEA3 independently regulates Notch-1 without the aid of other factors. Other consensus sites on the Notch-1 promoter near PEA3 sites are putative NFκB and SP-1 sites (*data not shown*) as determined by promoter scanning using NCBI Entrez Gene bank. These factors have not been significantly tested *in vitro* to discern if they interact with PEA3, but are emerging as transcription factors of interest in the regulation of Notch-1. Moreover, if in addition to directly regulating Notch-1 transcripts, PEA3 carries factors with chromatin remodeling capabilities, then the dynamics of PEA3 regulation becomes more fascinating allowing for remodeling events (Clapier and Cairns, 2009; Zhao et al., 2009) to occur, exposing silent regulatory sites,
and recruiting other transcription factors possibly the factors mentioned above to regulate the Notch-1 receptor or even other Notch receptors as well. This requires further studies to address, but becomes an additional hypothesis. Taken together, PEA3 harbors very interesting properties in response to cellular stimuli, not only limited to its effects on gene regulation, but also its effects mediated by pharmacological intervention.

It was demonstrated that dual inhibition of Notch via a gamma-secretase inhibitor (MRK-003) and PEA3 via siRNA, reduced proliferation (Figure 38), anchorage-independent growth (Figure 41 and Figure 42), and increased apoptosis in MDA-MB-231 cells (Figure 39). The enhanced sensitivity of MDA-MB-231 cells towards the GSI in the presence of PEA3 siRNA could be a result of targeting both the transcription and activity of the Notch pathway (Figure 47). GSI treatment inhibits the third proteolytic cleavage and attenuates the signaling cascade (Weijzen et al., 2002). However, more receptors are continually being transcribed at the gene level without control. Thus, it could be hypothesized that inhibiting at the level of gene transcription and at the level of receptor cleavage could greatly enhance the inhibition of Notch signaling overall. It was observed that PEA3 inhibition alone was not sufficient to reduce anchorage dependent and affect anchorage independent proliferation (Figure 38 and Figure 42) most likely due to the fact that other regulatory factors (Alimirah et al., 2007; Cai et al., 2009; Pajerowski et al., 2009; Wu and Bresnick, 2007; Wu et al., 2005) including AP-1 (Wu and Bresnick, 2007; Wu et al., 2005) may be important in regulation of Notch transcripts.

PEA3 regulation of Notch-1 and Notch-4 transcripts was observed among other subtypes of breast cancer (Figure 44) in vitro. Notch-4 transcripts were affected in the
presence of exogenous PEA3 expression (Figure 44). This could be in part due to the differences in AP-1 activity in the cell types (Figure 45), or that the endogenous levels of PEA3 differ (Figure 43). BT474 cells have endogenously high activity of AP-1, and SKBr3 have lower activity (Figure 45). However, in SKBr3 and BT474 cells, in which PEA3 is endogenously low, exogenous expression of PEA3 increased Notch-4 transcripts (Figure 46). This suggests together with the Luciferase results seen prior (Figure 27) that PEA3 is a potent activator of Notch-4 transcription and the levels of AP-1 activity, although important, may have a secondary role in Notch-4 transcription.

It was observed that PEA3 or GSI alone was able to reduce colony formation by approximately 40-50% (Figure 42), but the combination was able to reduce colony formation by more than 60% (Figure 42). When Notch-1IC was transfected into MDA-MB-231 cells, restoration of colony formation number was quantified (Figure 42), indicating that Notch-1 may be sufficient to restore the effects of PEA3, GSI, and dual inhibition. Notch-4IC transfection also restored colony formation but not as sufficiently as Notch-1IC (Figure 42). Initially, the effects of treatment/transfection appear to be mediated through Notch-1. However upon closer observation, although Notch-1 exogenous expression produced similar colony numbers as compared to control, the colonies were smaller in size when visualized under a 40X magnification of a light microscope (Figure 41). This observation was not quantified, but merely observed. Notch-4 exogenous expression did not rescue colony number as sufficiently as Notch-1, but the phenotype of the colonies (not quantified data, mere observation) were similar to control; the colonies were repeatedly observed to appear more robust and similar in size
to control (Figure 41). Interestingly, dual exogenous expression not only restored the colony formation number, but also the phenotype of the colonies when compared to control (not quantified data, mere observation) (Figure 41). This could be explained by the notion that Notch-1 along with its ligand, Jagged-1, are highly regulated receptors and have been implicated in cell proliferation and growth (Dickson et al., 2007; Reedijk et al., 2005). Notch-4 has been implicated in mammary cancer stem cells (Harrison et al., 2010). Taken together, Notch-1 exogenous expression could be responsible for proliferation of the cells thus acquiring similar colony numbers (in agreement to what was observed using a peptidyl γ-secretase inhibitor, z-Leu-Leu-Nle-CHO reviewed in Chapter II and (Clementz and Osipo, 2009), in MDA-MB-231 cells (Lee et al., 2008)), but Notch-4 could be responsible for stimulating the mammary cancer stem cells (Harrison et al., 2010) creating a more robust colony from mere observation. Together, they restore the effects of PEA3 and Notch inhibition by possibly stimulating both proliferating cells and stem cells.

In addition, Harrison et al. has recently implicated Notch-4 in mammary tumor stem cell survival and self-renewal in which they demonstrated that targeting Notch-4 via siRNA interference specifically was more effective than a GSI in inhibiting the Notch pathway (Harrison et al., 2010). In these studies, Notch-4 gene transcription was more sensitive to PEA3 inhibition. Given this fact, the sensitivity obtained in our MDA-MB-231 system by the dual inhibition of PEA3 and Notch towards reduced viability (Figure 40) may be explained by the notion that we may be not only targeting proliferation and
survival of bulk cancer cell populations but also the cancer stem cell population (Harrison et al., 2010; Korkaya and Wicha, 2007; Korkaya and Wicha, 2009).

Notch is a cellular fate determinant, can induce proliferation, and/or differentiation depending on the cellular environment (Kopan and Ilagan, 2009). PEA3 has been linked to invasion, migration, and aggressiveness of tumor cells (Coutte et al., 1999). The dual inhibition of Notch by the GSI, and the inhibition of PEA3 by siRNA could act by preventing two vital arms of cancer progression, namely proliferation and possibly metastatic potential in vivo. Emerging nanotechnology as a means to direct siRNA therapies (Del Pino et al., 2010), recent advances in targeted siRNA treatments in vivo (Ueyama et al., 2010), and the advent of stapled interface peptides (Moellering et al., 2009) that disrupt transcription factor complexes transforms the notion for specific targeting of PEA3 into a potential reality.

Here, these studies have provided evidence of a novel combination treatment to be exploited for the treatment of triple-negative breast cancer, and potentially other breast cancer subtypes where PEA3 regulates Notch-1 and Notch-4. The findings offer a potential mechanism that has identified better strategies for future pre-clinical, in vivo studies which are currently underway. Enhanced sensitivity towards current GSIs in future clinical trials by inhibition of PEA3 by nanoparticles (Del Pino et al., 2010), small molecule inhibitors, or future siRNA approaches (Ueyama et al., 2010) may increase patient response to treatment and could reduce or eliminate recurrence if stem cell populations are eliminated (Korkaya and Wicha, 2009). Inhibiting PEA3 in combination with the GSI may also allow for a larger therapeutic window for the use of a GSI,
enabling the reduction of pharmacological doses given to the patient and thus limiting the resulting undesirable side effects such as gastrointestinal toxicity (Staal and Langerak, 2008; van Es et al., 2005).

Furthermore, the implications of these results lead to many areas of active research within the clinic. PEA3 expression in breast cancer could become a prognostic indicator of how a patient may respond to Notch inhibition by a GSI since endogenous levels of PEA3 (high or low) could determine whether or not a combination treatment is desirable. From the results observed when PEA3 was endogenously elevated, inhibition of PEA3 sensitized the cells towards the GSI (Figures 38-42). Therefore, if a patient presents in the clinic with elevated endogenous levels of PEA3, it may be beneficial to inhibit both PEA3 and Notch to achieve tumor regression. Inversely, if a patient presents with endogenously low levels of PEA3, Notch inhibition via a GSI may be only required as signal targeted therapeutic strategy. Taken together, a patient may acquire a more unique chemotherapeutic strategy and reduce unnecessary combinatorial strategies as demonstrated with these results. Oncogenic gene profiling acquired by a primary biopsy is currently measured to decide what appropriate treatment is beneficial to the patient (Zanetti-Dallenbach et al., 2006). Diagnosis of HER2, estrogen receptor α, and progesterone receptor expression are already used in the clinic (Carter et al., 1989; Galea et al., 1992; Mirza et al., 2002). Thus, PEA3 could be another protein to screen to determine the necessary therapeutic strategy if methods of PEA3 are available. Moreover, AP-1 activity independently may also prove to be significant in deciding treatment, since the results indicate that PEA3 and AP-1 are interactive partners in certain breast cancers.
**The Model**

This investigation has led to the development of a model of targeting Notch both at production (transcription of Notch) and activation (formation of the active intracellular Notch) (Figure 47):

1. **Production**

It was determined that PEA3 transcriptionally activates Notch-1 independently where as PEA3 partners with c-JUN on Notch-4 (Figure 22 and Figure 31, 32). The results also suggest that Fra-1 is an activator and c-FOS a repressor of Notch-4 transcription (Figure 35). This stage involves direct regulation at the level of the Notch gene. Many events including transcriptional complex formation, active transcriptional hubs (areas of three dimensional gene locus looping as seen in the CFTR gene (Ott et al., 2009)), and chromatin remodeling (Zhao et al., 2009) are brought to fruition to successfully regulate the production of the mature Notch receptor. The dynamic interplay of these factors identified here, such as AP-1 and PEA3 as well as other potential players unidentified including histone acetyltransferases (Liu et al., 2004; Matsui et al., 2006), SP-1 (determined by promoter scanning using NCBI Entrez Gene bank), and/or basic transcriptional machinery (Firlej et al., 2005; Hesselbrock et al., 2005) are working to tightly regulate Notch gene transcription. This transcriptional stage provides the first area for potential targeted therapy, namely inhibition of PEA3, to abrogate Notch production. Once cells acquire the appropriate cellular stimuli, Notch is
Figure 47: Model of the Transcriptional Regulation of Notch-1 and Notch-4 by PEA3.

**I. Production:** PEA3 transcriptionally activates Notch-1 independently where as Notch-4 in a dynamic partnership with AP-1 notably c-JUN, Fra-1 (Activators) and c-FOS (Repressor). Notch is then processed and shuttled to the cellular membrane. **II. Activation:** Upon engagement of ligand, Notch undergoes two subsequent cleavages. The last cleavage by the gamma-secretase complex is inhibited by GSI, MRK-003, to attenuate Notch signaling and prevent the formation of intracellular Notch (N\text{IC}), responsible for displacing co-repressors and recruiting co-activators on CBF-1 driving target genes involved in cell fate, proliferation, and differentiation. Dual targeting of Notch via GSI and PEA3 via siRNA, reduces tumorigenicity by attenuation of Notch signaling at both its production and activation stages resulting in overall growth arrest and increased apoptosis. This combinatorial inhibition may provide a more effective targeted therapy for those with triple negative breast cancers, and could provide addition means of targeting in other breast cancer subtypes.
then processed and shuttled to the cellular membrane where it awaits engagement with its ligand (Kopan and Ilagan, 2009).

2. Activation

Upon engagement of ligand, Notch undergoes two subsequent cleavages. The last cleavage by the gamma-secretase complex is inhibited by GSI (MRK-003) to attenuate Notch signaling and prevent the formation of intracellular Notch (N\textsuperscript{IC}), responsible for displacing co-repressors and recruiting co-activators such as Mastermind on CBF-1 driving target genes involved in cell fate, proliferation, and differentiation (Callahan and Raafat, 2001). This step is critical for activation of Notch at the post-translational level and provides a second area for targeted treatment, namely GSI treatment (Weijzen et al., 2002).

The hypothesis remains that two critical functional areas are open for direct targeting: transcription and activation. GSI targets the mature receptor (Kopan and Ilagan, 2004; Weijzen et al., 2002), but production of the receptor is continuing to form. PEA3 inhibition was only successful at reducing transcripts by 50-80% (Figure 14) leaving some receptor formation to continue to be produced. If both function areas are successfully targeted, Notch activation can be attenuated by a GSI and more importantly the continual production of Notch is dampened by PEA3 siRNA. Therefore, the “global” abrogation of Notch signaling will reduce the oncogenic effects more successfully than targeting one or the other independently. Dual targeting of Notch (GSI) and PEA3 (siRNA) reduces tumorigenicity by attenuation of Notch signaling at both production and
activation resulting in overall growth arrest and increased apoptosis. This combinatory inhibition may provide a more effective targeted therapy for those with triple negative breast cancers, and could provide additional means of targeting in other breast cancer subtypes where PEA3 regulates Notch-1 and Notch-4.

**Future Investigations**

In summary, several areas can be investigated to understand the regulation of Notch by PEA3. It would be interesting to explore further the notion of PEA3 acting as a repressor on the Notch-2 promoter. Properly designing primers flanking the Ets sites and performing subsequent chromatin immunoprecipitations will determine if PEA3 directly regulates Notch-2. Further confirmation regarding the necessity of certain Ets sites could be explored by designing promoter luciferase constructs. Some may contain the intact Ets sites and the other ablated Ets sites via site-directed mutagenesis. Dual luciferase could determine the importance of each particular site in regulating Notch-2.

PEA3 enrichment on the Notch-1 promoter was independent from AP-1. Other sites have been identified on the Notch-1 promoter including SP-1 and NFkB. It would be interesting to understand what additional factors may be regulating Notch-1 in conjunction with PEA3. Co-immunoprecipitation studies could determine if PEA3 is in complex with SP-1, NFkB, or p300, for example. Moreover, an in depth analysis of the Notch-1 promoter may reveal other sites of importance. Continuing this idea, it would be interesting to determine what other partners work with PEA3 and AP-1, including classic transcriptional machinery, co-activators, co-repressors, etc.
With the effects observed on cellular proliferation and apoptosis upon dual inhibition of PEA3 and Notch in vitro, an in vivo study could be formulated. Xenografts of MDA-MB-231 transfected with PEA3 siRNA could be injected into the mammary fat pads of nude mice and immediately administered MRK-003 GSI via oral gavage. Tumor burden and size could be recorded to determine if dual inhibition has the same effects on tumor proliferation in vivo.

These are just a few areas that could be explored in the future to continue understanding the role of PEA3 on Notch regulation. What is clear from these studies is that PEA3 is a transcriptional activator of Notch-1 and Notch-4 in MDA-MB-231 cells, an example of triple-negative breast cancer. PEA3 inhibition could help MDA-MB-231 cells respond better to a gamma-secretase inhibitor in combination as indicated by these studies (Figures 38–42). Dual inhibition reduces both anchorage dependent and independent proliferation (Figure 38, 41, 42) and increases apoptosis (Figure 39). PEA3 may be a great candidate in addition with Notch inhibition for the targeted treatment of triple-negative breast cancers.
CHAPTER VI
CLOSING REMARKS

As of yet, there is no known FDA approved, targeted therapy for women diagnosed with triple-negative breast cancer. They frequently present with metastatic tumors and have the worst overall prognosis. These studies provide the first evidence of transcriptional regulation of Notch-1 and Notch-4 by PEA3 in triple-negative and other breast cancer subtypes. It was demonstrated that Notch-4 gene regulation is in part due to the presence of AP-1, specifically c-JUN and Fra-1, and PEA3 depending on cellular context. PEA3 regulates the Notch-1 promoter, but the additional factors that aid in the regulation are still being investigated. Further evidence reveals that PEA3 inhibition helps sensitize MDA-MB-231 cells to a gamma-secretase inhibitor, showing promise in significantly reducing both anchorage dependent and independent growth and increasing apoptosis. Thus, PEA3 emerges as potential target of inhibition in combination with inhibiting Notch activity to provide a novel and combined targeted therapy for triple-negative cancer.

The building blocks of human life, their innate blueprint, rests within the genome. The genome is a dynamic world of regulation involving opening and closing of DNA segments, chromatin remolding, histone modifications, and epigenetics. This research provides insight into gene regulation that can be applied to other genes, systems, and
disease states. Complex interplay of transcription factors and cellular stimuli when altered significantly can lead to the disruption of homeostasis and the development of human disease. PEA3, AP-1, and NOTCH are just a few proteins among myriads of others aberrantly expressed during disease. Many thousands of proteins are known to be active within the cell and their genomic regulation is just as vast. This research aims to provide another stepping stone to understand gene regulation and breast cancer disease state. Eradication of the breast cancer disease begins here –at the transcriptome.
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The author, Anthony G. Clementz, was born in Aurora, IL on July 14th, 1983. He is the last child of five children of Peter and Toni Clementz. He has four amazing siblings all sharing interest in the sciences: Gina, a doctor of dermatology; Peter, an electrician; Mia, a science teacher; and Andria, a registered nurse.

Anthony graduated as a Cadet First Lieutenant at Marmion Military Academy in 2001. He graduated from Northern Illinois University with high recognition with his bachelors in Chemistry and Biochemistry in 2005. Anthony received numerous awards while in school including the American Chemical Society Award in Analytical Chemistry, Scholastic Excellence Award, Golden Key International Honor Society, National Society of Collegiate Scholars, CRC Press Frosh Chemistry Achievement Award, and the National Deans List.

After graduation, he worked for Flavors of North America (FONA) as a flavor scientist and technician. Anthony entered the doctoral program in the Department of Cell Biology, Neurobiology, and Anatomy in the Division of Molecular and Cellular Biochemistry at Loyola University Medical Center in August 2006. He joined Dr. Clodia Osipo’s laboratory in June 2007 where he began his work on novel crosstalk between Notch and ErBB2 signaling and transcriptional regulation of Notch receptors. While at Loyola
University he has been a graduate school council representative for the Division of Molecular and Cellular Biochemistry, and has participated in assisting students with disabilities. Anthony has taught lectures on Hormone Signaling in both graduate student courses: *Molecular Oncogenesis* and *Signal Transduction*. He also was an active facilitator for the first year medical students in their course: *Cell Molecular Biology and Genetics*. Anthony is an active member as an Associate Member of American Association for Cancer Research and an American Chemical Society affiliate. He has also been an active judge for the Chicago Public Schools Science Fairs. Anthony’s research presented here has been submitted and is under review for a novel patent with Merck Pharmaceuticals.

Anthony G. Clementz also has a musical artistic side for he is an accomplished pianist and singer having played and sung various musicals and events throughout the Chicagoland area. He is also a graduate of iO!, formerly Improv Olympic!, and Chicago’s Second City and actively performs improv and sketch comedy.

Anthony G. Clementz has recently accepted a position as adjunct professor in the Department of Chemistry at DePaul University. Also, following completion of his dissertation, he will continue his career in transcription regulation as a post-doctoral researcher at Northwestern University Children’s Memorial Center in the laboratory of Dr. Ann Harris, professor and director of the Human Molecular Genetics Program. He plans to investigate the “transcription hubs” of the CFTR gene known to be responsible for the development of Cystic Fibrosis. He also plans to use his knowledge of
oncogenesis to elucidate the role of Collagen XV as a tumor suppressor in pancreatic cancer both \textit{in vitro} and \textit{in vivo}. 
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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_______________________   ________________________________
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