Novel Role of ErbB-2 in Inhibition of Jagged-1-Mediated Trans-Activation of Notch in Breast Cancer

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LOYOLA UNIVERSITY CHICAGO

NOVEL ROLE OF ErbB-2 IN INHIBITION OF JAGGED-1-MEDIATED TRANS- ACTIVATION OF NOTCH IN BREAST CANCER

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

PROGRAM IN MOLECULAR BIOLOGY

BY
KINNARI PANDYA
CHICAGO, ILLINOIS
AUGUST 2013
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of life from these excellent people; and I am extremely grateful to them for being there for me. I attribute my behavioral gifts to five amazing people from my family who have taught me and help me carry a part of each of them within me: Among so many other things, my father has taught me determination, perseverance, and problem solving and my mother instilled respect, time management, and patience in me. My incomparable Grandfather and Father-In-Law, who encouraged me to work hard, acquire higher education and taught me dedication, sincerity and seriousness of purpose. And finally, my husband supported me throughout my journey with his wisdom, prayer, hopes and always believed in me and saw my potential before I could even see it in myself.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ADAM17/TACE</td>
<td>Tumor Necrosis Factor α Converting Enzyme</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AKT</td>
<td>Activated Protein Kinase B</td>
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<td>ANK</td>
<td>Ankyrin Repeats</td>
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<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>AREG</td>
<td>Amphiregulin</td>
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<tr>
<td>AS-C</td>
<td>Achaete-Scute Complex</td>
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<tr>
<td>BCS</td>
<td>Breast Cancer Stem cells</td>
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<td>Brd</td>
<td>Bearded</td>
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<td>Cdk2</td>
<td>Cyclin Dependent Kinase - 2</td>
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<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
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<td>CS cells</td>
<td>Cancer Stem cells</td>
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<tr>
<td>Dll1</td>
<td>Delta-like 1</td>
</tr>
<tr>
<td>Dll3</td>
<td>Delta-like 3</td>
</tr>
<tr>
<td>Dll4</td>
<td>Delta-like 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR or ErbB-1</td>
<td>Epidermal Growth Factor Receptor -1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EphA2</td>
<td>ephrin type-A receptor 2</td>
</tr>
<tr>
<td>EpoR</td>
<td>Erythropoietin Receptor</td>
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<td>ErbB-2</td>
<td>Epidermal Growth Factor Receptor -2</td>
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<td>ErbB-3</td>
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<td>ErbB-4</td>
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<td>ERα</td>
<td>Estrogen Receptor</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FISH</td>
<td>Fluorescent In Situ Hybridization</td>
</tr>
<tr>
<td>FoxO3a</td>
<td>Forkhead Box O3a</td>
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<td>GSI</td>
<td>γ-secretase Inhibitor</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>Hey</td>
<td>Hairy/Enhancer of Split</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
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<td>HRG</td>
<td>Heregulin</td>
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<tr>
<td>hRPL13A</td>
<td>Human ribosomal protein L 13A</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis</td>
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<tr>
<td>ICD</td>
<td>Intracellular Domain</td>
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<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
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<td>IGF-1R</td>
<td>Insulin-like Growth Factor-1 Receptor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>IMEM</td>
<td>Iscove’s Minimal Essential Media</td>
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<td>Jag-1</td>
<td>Jagged-1</td>
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<tr>
<td>Jag-1i</td>
<td>Jagged-1 siRNA</td>
</tr>
<tr>
<td>Jak2</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MAML</td>
<td>Mastermind-like 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>Mib-1</td>
<td>Mindbomb-1</td>
</tr>
<tr>
<td>Mib-1i</td>
<td>Mib-1 siRNA</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin1</td>
</tr>
<tr>
<td>MUC4</td>
<td>Mucin4</td>
</tr>
<tr>
<td>NCR</td>
<td>Cytokine Response Element</td>
</tr>
<tr>
<td>NEC</td>
<td>Notch Extracellular Domain</td>
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<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
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<tr>
<td>Neur</td>
<td>Neuralized</td>
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<td>NEXT</td>
<td>Notch Extracellular Truncation</td>
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<tr>
<td>NFB</td>
<td>Nuclear Factor kappa B</td>
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<td>NHR</td>
<td>Neuralized Homology Repeats</td>
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<td>Acronym</td>
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<tr>
<td>NICD</td>
<td>Notch Intracellular Domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>NRR</td>
<td>Negative Regulatory Region</td>
</tr>
<tr>
<td>NTM</td>
<td>Notch Transmembrane Domain</td>
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<tr>
<td>O-fut</td>
<td>O-Fucosyl Transferase I</td>
</tr>
<tr>
<td>ORR</td>
<td>Overall Response Rate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline/Glutamic acid/Serine/Threonine</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol3-Kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-di-phosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-tri-phosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAM</td>
<td>RBP-JK Associated Molecule</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SCBi</td>
<td>Scrambled control siRNA</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulator</td>
</tr>
<tr>
<td>SFK</td>
<td>Src Family Kinase</td>
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<td>SH2</td>
<td>Src homology</td>
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TAD  Transcription Activation Domain
T-ALL  T-cell Acute Lymphoblastic Leukemia
T-DM1  Trastuzumab-DM1
TGFα  Transforming Growth Factor-α
TGFβ  Transforming Growth Factor-β
TKI  Tyrosine Kinase Inhibitor
TLR  Toll-like Receptor
Ub  Ubiquitin
UIM  Ubiquitin Interaction Motif
VEGF  Vascular Endothelial Growth Factor
ABSTRACT

The *ErbB-2* gene is amplified and the resulting protein product overexpressed in 15-30% of breast tumors, and associated with aggressive behavior and poor overall survival. Currently, there are two FDA approved therapies targeting ErbB-2 for the treatment of ErbB-2 positive breast cancer: trastuzumab, a humanized monoclonal antibody is directed against the extracellular domain of ErbB-2 and lapatinib, a dual EGFR/ErbB-2 tyrosine kinase inhibitor. Unfortunately, anti-ErbB-2 therapy resistance remains a major problem in metastatic breast cancer. Our data suggested that gene amplification or overexpression of ErbB-2 inhibits Notch-1 transcriptional activity and trastuzumab or lapatinib increased Notch-1 transcriptional activity. Furthermore, Notch-1 is a breast oncogene and a novel target for the treatment of trastuzumab resistant ErbB-2 positive breast cancer *in vitro*.

The Notch-1 receptor is overexpressed with its ligand Jagged-1 in breast cancers with the poorest overall survival. We showed that ErbB-2 inhibition activates Notch-1 which results in a compensatory increase in Notch-1-mediated proliferation. However, we do not yet know the mechanism by which ErbB-2 overexpression suppresses Notch-1 activity and whether inhibition of Notch-1 would reverse resistance to trastuzumab *in vivo*. Our results demonstrated that trastuzumab or lapatinib treatment of SKBr3 cells increased the cell surface protein expression of Jagged-1 by flow cytometry and cell surface biotinylation. Moreover, confocal studies indicated that Jagged-1 and Notch-1 co-localized possibly in early endosomal antigen-1 (EEA-1) positive vesicles. However,
upon treatment with trastuzumab, Jagged-1 and Notch-1 don’t co-localize. Jagged-1 is present at the plasma membrane and Notch-1 is distributed throughout the cell. In SKBr3 and MCF-7/HER-2 breast cancer cells, ErbB-2 stabilizes the protein levels of Jagged-1. We demonstrated for the first time that Jagged-1 inhibits Notch in cis. More interestingly, ErbB-2 prevents Jagged-1-mediated trans-activation of Notch signaling by limiting the association of Jagged-1 and Mib-1 and subsequent ubiquitylation of Jagged-1. Moreover, Mib-1 is the E3 ubiquitin ligase required for lapatinib-mediated ubiquitylation of Jagged-1 and induction of Notch activity. Additionally, ErbB-2 promotes an association between Jagged-1 and PKCα. Further, PKCα inhibits Notch transcriptional activity. Importantly combined inhibition of Jagged-1 by siRNA and ErbB-2 by trastuzumab significantly growth arrested SKBr3, BT474 HS, and BT474 HR cells in G1 phase of the cell cycle and induced cell death in vitro. Combined inhibition of Notch and ErbB-2 signaling pathways could decrease recurrence rates for ErbB-2 positive breast tumors and may be beneficial in the treatment of recurrent trastuzumab-resistant disease. Our studies will elucidate the mechanism by which ErbB-2 and Notch pathways crosstalk in ErbB-2 positive breast cancer cells. Mechanisms underlying trans-activation and cis-inhibition of Notch by its ligand even though not well characterized yet are critical processes regulating Notch activity. These findings will provide a mechanism and functional relevance of Jagged-1–Notch interactions in ErbB-2 positive breast cancer cells. Furthermore, these studies will identify Jagged-1 as a novel and better therapeutic target for the treatment of ErbB-2 positive breast cancer. Finally, these studies will provide a preclinical proof of concept for future clinical trials using combination of
trastuzumab or lapatinib and a Notch pathway inhibitor (GSI or Jagged-1 targeted therapy) for the treatment of ErbB-2 positive breast cancer.
CHAPTER I

INTRODUCTION

1. Anatomy of the Human Breast

In women, the breast organ develops after birth but grows rapidly at puberty to produce tree-like structures referred to as terminal end buds. The tree-like structures are composed of an inner layer of luminal cells surrounded by an outer layer of basal or myoepithelial cells. The luminal cells (comprising of ductal and alveolar epithelial cells) differentiate further into milk producing cells and the basal cells facilitate the movement of the milk from the alveolar epithelial cells into the ducts to be ejected from the nipples. Interestingly, during each menstrual cycle and pregnancy, the breast epithelium expands in number and subsequently regresses. Upon weaning, the tree-like structures referred to as terminal end buds regresses to their pre-pregnant stage through a process known as involution. These repeated cycles of expansion and regression of the breast epithelial cells suggest the existence of adult breast stem cells. In mice, the breast organ is called a mammary gland and normal mammary stem cells are able to reconstitute an entire functional gland at the single-cell level, displaying regenerative potential (Shackleton et al., 2006; Stingl et al., 2006). Moreover, the mouse mammary gland has been reported to be enriched in multi-potent progenitor cells (Alvi et al., 2003; Welm et al., 2002)
whereas, in human breast tissue, bi-potent cells capable of generating both luminal and myoepithelial cells \textit{in vitro} have been identified (Dontu et al., 2004; Gudjonsson et al., 2002; Stingl et al., 2001). Multiple steps during normal development and pregnancy could be susceptible to mutations, which, in turn, transform normal breast epithelial cells to a malignant phenotype.

2. \textit{Breast Cancer}

Breast cancer continues to be the second leading cause of cancer-related deaths among women. Breast cancer accounts for 22.9\% of all cancers in women worldwide. Although the mortality rate due to breast cancer has decreased in western populations due to early detection, the American Cancer Society estimated that 229,060 (226,870 women, 2,190 men) new breast cancer cases would be identified in the United States in 2012 with an estimated death rate of 39,920 (39,510 women, 410 men).

Until recently, for many years it was believed that the majority of the cells in a tumor have the potential to extensively proliferate and the treatment should eliminate all the proliferating cells and cure the disease. However, recent studies have suggested that the ability of tumors to proliferate relies on a small subpopulation of cells which exhibit stem cell-like properties. This limited subpopulation of cells was termed as cancer stem (CS) cells or tumor initiating cells. Emerging evidence suggests that breast cancer also arise from a small sub-population of cells termed as breast cancer stem (BCS) cells identified by CD44$^+$/CD24$^-$ cell surface markers. CD44$^+$/CD24$^-$ BCS cells show tumor-initiating properties, invasive features, and are resistant to most targeted and cytotoxic agents (Farnie and Clarke, 2007).
3. An Introduction to Breast Cancer Subtypes

Breast cancer is a heterogeneous disease exhibiting considerable diversity both biologically and clinically. Based on gene expression profiling and clinical outcomes, Sorlie and Perou described five distinct subtypes: luminal A, luminal B, ErbB-2, normal breast-like and basal-like (Sorlie et al., 2001; Sorlie et al., 2003). The most important determinants of these subtypes are the presence or absence of expression of the estrogen receptor alpha (ERα) or the progesterone receptor (PR), or the amplification of the Epidermal Growth Factor Receptor-2 or ErbB-2 proto-oncogene. Despite the ability of these subtypes to predict outcome, patient response to targeted therapy remains variable. The heterogeneity was also reflected by differences in histological staining of sections of patient samples: keratin 5 and 17 positive tumors (basal-epithelial origin), and keratin 8 and 18 positive tumors (luminal origin) (Perou et al., 2000). Further classifications are continuing to emerge such as the molecular apocrine and claudin-low breast cancer subtypes. Although divided into different categories, sometimes the classification of breast cancer subtypes tends to overlap with their gene expression profile.

3.1. Basal-like, triple negative breast cancers

Most triple negative breast tumors are classified as basal-like because the tumor cells arise from the basal or myoepithelial cells of the breast (Jones et al., 2004). The triple negative breast cancer subtype acquires its name because of the lack of expression of ERα, PR, and ErbB-2 in tumor cells (Sorlie et al., 2001). The triple negative subtype makes up approximately 15-20% of all breast cancer cases. The tumors express basal markers such as cytokeratin 5 and 17 (Perou et al., 2000). These tumors are highly...
proliferative, very aggressive in nature, have high histological grade, and high rates of metastasis (Nielsen et al., 2004). Approximately 85% tend to harbor TP53 mutations (Sorlie et al., 2001; Weigelt et al., 2009) and up to 60% overexpress the Epidermal Growth Factor Receptor-1 (EGFR) or HER-1 (Nielsen et al., 2004; Reis-Filho et al., 2006). Women diagnosed with triple negative breast cancer have lower overall survival than those who present with luminal type tumors. Furthermore, the majority of the patients with triple negative breast cancer never make their five year survival mark. Currently, there is no known targeted therapy available for the treatment of triple negative breast cancer. The standard of care is a combination of cytotoxic drugs including cyclophosphamid, doxorubicin, and fluorouracil (Hortobagyi, 1998). Thus, there is an immediate need to identify novel targets to treat these patients and increase their survival.

3.2. Luminal A breast cancers

The luminal A subtype is the most common form of breast cancer accounting for approximately 60-70% of all breast cancers. This subtype of breast cancer originates from the epithelial cells within the lumen of the breast. The luminal A subtype exhibit distinct characteristic gene expression profile which is summarized as follows: positive for the expression of luminal markers – ERα and PR; negative for the overexpression or gene amplification of ErbB-2 (Sorlie et al., 2001); lack expression of basal markers such as cytokeratin 5/6; express GATA3 (Chou et al., 2010; Usary et al., 2004), show TP53 mutation in 13% of the cases (Langerod et al., 2007), and low expression of a proliferation marker (Ki-67). Luminal A breast tumors usually present with a low histological grade and are responsive to anti-estrogen therapy, have lower risk of
recurrence, and have overall good prognosis (Langerod et al., 2007). Currently, pre-menopausal women diagnosed with this subtype of breast cancer are treated with the Selective Estrogen Receptor Modulator (Tamoxifen). However, post-menopausal women diagnosed with ERα+ breast cancer are treated preferably with an aromatase inhibitor (Letrozole, anastrozole, or Exemestane) or Tamoxifen (Swaby and Jordan, 2008). The major differences in the two targeted agents are that Tamoxifen is a competitive inhibitor of ERα and an aromatase inhibitor blocks the synthesis of 17β-estradiol from the androgen, androstenedione. Although luminal A breast cancer have the longest survival rates when compared to other subtypes, intrinsic and/or acquired resistance to anti-estrogen therapy is still a major problem showing a 63% rate of recurrence (Peppercorn et al., 2008).

### 3.3. Luminal B breast cancers

Like luminal A, the luminal B subtype makes up 55% of breast cancers and originates from luminal epithelial cells of the breast. However, the luminal B gene expression profile differs from the luminal A and is characterized as follows: positive for the expression of hormone receptors, ERα and/or PR, and ErbB-2 (Sorlie et al., 2001); lack expression of basal markers such as cytokeratin 5/6; express GATA3 (Usary et al., 2004), and have a 40% – 70% rate of TP53 mutation (Langerod et al., 2007; Peppercorn et al., 2008). These tumors are more aggressive in nature than luminal A tumors, have high expression of proliferation-associated genes and high histological grade. Non-metastatic luminal B breast cancer patients have overall good survival due to the availability of a combination treatment strategy targeting ERα using an anti-estrogen and
ErbB-2 using anti-ErbB-2 therapy (Amar et al., 2009; Bedard et al., 2009; Dean-Colomb and Esteva, 2008). Unfortunately, despite the available treatment options, intrinsic or acquired resistance to anti-estrogen or anti-ErbB-2 therapy remains problematic as evidenced by a 81% rate of recurrence (Peppercorn et al., 2008).

3.4. Normal-like breast cancers

Normal-like breast cancer, although not well-characterized, is known to be ERα positive or negative, PR status undetermined, and ErbB-2 negative. The expression of basal markers leads one to believe that it arises from the basal-like subtype. The grade, presence of TP53 mutations, and expression of proliferative markers such as Ki-67 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).

3.5. Claudin-low breast cancers

Claudin-low breast cancer show reduced expression of genes associated with tight junctions and cell-cell adhesion. This subtype lacks expression of ERα, PR, or ErbB-2 suggesting a possible link with the triple negative subtype. Like the triple negative subtype, the claudin-low subtype has high histological grade and high levels of genes responsible for proliferation. The TP53 mutation status is unknown. However, what differentiates this subtype from the triple negative is that they appear as ‘cancer stem cell-like’ based on their gene expression profile (Hennessy et al., 2009). Investigations into this subtype may prove to be advantageous and interesting to the field of breast cancer stem cell research.
3.6. Molecular apocrine breast cancers

Molecular apocrine tumors represent 8–14% of breast cancers. Molecular apocrine breast cancer is believed to be ErbB-2 positive, ERα and PR negative, and androgen receptor (AR) positive. Although, what distinguishes it from the other ErbB-2+ and ERα- tumors is the presence of an androgen receptor (AR) and its subsequent target genes (Farmer et al., 2005). In addition, relative levels of genes responsible for proliferation are high. High level of AR expression may serve as a novel target for the treatment of this type of breast cancer.

4. ErbB-2 Positive Breast Cancer

The ErbB-2 or HER-2 protein is a member of the ErbB family of type I transmembrane receptor tyrosine kinases (RTKs), which includes ErbB-1 or HER-1, ErbB-3 or HER-3, and ErbB-4 or HER-4. The ErbB family members contain an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Figure 1) (Citri et al., 2003). The crystal structure of the extracellular domain of ErbB receptors identified four subdomains that are critical for ligand binding in the case of ErbB-1, ErbB-3, and ErbB-4 (cysteine-free subdomains I: L1 and subdomain III: L2), and receptor dimerization (cysteine-rich subdomains II: S1 and IV: S2). Protrusion of the S1 dimerization loop is critical for ErbB family homo- and hetero-dimerization and subsequent receptor activation. In the absence of ligand, the S1 and S2 subdomains of ErbB-1, ErbB-3, and ErbB-4 receptors interact with each other to achieve an “inactive” conformation.
Figure 1: Schematics of Ligand Induced Receptor Heterodimer
However, binding of a ligand to the L1 and L2 subdomains of the ErbB receptors rearranges the conformation of the extracellular domain, leading to protrusion of the S1 dimerization loop. The crystal structure of ErbB-2 revealed that its L1 and L2 subdomains interact and are already in a “ligand bound” conformation, allowing protrusion of the S1 dimerization loop even in the absence of ligand binding (Citri et al., 2003). Heterodimerization between a ligand-bound ErbB-3 and ErbB-2 receptors is mediated primarily by the S1 dimerization loop and secondarily by the S2 subdomain. Additionally, interactions between the transmembrane and tyrosine kinase domains may also play a role in stabilizing the heterodimer. Binding of ligands to the extracellular region of ErbB-1, ErbB-3, or ErbB-4 induces homodimerization or heterodimerization of receptors followed by cis-phosphorylation and trans-phosphorylation of several tyrosine residues within the C-terminal region of the receptor or its dimer partner, respectively. These phosphorylated tyrosine residues, in turn, serve as docking sites for a number of src homology (SH2) domain containing adaptors and signal transducers (i.e. Grb-2 through, Shc, and the regulatory subunit of the phosphoinositide 3-kinase (PI3-K), p85) (Citri et al., 2003; Linggi and Carpenter, 2006; Wieduwilt and Moasser, 2008; Yarden and Sliwkowski, 2001). ErbB RTK dimerization is required for activation and subsequent downstream signaling, mediated primarily by the PI3-K and mitogen-activated protein kinase (MAPK) pathways that mediate cell survival, proliferation, and the transforming effects of these receptors (Figure 2).
Figure 2: ErbB-2 Signaling Pathway
ErbB-2 is the preferred dimer partner for ErbB-1, ErbB-3, or ErbB-4 in breast cancer as it is the most stable RTK protein when overexpressed. The ErbB family of RTKs, except ErbB-2, is activated by growth factor binding to the EGF repeats of the N-terminal extracellular domain. ErbB-1 family of growth factors includes EGF, TGF-α, AREG, and ampiregullin. ErbB-3 or ErbB-4 bind the Heregulin (HRG) family of growth factors which comprise HRG-1, -2, -3, and -4. The choice of which ErbB receptor will heterodimerize with ErbB-2 is primarily determined by the availability and/or abundance of growth factors. Each unique heterodimer has a predicted signaling potency based on the partners. For example, the ErbB-2–ErbB-3 heterodimer has the most transforming ability based on the high numbers of tyrosines that can be phosphorylated (Yarden and Sliwkowski, 2001). ErbB-2 mediated trans-phosphorylation of ErbB-3 recruits at least 8 p85 regulatory subunits of PI-3K, which in turn potentially forms 8 active PI-3Kinas to activate protein kinase B (AKT) survival pathway (Alimandi et al., 1995; Lee-Hoeflich et al., 2008; Ritter et al., 2007; Wallasch et al., 1995; Yakes et al., 2002).

ErbB-2 positive breast cancers, which account for 20–30% of all breast cancers, contain gene amplification of ErbB-2 on chromosome 17q12 which results in protein overexpression. ErbB-2 gene amplification can be determined by fluorescence in situ hybridization (FISH) and protein overexpression can be detected by immunohistochemistry (IHC), respectively (Press et al., 2008). ErbB-2 positive breast cancers have an aggressive phenotype, (Slamon et al., 1987; Slamon et al., 1989), high expression of proliferation-associated genes compared to luminal A tumors (Peppercorn et al., 2008), high histological grade and harbor a 71% rate of TP53 mutations.
(Peppercorn et al., 2008; Weigelt et al., 2009). ErbB-2 positive 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). Currently, there are two FDA approved therapies targeting ErbB-2 for the treatment of ErbB-2 positive breast cancer: trastuzumab, a recombinant, humanized, monoclonal antibody directed against the extracellular juxtagdomain of ErbB-2 (Carter et al., 1992) and lapatinib, a small molecule dual EGFR/ErbB-2 tyrosine kinase inhibitor (Chen et al., 1997; Geyer et al., 2006; McArthur, 2009). The therapeutic effect of trastuzumab is possibly due to number of mechanisms including inhibition of receptor-receptor interaction, endocytic-mediated receptor down-regulation, blocking cleavage of the extracellular domain of the receptor, activation of antibody-dependent cell-mediated cytotoxicity, and induction of p27 mediated G1 cell cycle arrest (Hudis, 2007). In the treatment of ErbB-2 positive breast cancer, trastuzumab showed significant efficacy in the adjuvant settings with an overall response rate (ORR) of 26%, which increased to about 90% when combined with chemotherapeutic agents (Amar et al., 2009; Bedard et al., 2009; Dean-Colomb and Esteva, 2008; Hall and Cameron, 2009). However, despite its proven efficacy and dramatic effects on survival, 20–50% of women with ErbB-2–positive, metastatic breast cancer exhibit intrinsic resistance, indicating that they do not respond to trastuzumab (Cobleigh et al., 1999; Vogel et al., 2002). Furthermore, 10–15% of the women treated with trastuzumab plus chemotherapy in the adjuvant setting developed acquired resistance within the first year, which means that they initially responded to trastuzumab, but became resistant during treatment (Amar et al., 2009; Romond et al., 2005). In ErbB-2 positive breast cancer, lapatinib is a potent inhibitor of ErbB-2’s activity and strongly
inhibits downstream PI-3K–AKT and MAPK signaling pathways. Lapatinib, a dual EGFR/ErbB-2 tyrosine kinase inhibitor, is approved for the treatment of ErbB-2 positive breast cancer that has advanced during or after trastuzumab treatment (Chen et al., 1997; Geyer et al., 2006; McArthur, 2009). Recently, it was shown that in patients with metastatic breast cancer, treatment with lapatinib induced apoptosis (Spector et al., 2005). Further, in a neoadjuvant study, lapatinib showed significant inhibition of cell proliferation in patients with newly diagnosed ErbB-2 positive breast cancer (Dave et al., 2011). Even though patients treated with lapatinib showed significant clinical benefit, it is clear that a fraction of the patients will become resistant. This suggests that tumors harbor intrinsic or acquired mechanisms of resistance to anti-ErbB-2 targeted therapies.

T-DM1 (trastuzumab-DM1) consists of trastuzumab conjugated to a derivative of maytansine (an inhibitor of microtubule polymerization) via a non-cleavable linker. T-DM1 increased clinical response rate by 25% when used as a single agent in patients with metastatic disease and who has progressed after trastuzumab-, lapatinib-, taxanes-, and anthracyclines-based therapy (Burris et al., 2011; Krop et al., 2010). The clinical benefits exhibited by T-DM1 in lapatinib resistant xenografts were due to inhibition of downstream signaling and induction of an ADCC response. Thus, understanding the mechanisms responsible for resistance to ErbB-2 targeted therapies is critical to identify novel targets to prevent and/or reverse the resistant phenotype that is responsible for disease progression and the majority of deaths.
5. Potential Mechanisms Responsible for Resistance to Trastuzumab

Extensive efforts have been made to understand the exact mechanism of intrinsic or acquired resistance to anti-ErbB-2 targeted inhibitors using cell culture models, preclinical models, and in some cases, patient-derived tumor samples. Identification of signaling pathways that are responsible for resistance has allowed and will allow for the development of novel therapeutic agents (Table 1). Each of the novel agents, either alone or when combined with trastuzumab or lapatinib, could potentially open new horizons that may help to replace trastuzumab or lapatinib or increase the efficacy of anti-ErbB-2 therapy to prevent and/or reverse resistance.

Resistance to anti-ErbB-2 therapy occurs at three levels: 1. resistance that involves ErbB-2 gene mutations; 2. resistance that involves activation of parallel and/or bypass signaling pathways; and 3. resistance that arises from alterations in the apoptotic pathway in tumor cells.

5.1. ErbB-2 Mutations

Mutations in the tyrosine kinase domain or the juxtamembrane region of ErbB-2 could result in decreased affinity of drug binding and resistance to ErbB-2 directed inhibitors. Mutations in the kinase domain decrease the affinity of the TKI to bind to the kinase domain and thus decrease the therapeutic efficacy of TKIs. However, mutations in the kinase domain do not alter the affinity of trastuzumab to bind to the juxtamembrane region of ErbB-2. In contrast, mutations in the juxtamembrane region decrease binding of trastuzumab but retain kinase activity and thus the ErbB-2 RTK is susceptible to TKI activity. Kinase domain mutations in ErbB-2 have been observed in patients with lung cancer, CML and gastrointestinal stromal tumors treated with an EGFR TKI or a c-ABL...
TKI, imatinib, respectively (Anido et al., 2006; Engelman et al., 2006; Gorre et al., 2001; Kobayashi et al., 2005; Kosaka et al., 2006; Pao et al., 2005; Tamborini et al., 2004). Moreover, patients harboring a truncated form of ErbB-2 (p95HER2), which lacks the entire N-terminal extracellular region including the trastuzumab binding region, are resistant to trastuzumab but retain sensitivity to lapatinib (Anido et al., 2006). Furthermore, D16 ErbB-2, a splice variant that retains the trastuzumab binding epitope but stabilizes ErbB-2 homodimers, has been identified to cause resistance to trastuzumab in breast cancer cell lines (Castiglioni et al., 2006; Mitra et al., 2009). A small number of point mutations or small insertions in the ErbB-2 gene have been identified in non-small-cell lung, gastric, colorectal, head and neck, and ErbB-2 overexpressing human breast cancers (Mounawar et al., 2007; Shigematsu et al., 2005; Stephens et al., 2004; Willmore-Payne et al., 2006a, b).

Another emerging mechanism that could possibly contribute to trastuzumab resistance is altered interaction between ErbB-2 and trastuzumab by co-expression of another protein that binds to ErbB-2. For example, Mucin-4 (MUC4), a membrane-associated proteoglycan, is up-regulated during acquired trastuzumab resistance (Nagy et al., 2005). MUC4 directly interacts with ErbB-2 via an EGF-like domain, masking the trastuzumab binding site on ErbB-2 (Carraway et al., 2001; Price-Schiavi et al., 2002). MUC4 activates ErbB-2, but does not affect the expression of ErbB-2 (Carraway et al., 2001; Price-Schiavi et al., 2002). Knockdown of MUC4 increased trastuzumab binding and sensitized the resistant JIMT-1 breast cancer cells to trastuzumab (Nagy et al., 2005). Studies have also shown that MUC1, another family member, interacts with ErbB-2 (Li et al., 2003), initiates heterodimerization and induces downstream signaling that
promotes survival and proliferation (Hikita et al., 2008; Mahanta et al., 2008). Thus, novel agents targeting expression and/or function of MUC family members in combination with trastuzumab might prove to be advantageous in the treatment of resistant breast tumors.

5.2. Activation of compensatory signal transduction pathways

5.2.1. Role for receptor tyrosine kinases

ErbB family members have some redundant functions but are also unique as they can form homodimers and amplify diverse signaling pathways. Trastuzumab inhibits ErbB-2 phosphorylation but it rarely blocks the heterodimerization of ErbB-2 with other ErbB family members. Therefore, signaling initiated by other ErbB family members can trans-activate ErbB-2 and drive proliferation and survival, thus bypassing the anti-proliferative effects of lapatinib or trastuzumab. Recently, elevated ErbB-1 or ErbB-3 expression has been reported when ErbB-2–overexpressing breast cancer cells (T47D, UACC812, UACC893, MDA-MB-453, MDA-MB-361) were treated with trastuzumab for long durations (Narayan et al., 2009). This suggests that alternate ErbB family dimers, such as ErbB-1/ErbB-1 homodimers or ErbB-1/ErbB-3 heterodimers, could possibly rescue the inhibitory effects of trastuzumab. Moreover, in ErbB-2–overexpressing MCF-10A and BT474 cells, TGF-β has been shown to activate ErbB-3 and, subsequently, the PI3-K pathway by activating ADAM17. This result in an increase in ErbB ligand shedding and decreased sensitivity to trastuzumab (Wang et al., 2008). A gene signature of TGF-β activity, derived from breast cancer cells expressing a constitutively active mutant form of the TGF-β type I receptor, correlates with resistance to trastuzumab and poor clinical outcome in patients (Wang et al., 2008). Interestingly,
from ErbB-1 and ErbB-3 receptor knockdown studies, ErbB-3 has been shown to play a crucial role over ErbB-1 in ErbB-2 positive breast cancer (Lee-Hoeflich et al., 2008) and ErbB-3 signals preferably by heterodimerizing with ErbB-2 (Hynes and Lane, 2005). Therefore, the promising approach to treat trastuzumab resistance would be to design monoclonal antibodies that can target dimerization among the ErbB family members. Pertuzumab, another humanized, monoclonal antibody against ErbB-2, prevents heterodimerization of ErbB-2 with ErbB-3 and has shown a 6-month clinical benefit when used in combination with trastuzumab in patients who had progressed after trastuzumab-based therapy (Franklin et al., 2004; Nahta et al., 2005). Also, multi-targeted tyrosine kinase inhibitors, including ErbB-1/ErbB-2–specific inhibitors lapatinib, neratinib, and BIBW 2992, have shown significant efficacy in trastuzumab-resistant disease (Allen et al., 2002; Rusnak et al., 2001).

Crosstalk with other RTKs has also been implicated as a means to bypass trastuzumab action. Insulin-like growth factor-1 receptor (IGF-1R) is another type I transmembrane receptor tyrosine kinase that is activated by IGF-1 and a related growth factor, IGF-2 (Lu et al., 2001). Up-regulation of IGF-1R or an increase in levels of IGF-1R/ErbB-2 heterodimers and subsequent trans-phosphorylation can strongly activate the PI3-K – AKT pathway and contribute to trastuzumab resistance (Lu et al., 2001; Nahta et al., 2005). In a neo-adjuvant study of trastuzumab plus chemotherapy, high level expression of IGF-1R correlated with a poor clinical outcome (Harris et al., 2007). Interestingly, IGF-1R has been found to form a heterotrimeric complex with ErbB-2 and ErbB-3 (Huang et al., 2010). IGF-1R blockade mediated by IGF-binding protein 3, neutralizing antibody, or a TKI resensitized SKBR3 cells to trastuzumab (Nahta et al.,
These results suggest a possible role for the IGF-1R signaling pathway in trastuzumab resistance, and that IGF-1R inhibitors in combination with trastuzumab may help to improve the efficacy of trastuzumab. CP-751871, a monoclonal neutralizing antibody, and NVP-AEW541, an IGF-1R tyrosine kinase inhibitor, should be tested either in combination or sequentially with trastuzumab in women with ErbB-2 positive, metastatic breast cancer. Recently, targeting IGF-1R with metformin was also shown to overcome resistance to trastuzumab (Liu et al., 2011a).

Importantly, the c-Met (mesenchymal-epithelial transition factor) receptor is another type I transmembrane tyrosine kinase receptor that is activated by binding to its ligand, hepatocyte growth factor (HGF) (Bottaro et al., 1991). c-Met and HGF are often overexpressed in breast cancer, including a subset of ErbB-2 positive breast cancer (Edakuni et al., 2001; Kang et al., 2003; Lindemann et al., 2007; Nagy et al., 1996; Yamashita et al., 1994). The aberrant expression of c-Met correlates with decreased overall survival and poor patient prognosis (Camp et al., 1999; Ghoussoub et al., 1998). Further, over-expression of c-Met and its ligand, HGF, were reported in ErbB-2 positive breast cancer patients who did not respond to trastuzumab plus chemotherapy (Shattuck et al., 2008). Activated c-Met has been shown to dampen the growth-inhibitory effects of trastuzumab and promote survival of ErbB-2 overexpressing BT474 and SKBR3 breast cancer cells. The proliferative effects exhibited by activated c-Met are mediated through inhibition of a cyclin dependent kinase inhibitor (p27Kip1) expression (Shattuck et al., 2008). Therefore, inhibition of c-Met resensitized BT474 and SKBR3 cells to trastuzumab (Shattuck et al., 2008). Consistent with this finding is that trastuzumab treatment rapidly increases expression of c-Met in ErbB-2 overexpressing breast cancer
cells (Shattuck et al., 2008). This is important, as simultaneous overexpression of both c-Met and ErbB-2 has been shown to collaborate to promote cellular invasion, suggesting that tumors expressing both receptors may present with aggressive behavior. Thus, it is certainly possible to predict that a subset of women with co-overexpression of ErbB-2 and c-Met might benefit from a combined therapy targeting both ErbB-2 and c-Met receptors.

Overexpression of the EphA2 receptor was found to predict for reduced disease free survival in a cohort of patients with ErbB-2 positive breast cancer. Trastuzumab resistant, ErbB-2 positive breast cancer cells showed increased activation of EphA2, and treatment with an EphA2 neutralizing antibody restored trastuzumab sensitivity (Zhuang et al., 2010).

Moreover, co-expression of erythropoietin receptor and ErbB-2 was noted in ErbB-2 positive breast cancer cells and primary tumors. In these cell lines, concurrent treatment with recombinant human erythropoietin conferred trastuzumab resistance. This was mediated via Jak2 and c-Src signaling, leading to inactivation of PTEN, a tumor suppressor and negative regulator of AKT. Finally, in patients with ErbB-2 positive metastatic breast cancer, the concurrent treatment of erythropoietin and trastuzumab correlated with a shorter overall survival compared to patients who did not receive erythropoietin (Liang et al., 2010).

Finally, in a model of acquired resistance to lapatinib, the AXL receptor tyrosine kinase was found up-regulated. The phosphorylated tyrosines in the C-terminal region of AXL recruited the p85 regulatory subunit of PI3-K to activate PI3-K and
bypass the effects of either lapatinib or trastuzumab. A kinase inhibitor targeting AXL activity restored sensitivity to ErbB-2 antagonists (Liu et al., 2009).

5.2.2. Role for intracellular kinases

Activating mutations in the components of the PI3-K–AKT pathway are frequent and can confer resistance to ErbB-2 inhibitors. These include gain-of-function mutations in the p110 catalytic subunit of PI3-K, AKT1, the p85 regulatory subunit of PI3-K or amplification of AKT2. In addition, loss-of-function mutations have been identified in tumor suppressors such as PTEN (Bachman et al., 2004; Bellacosa et al., 1995; Campbell et al., 2004; Carpten et al., 2007; Gewinner et al., 2009; Li et al., 1997; Saal et al., 2005).

PI3-K activating mutants and consequently, constitutive AKT kinase activity has also been shown to promote growth and proliferation of breast tumors (Yakes et al., 2002). Concurrently, loss or decreased expression of PTEN has been reported in ErbB-2+ breast tumors and is associated with poor response to ErbB-2–targeted therapy (Berns et al., 2007; Esteva et al., 2010; Nagata et al., 2004). PTEN is a lipid phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3) to PIP2 and thus negatively regulates the AKT pathway. In addition, human breast cancer cell lines containing endogenous mutations in PI3-K are intrinsically resistant to trastuzumab (Ginestier et al., 2007; O'Brien et al., 2010). Human breast cancer cell lines in which activating PI3-K mutations are ectopically expressed exhibit an attenuated response to lapatinib (Eichhorn et al., 2008). ErbB-2 overexpressing BT474 breast cancer cells that have heightened PI3-K/AKT signaling and reduced PTEN expression were shown to be sensitive to PI3-K or Mammalian target of rapamycin (mTOR) inhibitors, and these
inhibitors were able to reverse trastuzumab resistance both in vitro and in vivo (Chan et al., 2005; Nagata et al., 2004). Furthermore, combinations of trastuzumab plus PI3-K inhibitor XL147, or trastuzumab or lapatinib plus the dual PI3-K-mTOR inhibitor BEZ235, inhibit growth of PI3-K mutant xenografts resistant to anti-ErbB-2 therapies (Chakrabarty et al., 2010; Eichhorn et al., 2008; Serra et al., 2008). In a model of trastuzumab resistance caused by PTEN loss, targeting mTOR or AKT was able to at least partially overcome resistance (Lu et al., 2007). These results suggest that loss or low expression of PTEN and subsequent high AKT kinase activity serve as predictors of trastuzumab response, and those inhibitors of the PI3-K/AKT/mTOR signaling pathway need to be explored in combination with trastuzumab to prevent possible trastuzumab resistance. For example, in a phase II study, the combination of the everolimus, a TORC1 inhibitor, with trastuzumab and chemotherapy showed a partial response rate of 19% in patients with ErbB-2 positive metastatic breast cancer that had progressed on trastuzumab plus taxanes.

Up-regulation of the non-receptor tyrosine kinase, Src (SFK) activity, has been reported in several trastuzumab and lapatinib resistant cell lines. Src kinase mediated anti-ErbB-2 therapy resistance may be partly through activation of erythropoietin receptor. EpoR activates Src via Jak2. Activated Src associates with ErbB-2, where it is proposed to phosphorylate and inhibit PTEN. The inhibition of PTEN, in turn, up-regulates PI3-K, providing a viable mechanism for attenuation of trastuzumab action (Liang et al., 2010). Combination of SFK inhibitor (dasatinib) and lapatinib partially blocked PI3-K activation in the resistant cells and restored sensitivity to lapatinib in BT474 xenografts (Rexer et al., 2011). Combinatory treatment strategy including Src
inhibitor and trastuzumab overcame trastuzumab resistance conferred by the D16 ErbB-2 splice variant (Mitra et al., 2009; Zhang et al., 2011). Finally, the activation of EphA2 seen after chronic trastuzumab treatment and onset of trastuzumab resistance is also mediated through Src (Zhuang et al., 2010).

5.3. Defects in apoptosis and cell cycle control

ErbB-2 is the oncogenic driver promoting survival and proliferation signals. In these breast tumors, inhibiting ErbB-2 activity induces growth arrest and apoptosis. Therefore, alterations in the normal apoptotic machinery acquired during the course of trastuzumab treatment, in turn can cause resistance to ErbB-2-targeted therapies. In a recent report, levels of the pro-apoptotic BH3-only Bcl-2 family member BIM were shown to be predictive of response to a TKI in an ErbB-2 addicted breast cancer. Although lapatinib potently inhibited ErbB-2 activity in all ErbB-2 positive breast cancer cells, only the cell lines expressing high levels of BIM underwent apoptosis (Faber et al., 2011; Tanizaki et al., 2011).

Survivin is a member of the inhibitor of apoptosis (IAP) protein family, which inhibits the activity of caspases, necessary effectors of programmed cell death. ErbB-2 positive breast cancer cells resistant to lapatinib showed up-regulation of ERα, which, in turn induced FoxO3a mediated transcription of survivin (Xia et al., 2006). Elevated levels of survivin and the anti-apoptotic Mcl-1 proteins were found in trastuzumab resistant cells. Treatment with a general kinase inhibitor that reduces levels of survivin and Mcl-1 inhibited the growth and survival of the ErbB-2 positive breast cancer cells (Valabrega et al., 2011). Knockdown of survivin in combination with trastuzumab restored sensitivity to trastuzumab (Oliveras-Ferraros et al., 2011). Furthermore, PI3-K
signaling regulates survivin expression in ErbB-2 gene amplified breast cancer cells. Thus, inhibition of ErbB-2 – PI3-K pathway reduces survivin expression and induces apoptosis in ErbB-2 overexpressing breast cancer cells (Faber et al., 2009; Tanizaki et al., 2011).

Trastuzumab induces a G1 cell cycle arrest and eventually apoptosis within the breast tumor by enhancing the association of p27\textsuperscript{Kip1} with cyclin E/Cdk2 complexes. This association increases the half-life of p27\textsuperscript{Kip1} and prevents the phosphorylation of p27\textsuperscript{Kip1} by Cdk2 and subsequent ubiquitin-dependent degradation (Le et al., 2003; Nahta et al., 2004). Thus, altered control of cell cycle progression in response to trastuzumab may play a critical role in resistance. Decreased p27\textsuperscript{Kip1} levels and amplification of Cdk2 have been reported in trastuzumab resistant breast cancer (Nahta et al., 2004; Scaltriti et al., 2011). In a cohort of patients with ErbB-2 positive breast cancers treated with trastuzumab, amplification of cyclin E was associated with decreased response to trastuzumab. Further, Cdk2 inhibitors reduced growth of trastuzumab resistant xenografts. Depletion of p27\textsuperscript{Kip1} using either antisense or siRNA with a resulting increase in Cdk2 activity prevented trastuzumab induced growth inhibition in ErbB-2 positive SKBR3 breast cancer cells. Conversely, overexpression of p27\textsuperscript{Kip1} or preventing p27\textsuperscript{Kip1} degradation using a proteasome inhibitor MG132 resensitized resistant cells derived from the SKBR3 cell line to trastuzumab. These results suggest that low level expression of p27\textsuperscript{Kip1} and increased Cdk2 activity are also critical trastuzumab resistant markers. Additionally, modulation of p27\textsuperscript{Kip1} levels appears to be a common downstream mechanism for several of the resistance pathways described previously, including IGF-1R and c-MET (Lu et al., 2001; Nahta et al., 2005; Shattuck et al., 2008).
6. Conclusion

Several mechanisms of resistance to both trastuzumab and lapatinib have been identified in preclinical and clinical settings. Emerging preclinical and clinical data suggest the use of combinations of therapies targeting the ErbB-2 signaling network at multiple points. However, there is little to no consideration of crosstalk between ErbB-2 and other oncogenic pathways that may play a role in development and maintenance of ErbB-2 positive breast cancer and resistance phenotype. Thus, open ended molecular approaches must be utilized to identify biomarkers and/or effectors of resistance to anti-ErbB-2 therapies.
<table>
<thead>
<tr>
<th>Targeted Therapies</th>
<th>Target</th>
<th>Mechanism</th>
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<tr>
<td>Lapatinib, Neratinib or Afatinib</td>
<td>ErbB-2 and ErbB-1</td>
<td>TKI</td>
</tr>
<tr>
<td>Pertuzumab</td>
<td>ErbB-2</td>
<td>Dimerization inhibitor</td>
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<td>T-DM1</td>
<td>ErbB-2</td>
<td>Neutralizing antibody - microtubule inhibitor fusion</td>
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<tr>
<td>Perifosine</td>
<td>AKT</td>
<td>Plasma membrane translocation inhibitor</td>
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<td>MUC4, MUC1 monoclonal antibody</td>
<td>MUC4</td>
<td>Neutralizing antibody</td>
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<td>Dasatinib</td>
<td>Src</td>
<td>TKI</td>
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<tr>
<td>Everolimus, Temsirolimus, Sirolimus</td>
<td>mTOR</td>
<td>TKI</td>
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<td>AP23573</td>
<td>mTOR</td>
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<td>EphA2 antibody</td>
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<td>AXL inhibitor</td>
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Table 1: Novel therapeutic strategies to overcome trastuzumab resistance in combination with anti-ErbB-2 therapies.
NOTCH: A LITERATURE REVIEW

The molecular profile of ErbB-2 positive breast cancer has identified several crosstalk mechanisms as described previously that limit the effectiveness of ErbB-2 directed therapy. The tumor cells acquires bypass signaling through other receptor or intracellular signaling pathways which is then responsible for the proliferation, survival, resistance to apoptosis and/or metastasis. Oncogenic pathways that increase proliferation and prevent apoptosis have emerged as potential targets for the treatment of breast cancer. For example, Notch signaling has been presented as a novel biomarker of trastuzumab resistance. Activated Notch in response to ErbB-2 inhibitors could promote a survival advantage and contribute to resistance. Thus, a thorough investigation of the role of Notch signaling in ErbB-2 positive breast cancer and trastuzumab resistance would provide an evidential rationale of whether targeting the Notch pathway either alone or in combination with therapies targeting ErbB-2 will be effective in overcoming or preventing resistance.

1. Introduction

The Notch pathway was identified early in the twentieth century through a genetic mutation screen in Drosophila melanogaster. Affected flies demonstrated a “notched” wing margin that was passed on from parent to progeny (Morgan et al., 1917). Since the identification of the Notch gene, continuous work on understanding Notch signaling has been done which eventually led to the discovery of the Notch pathway. We now know
that Notch signaling plays critical roles during development and cancer in a variety of organisms. These include stem cell maintenance, cell proliferation, survival, apoptosis, and differentiation.

2. Notch Function

The development of the breast in humans and the mammary gland in mice is governed by a variety of signaling pathways involved in cell fate determination and differentiation. Notch signaling plays diverse and essential roles in the regulation of cell-fate decisions during mammary gland development. Cell fate decisions occur through ligand – receptor interactions; Receptor activation leads to the specific fate of a cell, whereas receptor inhibition leads to an alternate fate. Notch also acts as a regulator of cell survival and proliferation in a context-dependent manner (Artavanis-Tsakonas et al., 1995; Greenwald, 2012; Politi et al., 2004). Recently, it has been demonstrated that pathways such as Notch which regulate development during embryonic life and adult stem cells, can be reactivated in malignancies (Nickoloff et al., 2003; Radtke and Raj, 2003) and support the tumor initiating cells (Song and Miele, 2007).

2.1. Notch receptors

The Notch heterodimer is comprised of three domains: extracellular, transmembrane, and intracellular (Figure 3). The N-terminal extracellular portion ($N^{EC}$) of Notch receptors contains the ligand binding domain, which comprises of 29-36 tandem epidermal growth factor (EGF) – like repeats. Productive interactions with ligands to receptors presented in trans require EGF repeats 11-12. In contrast, cis-inhibition of Notch receptors by ligands expressed in the same cell requires EGF repeats 24-29. Notch integrates cis-trans activities to regulate and determine cell fate specifications. From
each extracellular domain extend six cysteine residues which form three intra-domain disulfide bridges. Following the extracellular domain is the transmembrane domain (N\textsuperscript{TM}) which comprises two components: the unique negative regulatory region (NRR) composed of three cysteine-rich Lin-12/Notch repeats (LNR) which prevent ligand-independent interactions, and the heterodimerization domain which maintains the Notch receptor in an inactivated state. In the absence of ligand, the second cleavage site (S2) is buried within the NRR thus preventing receptor activation in the absence of ligands. The transmembrane portion (N\textsuperscript{TM}) contains the S3 cleavage site for the γ-secretase complex. Finally, the C-terminal region of the Notch receptor is the intracellular domain (N\textsuperscript{TM}) which extends from the inner cell membrane into the cytoplasm. The N\textsuperscript{TM} comprises four components: a RAM domain (RBP-JK associated molecule; interacts with the DNA-binding protein CSL), seven cdc10/ankyrin repeats (ANK domain; associated with CSL to help recruit the co-activator Mastermind), two nuclear localization sequences (NLS) flanking the ANK domain, followed by a transcription activation domain (TAD) and a proline/glutamic acid-serine/threonine-rich PEST domain (regulates the stability of NICD). Lastly, Notch-1 and Notch-2 contain transcription activation domains (TAD) and cytokine response elements (NCR), whereas Notch-3 only contains a NCR region (Kopan and Ilagan, 2009). All these elements together are critical to the productive activation of Notch signaling.
Figure 3: Canonical Regulators of Notch Signaling Pathway
2.2. Notch ligands

In vertebrates, there are five type I, membrane spanning Notch ligands, Delta-like 1, 3, and 4 and Jagged-1 and 2 (Figure 3). The Notch ligands bind and activate the Notch receptor presented on the surface of an adjacent cell. Ligands of Notch receptors are composed of a large extracellular region and a 100-150 residue cytoplasmic tail. Notch ligands are characterized by three structural motifs in their extracellular domain: an N-terminal DSL (Delta/Serrate/LAG-2) motif, specialized tandem EGF repeats called the DOS domain, and EGF-like repeats. Both the DSL and DOS domains are involved in receptor binding, with the DSL domain involved in both trans- and cis- interactions with the Notch receptor. The Jagged ligands are longer than the Delta-like ligands and the length is determined by the 6-16 EGF-like repeats in the extracellular domain. A cysteine-rich area is located at the end of the EGF-like repeats. Notch ligands can be divided into two groups based on the presence (Jagged) or absence (Delta) of a cysteine-rich domain and the presence (Jagged-1, -2, and Delta-like 1) or absence (Delta-like 3 and 4) of a DOS domain (Cordle et al., 2008; D'Souza et al., 2008; Komatsu et al., 2008). The intracellular domain (C-terminal) of each ligand has a shorter cytoplasmic tail than the extracellular domain and contains a PDZ (PSD95/Dlg1/ZO1)-binding motif which aids in intracellular protein-protein interactions. Popovic et al has identified several residues in the intracellular region of Jagged-1 (T1197, S1207, S1210, Y1216) as potential targets for protein kinases and to play a role in the modulation of protein-protein interactions (Popovic et al., 2011).
In Drosophila, both Delta-like (Delta) and Jagged (Serrate) ligands can be proteolytically cleaved by metalloproteinases and cause extracellular domain shedding (Klüeg et al., 1998; Sun and Artavanis-Tsakonas, 1997). Although, cleaved or soluble DSL ligands can activate Notch signaling, they are less active than membrane bound ligands. Soluble ligands may require clustering or attachment to extracellular matrix or cell surface to efficiently activate Notch signaling. In fact, endocytosis deficient prefixed Delta-expressing cells can activate Notch target genes (Itoh et al., 2003; Parks et al., 2000). Perhaps the pulling force required for Notch activation is generated by cell detachment or Notch endocytosis upon ligand–receptor interaction. This mechanism may account for the signaling activity of naturally occurring soluble DSL ligands identified for C. elegans (Chen and Greenwald, 2004; Komatsu et al., 2008). Alternatively, ligand shedding could represent a mechanism for down-regulation of activated ligand (Kopan and Ilagan, 2009; Weinmaster, 1997).

3. An Overview of Canonical Notch Signaling Pathway

Notch signaling is an evolutionary conserved signaling pathway (Figure 4) that has emerged as a target for the treatment of breast cancer. There are four mammalian Notch receptors (Notch-1, -2, -3, and -4) (Blaumueller and Artavanis-Tsakonas, 1997) with five associated ligands (Delta-like 1, 3, and 4 and Jagged-1 and -2) (Dunwoodie et al., 1997; Lindsell et al., 1995; Shawber et al., 1996). Notch ligands and receptors are type I cell surface proteins (Blaumueller and Artavanis-Tsakonas, 1997). Notch receptor-ligand interactions promote the expansion one cell fate at the expense of another and the specific outcome is often context-dependent (Callahan and Raafat, 2001). Notch is synthesized as
a single, relatively large (>300 kDa) polypeptide in the endoplasmic reticulum. The newly translated Notch receptor proteins are then glycosylated. Addition of O-linked fucose to the EGF-like repeats is mediated by the enzymes O-fucosyl transferase I (O-fut) and Rumi, which is the first step in the production of a mature receptor. Glycosylated Notch receptors undergo a series of proteolytic cleavages by the PC5/furin, tumor necrosis factor-α-converting enzyme (TACE/ADAM 10/17) and γ-secretase complex (comprised of presenilin-1/2, nicastrin, Pen-2, and Aph-1). After the initial addition of O-linked fucose, the Notch pro-protein is chaperoned by the GTPase Rab-protein 6 through the secretory pathway to the trans-Golgi network. In the trans-Golgi, the first cleavage (S1) is mediated by PC5/furin-like convertase, which leads to production of a mature receptor. Enzymatic activity of O-fucose-specific β1,3-N acetylglucosaminyl-transferases Fringe (Lunatic, Manic, or Radical) then extends the O-fucose modification on Notch receptors, thereby altering the ability of specific ligands to activate Notch. Mature Notch receptors are composed of an extracellular subunit (N^{EC}) and transmembrane subunit (N^{TM}) held together by calcium cation mediated non-covalent interactions. Notch heterodimers are then trafficked to the cell surface where it relies on the ability of a ligand to bring about receptor proteolysis (Kopan and Ilagan, 2009). At the surface, Notch receptors are activated by binding to a ligand expressed on an adjacent cell. Ligand binding generates a mechanical force to promote a conformation change in the bound Notch receptor. This conformational change dissociates N^{EC} and N^{TM} subunits and the released N^{EC} domain is then trans-endocytosed by the ligand-expressing cell, exposing the second cleavage site (S2) in the Notch receptor. The second cleavage (S2)
is facilitated by TACE, which generates the membrane-anchored Notch extracellular truncation (NEXT) fragment, a substrate for the \( \gamma \)-secretase complex (Brou et al., 2000). The third and fourth cleavages (S3 and S4) are mediated by the \( \gamma \)-secretase complex (Kopan and Ilagan, 2004), which releases the active Notch intracellular domain (NICD) into the cytoplasm (Saxena et al., 2001). This final step can be pharmacologically inhibited by \( \gamma \)-secretase inhibitors (GSIs), which prevent the release of NICD, thus inhibiting Notch-mediated transcription and cell growth. GSIs are currently in clinical trials for the treatment of breast cancer and other solid tumors (Pannuti et al., 2010). GSIs currently undergoing clinical trials are listed in Table 2. NIC can subsequently enter the nucleus because of the presence of nuclear localization signals located within it (Lieber et al., 1993; Stifani et al., 1992). NICD forms an active transcriptional complex by displacing the corepressor proteins and histone deacetylases (HDACs) complex (Hsieh et al., 1999) and recruiting the protein mastermind-like 1 (MAML) (Wu et al., 2000) and histone acetyltransferases to the CSL (CBF-1/RBPjκ/Su(H)/Lag-1) complex. A few Notch target genes have been identified, including Hes (Hairy enhance of split) family (Iso et al., 2003), Hey (Hairy/enhancer of spit related with YRPW motif), nuclear factor-kappa B (NFκB) (Cheng et al., 2001), vascular growth factor receptor (VEGF), PI-3K – AKT - mammalian target of rapamycin (mTOR) (Gutierrez and Look, 2007; Palomero et al., 2007), cyclin D1 (Ronchini and Capobianco, 2001), c-myc (Weng et al., 2006), IGF-1R (Eliasz et al., 2010), p21, p27, etc, all of which have been well documented for their roles in tumor development and progression.
Figure 4: Canonical Notch Signaling Pathway
3.1. Notch signaling in mammary gland development

Murine mammary gland development is governed by a variety of signaling pathways involved in cell fate determination and differentiation. Notch signaling plays diverse and essential roles in the regulation of cell-fate decisions during mammary gland development. Cell fate decisions occur through ligand – receptor interactions; Receptor activation leads to the specific fate of a cell, whereas receptor inhibition leads to an alternate fate. Notch also acts as a regulator of cell survival and proliferation in a context-dependent manner (Artavanis-Tsakonas et al., 1995; Greenwald, 2012; Politi et al., 2004). Recently, it has been demonstrated that pathways such as Notch which regulate development during embryonic life and adult stem cells, can be reactivated in malignancies (Nickoloff et al., 2003; Radtke and Raj, 2003) and support the tumor initiating cells (Song and Miele, 2007).

A role for Notch in the normal mammary gland development has been highlighted in several key experiments. The Notch receptors, their ligands, and target genes play a critical role during different stages of mammary gland development. Among the Notch genes, mRNA levels of Notch-3, Jag-1, DLL3, and Hey-2 were highest during different stages of mammary gland development (Raafat et al., 2011). In addition, luminal cell commitment is defined by activation of Notch-3 and suppression of Notch-4 (Bouras et al., 2008; Raouf et al., 2008). Loss of the RBP-J in mammary progenitors disrupted luminal cell fate specification during pregnancy associated alveolar development (Buono et al., 2006). In vivo, transgenic mice expressing a constitutively active form of Notch-4 in the mammary gland fail to regulate branching morphogenesis of mammary epithelial cells, and subsequently develop mammary tumors (Soriano et al., 2000; Uyttendaele et
The Wicha group has demonstrated that Notch signaling plays a critical role in regulation of self-renewal and lineage specific differentiation of both adult stem cells and progenitor cells during normal mammary gland development (Dontu et al., 2004). This finding supports a role for Notch in normal breast development, and suggests that alterations in Notch signaling components might play a significant role in breast-cancer development by deregulating the self-renewal properties of normal mammary stem cells. Thus, identification and proper understanding of the role of Notch signaling in mammary cell fate decisions, will be of crucial interest for understanding breast cancer diversity and, ultimately, improving treatment options and breast cancer outcome.

### 3.2. Notch signaling in breast cancer

Emerging evidence suggests that the Notch signaling network is frequently deregulated in human malignancies including lung, colon, head and neck, breast, pancreas and renal carcinoma, acute myeloid leukemia, Hodgkin’s and large cell lymphoma (Wang et al., 2010). Most notably, deregulated Notch signaling has been frequently found in breast tumors, suggesting that Notch is a potent breast oncogene (Stylianou et al., 2006). Constitutive activation of Notch-1 through chromosomal translocation occurs in more than 50% of T-cell acute lymphoblastic leukemias (T-ALL) patients (Ellisen et al., 1991; Weng et al., 2004). As evidenced from literature, there is no known activating mutation or amplification of Notch gene reported in breast cancer. Rather, aberrant Notch activation occurs through transcriptional and post-translational regulation of core Notch pathway components which will be discussed in detail in chapter II (Lee et al., 2007; Stylianou et al., 2006).
The first evidence of aberrant Notch signaling in breast cancer comes from the murine mammary gland studies. The role of Notch-1, -3 or -4 in the development of mouse mammary tumors was established by using MMTV/neu transgenic mice. This data suggests that Notch-1, -3 or -4 has transforming potentials in vivo (Dievart et al., 1999; Gallahan and Callahan, 1997; Gallahan et al., 1987; Hu et al., 2006). Furthermore, both Notch-1 and Notch-4 genes were identified as a novel target for MMTV provirus insertional activation. MMTV insertion in the Notch-1 and Notch-4 locus induced the overexpression of mutant active form of Notch (NICD) protein that can transform HC11 mouse mammary epithelial cells in vitro (Dievart et al., 1999; Gallahan and Callahan, 1997; Gallahan et al., 1987). Transgenic expression of Notch-4/INT-3 RNA species encoding Notch-4 NICD (an active form) in non-malignant human mammary epithelial cell line MCF-10A enabled these cells to grow in soft agar suggested Notch-4 can transform MCF-10A cells (Imatani and Callahan, 2000). Furthermore, Wnt driven oncogenesis occurs through a Notch-dependent mechanism in human breast epithelial cells (Ayyanan et al., 2006). In addition, Notch-1 was found to be a mediator of oncogenic Ras (Weijzen et al., 2002). High level co-expression of Notch-1 and Jag-1 generates synergistic effect on overall survival of breast cancer patients (Dickson et al., 2007; Reedijk et al., 2005).

In addition, Notch-1 and Notch-3 NICD proteins correlated with increased transcription of its target genes in most breast cancer cell lines (Yamaguchi et al., 2008). In a recent report, knockdown of Notch-3 in breast cancer cells decreased osteoblast- and transforming growth factor (TGF)-β1 induced colony formations. Moreover, osteolytic lesions were significantly reduced following Notch-3 knockdown (Zhang et al., 2010).
These results suggest Notch-3 knockdown may stand as a novel mechanism for decreasing breast cancer derived bone metastasis.

Activated Notch-2 in normal mammary epithelial cells *in vivo* has not been reported. However, unlike other Notch members, Notch-2 expression correlated with better survival in patients with breast cancer (Parr et al., 2004). These data suggests that Notch-2 activation corresponds to well-differentiated and benign tumor states. Constitutively active form of Notch-2 NICD in the human adenocarcinoma line MDA-MB-231 increased apoptosis *in vitro* (O'Neill et al., 2007). These results demonstrated that Notch-2 signaling is a potent inhibitory signal in human breast cancer carcinogenesis.

### 3.3. Activation of Notch signaling in breast cancer stem (BCS) cells

Analogous to normal stem/progenitor cells, a role for Notch in BCS cells has been postulated, since Notch signaling pathway has key functions in the development of normal breast as well as breast cancer. Up-regulated Notch expression is found in BCS cells (Farnie and Clarke, 2007). BCS cells contain undifferentiated cells that exhibit tumor-initiating properties and invasive features (Farnie and Clarke, 2007). Active form of Notch-1 impairs mammary stem/progenitor cells and BCS cells self-renewal properties through cyclin-D1 (Ling et al., 2010) and ErbB-2 (Magnifico et al., 2009) dependent pathways, respectively. Moreover, Notch-1 has been demonstrated to interact with erythropoietin to maintain self-renewing capacity of BCS cells (Phillips et al., 2007). Notch-1 (luminal cells) (Raouf et al., 2008) and Notch-4 (basal cells and BCS-enriched population) (Harrison et al., 2010) are differentially expressed within normal breast epithelium suggesting that Notch-1 and Notch-4 may have different roles in maintenance
of BCS cells. Harrison et al found that inhibition of Notch-4 caused a significantly greater inhibition in mammosphere formation compared to affecting Notch-1 activity by \(\gamma\)-secretase which only partially inhibits mammosphere formation (Harrison et al., 2010). These findings can be recapitulated in vivo where Notch-4 activation, but not Notch-1, was not sufficient to generate mammary carcinogenesis in mice (Gallahan and Callahan, 1987). Notch-3 is critical for luminal cell commitment in vitro suggesting a possible role in BCS cells (Raouf et al., 2008).

**3.4. Role of Notch in novel crosstalk mechanisms in breast cancer**

In human breast cancers, the oncogenic effect of Notch activation is partly due to its crosstalk with other signaling pathways. For example, receptor tyrosine kinases (RTKs), developmental pathways (Wnt and HedgeHog), janus kinase/signal transducers and activators of transcription (Jak/STAT), transforming growth factor-\(\beta/\)Decapentaplegic (TGF-\(\beta\)), platelet-derived growth factor (PDGF/PDGFR), vascular endothelial growth factor (VEGF), phosphatidylinositol 3-kinase (PI-3K/Akt), Ras, mTOR, NF-\(\kappa\)B, HIF, cytokines IL-6, IL-1, and leptin plus ER\(\alpha\) signaling as well as microRNAs (Guo et al., 2011) have been described. Here we are going to focus on crosstalk between Notch signaling and the most critical pathways (ErbB-2, ER\(\alpha\), and PEA3) necessary for breast cancer development, progression, tumor recurrence, and the resistance phenotype. Elucidating the cross-talk between Notch and other pathways critical for breast cancer development is necessary to determine patients that would benefit from Notch inhibitors, agents to be combined with Notch inhibitors, and biomarkers that are indicative of Notch activity.
The existence of Notch-1 and ErbB-2 crosstalk in breast cancer has been established by our research group and many others since. We have shown that Notch-1 signaling is decreased in ErbB-2 gene amplified (BT-474 and SKBr3) and overexpressing (MCF-7/ErbB-2) breast cancer cells (Osipo et al., 2008). However, trastuzumab or a small molecule tyrosine kinase inhibitor similar to lapatinib reactivated Notch-1 (Osipo et al., 2008). More importantly, a GSI or specific down-regulation of Notch-1 by RNA interference increased the sensitivity of ErbB-2 positive breast cancer cells to trastuzumab mediated growth inhibition, indicating that Notch-1 signaling might contribute to trastuzumab resistance in vitro (Osipo et al., 2008). In contrast, Chen et al observed RBPJkappa binding sites in the ErbB-2 promoter and that RBPJkappa/Notch stimulated transcription from ErbB-2 promoter (Chen et al., 1997). Yamaguchi et al indicated that Notch-3 rather than Notch-1 signaling plays an important role in the proliferation of ErbB-2 negative breast tumor cells (Yamaguchi et al., 2008). Finally, Magnifico et al demonstrated that ErbB-2 overexpressing breast cancer stem cells display activated Notch-1 signaling. Moreover, Notch-1 siRNA or a γ-secretase inhibitor resulted in down-regulation of ErbB-2 expression and decrease in mammosphere formation (Magnifico et al., 2009). These studies together suggest critical interactions between Notch and ErbB-2 pathways, both of which are involved in the progression of breast cancer and regulation of breast cancer stem cells.

Recently, Clementz et al demonstrated for the first time that PEA3, an Ets family transcription factor, is a transcriptional activator of Notch-1 and Notch-4 in triple negative breast cancer (Clementz et al., 2011). While, PEA3 recruitment on the Notch-1 promoter was independent of AP-1 activity, PEA3 recruitment to the Notch-4 promoter
was dependent on AP-1 activity: positively regulated by c-JUN and Fra-1 and negatively regulated by c-Fos (Clementz et al., 2011). Finally, the findings from this study showed that down regulation of PEA3 using siRNA strongly inhibited triple negative breast cancer growth in vitro and in vivo (Clementz et al., 2011). Taken together, the results from this study demonstrated that combination therapy targeting PEA3 and Notch pathways might provide a new therapeutic strategy for triple-negative and possibly other aggressive breast cancer subtypes where PEA3 activates Notch transcription.

One of the most critical pathways necessary for survival of breast cancer cells is the ERα pathway. Rizzo et al demonstrated that Notch-1, Notch-4, and Jagged-1 were expressed at variable levels in ductal and lobular carcinomas (Rizzo et al., 2008a). In addition, a panel of breast cancer cell lines tested showed an inverse correlation between ERα or ErbB-2 and Notch activity. Specifically, triple negative MDA-MB-231 breast cancer cells exhibited the highest Notch activity, whereas, ERα positive MCF-7 and T47D:A18 breast cancer cells suppressed Notch activity (Rizzo et al., 2008a). Moreover, either tamoxifen or raloxifene blocked this effect, reactivating Notch (Rizzo et al., 2008a). In vivo, γ-secretase inhibitor treatment arrested the growth of ERα negative MDA-MB-231 tumors and, in combination with tamoxifen, caused regression of ERα positive T47D:A18 tumors (Rizzo et al., 2008a). Together, these data indicated that combination therapy targeting ERα and Notch pathways may be effective in the treatment of ERα positive breast cancers and that targeting Notch pathway alone may be effective in the treatment of ERα negative breast cancers. Moreover, Soares et al demonstrated a novel crosstalk between estrogen and Notch signaling in breast cancer and endothelial cells (Soares et al., 2004). In particular, the investigators demonstrated that estrogen
promoted a significant increase in Notch-1 and Jagged-1 gene expression in ER$^+/PR^+/ErbB-2^-$ breast cancer cells, MCF-7 and endothelial cells (Soares et al., 2004). In another report, Notch gene expression, together with HIF-1α, was up-regulated by estrogen (Soares et al., 2004). Overall, the crosstalk between Notch and the ERα signaling pathway plays a significant role in the development and progression of human breast cancer and angiogenesis.
CHAPTER II

REGULATION OF LIGAND – INDUCED NOTCH SIGNALING

Although simple at first glance, the complexity involved in the regulation of Notch activity is significant (Fortini, 2009; Fortini and Bilder, 2009; Kopan and Ilagan, 2009; Takeuchi and Haltiwanger, 2010). In particular, glycosylation of the $N^{EC}$ is necessary for proper folding and trafficking of the signaling competent Notch receptor to the plasma membrane. Glycosylation also modulates Notch interactions with ligands: glycosylation of Notch by glycosyltransferase Fringe increases its affinity for Delta ligands but reduces its affinity for Jagged ligands. Also, ubiquitylation of the Notch ligands by the evolutionary conserved E3 ligases Neuralized (Neur) and Mindbomb-1 (Mib-1) is required for Notch receptor activation. Similarly, the amount of Notch at the cell surface as well as the formation of NICD is also affected by Notch endocytosis and trafficking to endosomes. Several endocytic routes, including Notch removal from the membrane and control of NICD turnover have been proposed to avoid spurious, excessive or sustained activation. Here, we review the functional significance and molecular mechanisms of ligand mediated regulation of Notch activity because co-overexpression of Jagged-1 and Notch in primary breast cancer is associated with poorest overall survival (Reedijk et al.,
2005) and high Jagged-1 expression is associated with the basal phenotype and recurrence in lymph node negative breast cancer (Reedijk et al., 2008).

1. Regulation of DSL Ligand Expression

DSL ligand expression could be controlled at the levels of transcription and cell surface ligand expression. Notch signaling can regulate gene expression of both the receptor and the ligand. Moreover, signaling from transforming growth factor-β (TGF-β) (Niimi et al., 2007; Zavadil et al., 2004), vascular endothelial growth factor (Hainaud et al., 2006; Lawson et al., 2002; Patel et al., 2005; Ridgway et al., 2006), EGFR/MAP kinase (Izrailit et al., 2013), steroid hormones (Soares et al., 2004), NF-κB activation (Bash et al., 1999), and Toll-like receptor (TLR) activation (Foldi et al., 2010) also enhances DSL ligand expression.

2. Regulation of DSL Ligand Activity

Mechanisms that limit Notch signaling to one of the two adjacent cells are important to the Notch biology and determination of the correct cell fate during development. Notch signaling mediates such function through a process of “lateral inhibition”, a transcriptional feedback mechanism. While cells that receive the Notch signal induce developmental programs distinct from those sending the signal, the specific outcome is often context-dependent. The lateral inhibition mechanism is strengthened by two activities presented by Notch ligands: trans-activation of Notch in neighboring cells, and cis-inhibition of Notch in its own cell. If ligand and receptor are expressed on the same cell, then ligand-receptor interaction results in cis-inhibition of cell fate in that cell. If ligand and receptor are expressed on adjacent cells, then ligand-receptor interaction
results in trans-activation of Notch signaling in Notch receptor expressing cell. However, it remains unclear how Notch integrates these two activities and how the resulting system facilitate cell fate specifications. Lateral inhibition explains how small differences in the expression levels of Notch and DSL ligands can direct cell fate decisions for the initially equivalent cells. Initially equivalent cells are defined as both expressing similar amounts of Notch receptor, its ligand, and target genes of the E(Spl)/HES family. E(spl)/HES proteins inhibit activity of the achaete-scute complex (AS-C) genes, which has been shown to exhibit differential effects on expression of Notch and its ligands. Thus, a small stoichiometric difference in the expression levels of Notch and DSL ligands will drive the two interacting cells to function as either the signal-sending cell (up-regulating AS-C and Delta, down-regulating Notch and HES) or signal receiving cell (up-regulating Notch and HES, down-regulating AS-C and Delta) (Campos-Ortega, 1993; Fortini, 2009). These data suggests that a stable regulatory feedback loop between Notch and Delta is under a transcriptional control involving E(spl)/HES and the AS-C. In addition to the trans-activation of Notch, lateral signaling is also reinforced by cis-interactions between ligand and Notch expressed on the same cell. Whether cis-interactions between Notch receptor and its ligand occur at the cell surface or within the endosomes is not clear yet. The lateral inhibition mechanism explained here is a foundation of the Notch signaling and provides better understanding of Notch mediated cell fate specification events.

Several lines of evidence indicate that the endocytosis of DSL ligands within the ligand expressing cell is essential for trans-activation of Notch receptor on the signal receiving cell. It is clear that endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface and ligand induced Notch activation. Genetic
mosaic studies with the conditional *Drosophila shibire* mutant published in 1997 showed that dynamin-dependent endocytosis is required in both Notch signaling and receiving cells (Seugnet et al., 1997). Further evidence for DSL ligand endocytosis comes from antibody uptake assays performed in *Drosophila* tissue (Le Borgne and Schweisguth, 2003a, b). In vertebrates, DSL ligands defective in endocytosis (Delta proteins that lack the intracellular domain or with specific intracellular domain mutations) accumulate at the cell surface and are unable to activate Notch (Parks et al., 2000). Moreover, DSL ligands are detected predominantly in endocytic vesicles in *Drosophila*, zebrafish and mammalian cells, suggesting that Delta is indeed internalized from the cell surface (Itoh et al., 2003; Kramer and Phistry, 1996). A specialized endocytic pathway mediated by E3 ubiquitin ligases Neur and Mib-1 (ubiquitylate DSL ligands) and epsin liquid facets (clathrin associated sorting protein), has been identified to promote Notch ligand removal from the cell surface and potentiate ligand signaling activity in a non-autonomous fashion (Figure 5) (Bingham et al., 2003; Itoh et al., 2003; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003a, b; Pavlopoulos et al., 2001; Wang and Struhl, 2004). In addition, the ligand expressing cells also require proteins that function in clathrin-mediated endocytosis such as clathrin, dynamin, auxilin, and Eps15 for DSL ligand to signal effectively. These studies suggest that the productive Notch signaling relies on the ability of ligand to be ubiquitylated (Neuralized and Mindbomb) and routed to an endocytic pathway (epsin liquid facets) to generate the physical force needed to pull the N\textsuperscript{EC}, exposing the ADAM cleavage site and bring about receptor proteolysis.
Figure 5: Mib/Neur and Epsin Dependent Ligand Endocytosis and Notch Activation
The observation that the endocytosis of DSL ligand removes the ligand from the cell surface where it interacts and activates its receptor is seemingly paradoxical. Several models have been proposed to understand the role for ligand ubiquitination and endocytosis in activation of Notch signaling (Figure 5) and will be discussed in depth below.

2.1. E3 ubiquitin ligases – Neuralized and Mindbomb

2.1.1. Role for Mib and Neur in Notch activation

Mindbomb (Mib) and Neuralized (Neur), E3 ubiquitin ligases of the RING finger family, are known as being key regulators of ligand signaling activity. Loss of Neur and Mib results in phenotypes that are consistent with loss of Notch signaling. Additionally, in the Neur or Mib mutant cells, Notch ligands accumulated on the cell surface and were defective in activating Notch signaling (De Renzis et al., 2006; Deblandre et al., 2001; Lai et al., 2001; Le Borgne and Schweisguth, 2003b; Pavlopoulos et al., 2001). Neur has been previously reported to promote ligand degradation. However, Mib mediated ubiquitylation promoted ligand endocytosis but did not appear to promote ligand degradation (Deblandre et al., 2001; Lai et al., 2001). This finding indicates that the ubiquitylation of Delta by Neur may have a dual antagonistic role in promoting (signal sending cells) and inhibiting (signal receiving cells) ligand signaling activity. In signal receiving cells, Neur induced ubiquitylation of Delta promotes ligand endocytosis and subsequent degradation, thus removing cis-inhibiting ligand and allowing Notch to bind ligand in trans on adjacent cells (Deblandre et al., 2001; Lai et al., 2001). However, in
signal sending cells, Neur mediated ubiquitylation of ligand is not necessary for cis-inhibition of receptor, as evidenced from Neur deficient cells being able to inhibit Notch in adjacent cells, but can cis-inhibit Notch receptor (Glittenberg et al., 2006; Miller et al., 2009). However, overexpression of Neur suppresses cis-inhibition, possibly by removing ligand from the cell surface, leaving open the possibility that Neur may regulate Notch activation in receiving cells by modulating the amount of ligand available to cis-inhibit receptor (Glittenberg et al., 2006). Studies from both flies and frogs identified the Notch ligand Delta as a substrate for the Neur RING domain E3 ubiquitin ligase activity (Deblandre et al., 2001; Lai et al., 2001; Le Borgne and Schweisguth, 2003b; Pavlopoulos et al., 2001; Yeh et al., 2001). Drosophila Mib interacts with and endocytose both Delta and Jagged (Lai et al., 2005; Le Borgne et al., 2005), whereas zebrafish Mib only interacts with and regulate the endocytosis of Delta (Chen and Greenwald, 2004; Itoh et al., 2003). Importantly, Mib was required by the ligand signal sending cell to activate Notch signaling during lateral inhibition through cell transplantation procedures (Itoh et al., 2003). Furthermore, presence of a mono-ubiquitin on the ligand ICD is required for ligand to activate Notch. Together, these findings suggest a role for Neur and/or Mib mediated ubiquitylation in generating a competent ligand and enhance signaling potential by promoting ligand endocytosis. Fly, mouse and human genomes encode for two mib homologs, mib1 and mib2, suggesting conservation of this E3 ubiquitin ligase throughout metazoans. Indeed, disruption of mib1 gene alone but not neur1, neur2, and mib2 in developing mouse embryos produces the known Notch like mutant phenotypes. Cells lacking Mib-1 and expressing Notch ligands do not activate Notch reporters or target gene expression in mammalian co-culture assays
(Hansson et al., 2010; Yamamoto et al., 2010). Neur1-dependent ubiquitylation directs Jagged-1 for degradation, and this result in inhibition of Jagged-1-induced Notch signaling in co-culture assays (Koutelou et al., 2008).

2.1.2. Distinct and redundant functions of Neur and Mib

There are numerous studies suggesting functional redundancy among Neur and Mib. For example, Notch activation in the absence of Neur is due to the presence of Mib-1, and in situations where both E3 ligases are expressed, simultaneous reduction of both Mib-1 and Neur are essential to generate loss-of-Notch phenotypes (Lai et al., 2005; Le Borgne et al., 2005; Wang and Struhl, 2005). Despite numerous examples of functional redundancy, Mib-1 was unable to rescue the Neur specific neurogenic phenotype, identifying a unique requirement for Neur in this particular context (Le Borgne et al., 2005). Drosophila Mib2 has been reported to maintain muscle integrity and survival and neither Mib-1 nor Neur was able to rescue the muscle defects produced by Mib2, suggesting an additional function of Mib2 in this context (Carrasco-Rando and Ruiz-Gomez, 2008; Nguyen et al., 2007). There are studies in mammalian cells suggesting that Mib- and Neur-mediated ubiquitylation have distinct effects on Notch ligands. For example, it has been proposed that Mib-1-mediated ubiquitylation of Delta targets Delta for endocytosis, while ubiquitylation by Neur2 is needed for subsequent trafficking of Delta through an endosomal pathway (Song et al., 2006). Another study suggested a role for Neur2 in transcytosis of Delta ligand from the basolateral to apical domain of the plasma membrane in polarized mammalian epithelial cells but has yet to be linked to Notch signaling (Benhra et al., 2010). Additionally, whether Delta transcytosis is unique to Neur and not mediated by Mib has not been reported. In vertebrates,
mindbomb, but not neutralized, is required for Notch-dependent morphological phenotypes (Barsi et al., 2005; Koo et al., 2005).

2.1.3. Regulation of Neur and Mib activity

In Drosophila, a family of eight proteins called the Bearded (Brd) family binds Neur, and thereby inhibits Neur from accessing and ubiquitylating Delta (Bardin and Schweisguth, 2006; Chanet et al., 2009; De Renzis et al., 2006; Fontana and Posakony, 2009). Brd proteins contain at least one NxxN motif that binds to the neutralized homology repeats (NHRs) present in Neur. In Drosophila, Notch ligands Delta and Serrate contain a NxxN motif, and NxxN motif of Delta has been shown to be necessary for Neur binding and endocytosis of Delta (Fontana and Posakony, 2009). In addition, Brd family proteins cannot bind Mib-1 since Mib proteins lack NHRs (Bardin and Schweisguth, 2006). A recent study identified the cell polarity protein PAR-1 kinase phosphorylating Mib1 which results in auto-ubiquitylation and subsequent degradation of Mib-1 by the proteasome (Ossipova et al., 2009).

2.2. Epsin-dependent endocytosis in generating pulling forces

Endocytosis of ubiquitylated DSL ligand would generate pulling forces required to destabilize bound Notch heterodimer and result in activating proteolysis for downstream signaling. In Drosophila imaginal discs, Parks et al were the first to show that the NECD co-localizes with Delta in endosomes in signal sending cells known to activate Notch in signal receiving cells (Parks et al., 2000). Importantly, deficiencies in ligand endocytosis prevent NECD trans-endocytosis by signal sending cells and this correlates with Notch signaling defects in flies and mammalian cells (Nichols et al., 2007; Parks et al., 2000). Signaling potential of a ligand most probably relies on early
steps required for vesicle trafficking involving pulling force to bend the membrane, form and release endocytic vesicles. In particular, ligands could employ mechanical force intrinsic to endocytosis to pull on bound Notch and dissociate the preformed Notch heterodimer. It is clear that epsin, a clathrin associated sorting protein, is required for endocytosis of ubiquitylated ligand and functions downstream of ligand binding to Notch to participate in the generation of pulling force during the endocytic process (D'Souza et al., 2008; Nichols et al., 2007). Notch binding to ligand could induce ligand clustering, which would assemble multiple ubiquitin-binding sites for epsin by means of its UIM. Such an assembly of multiple UIM/monoubiquitin interactions will produce strong epsin/ubiquitylated DSL interactions (Barriere et al., 2006; Hawryluk et al., 2006), which may be necessary to overcome any resistance to DSL internalization when it is bound to Notch (Figure 2). These data suggests that multiple ubiquitin binding sites are required for ligand to activate Notch, possibly by providing stable interactions with epsin-positive endocytic vesicles. Another important feature about epsin structure is that it is a multi-domain protein known to interact with membrane phospholipids, clathrin, dynamin and actin cytoskeleton all of which have been implicated in generating pulling force to bend the plasma membrane during formation of endocytic vesicle (Liu et al., 2010; McMahon and Gallop, 2005). Importantly, studies in flies, worms and mammalian cells have indicated that these same endocytic components are required in signal sending cells to activate Notch signaling in receptor expressing cells. This may explain why ubiquitylated DSL ligands internalized in cells deficient in epsin are not able to activate Notch.
2.3. Ligand recycling

Ligand endocytosis, trafficking through endosomes and recycling back to the cell surface is required for Notch activation. However, it is still unclear how recycling defects affect ligand activity. The following events could happen upon DSL ligand recycling: Because clustering of cleaved DSL ligands is necessary for their activity, it is possible that endocytosis aid in clustering of the ligand, which is then recycled back to the plasma membrane (Hicks et al., 2002; Morrison et al., 2000). Recycling could also allow posttranslational modification (monoubiquitylation) of DSL ligands in the sorting or recycling compartments which may render the ligands more active (Wang and Struhl, 2004). In addition, recycling has also been proposed to generate an ‘active’ ligand by sorting ligand to an intracellular endocytic compartment; however, the specific molecular changes are not well defined (Wang and Struhl, 2004). The recycling model assumes that newly translated ligand trafficked to the cell surface fails to activate Notch signaling. Instead activation of Notch requires ligand endocytosis, trafficking through endosomes, ligand activation or processing within the endosomes and recycling back to the cell surface. Moreover, epsin has been shown to bind and target ubiquitylated Delta to a specific endosomal pathway to become activated, and only the active form is recycled back to the plasma membrane. However, to date no one has identified an activated or proteolytically processed form of Notch ligand. Nevertheless, the role for ligand recycling in Notch activation was reinforced by two papers proposing that in sensory organ development, the recycling proteins Sec15 and Rab11 may work in signal sending cells to promote Delta recycling and thereby signaling. Losses in Sec15, which functions with Rab11 in trafficking proteins from the recycling endosome to the plasma membrane,
also produce cell fate transformations indicative of defects in Notch signaling (Emery et al., 2005; Jafar-Nejad et al., 2005; Langevin et al., 2005; Wu et al., 2005); yet, it is still unclear how recycling affects ligand activity and if it is required to generate an active ligand. Interestingly, defects inDll1 recycling in bone marrow stromal cells exhibit Notch-independent phenotypic changes in lymphoid progenitors, suggesting that Notch ligand recycling is required to generate an active ligand (Nichols et al., 2007). However, direct evidence that Notch ligands actually recycle is lacking. Conversely, a few instances suggest that recycling is not the primary role of ligand endocytosis in Notch signaling. Particularly, in the germline or in the developing eye, loss of Rab11 activity was unable to affect Delta signaling (Banks et al., 2011; Windler and Bilder, 2010). Further, Rab5, a prerequisite for entry into the Rab11 recycling pathway, is also not necessary for Delta signaling in the germline or in the developing eye (Banks et al., 2011; Windler and Bilder, 2010). Alternatively, recycling could direct ligand to a specific plasma membrane microdomain or maintain optimal levels of DSL ligand at the plasma membrane. For example, Neur-dependent Delta transcytosis from the basolateral membrane to an apical region of the plasma membrane juxtaposes Delta with Notch on adjacent cells and thus enables it to engage and signal (Benhra et al., 2010; Rajan et al., 2009). Finally, recycling of DSL ligand could generate strong signal sending cells through asymmetric presentation of Neur that stimulates ligand activity by promoting ubiquitin–epsin-dependent endocytosis and trafficking of Notch ligand. In addition, it has been proposed that asymmetric localization of the recycling endosomes could regulate Delta levels or activity. However, the reported accumulation of Dll1 to recycling endosomes could decrease Dll1 at the cell surface, thereby accounting for the losses in
Notch signaling. In this case, the need for Dll1 to pass through the recycling endosome could reflect a mechanism to replenish cell surface ligand.

**Conclusion**

Breast cancer is a heterogeneous and complex disease and great effort is made in understanding the molecular biology and novel crosstalk mechanisms in breast cancer. This will ultimately aid in identification of better prognostic makers to design customized treatment strategies. We have identified a novel crosstalk mechanism between ErbB-2 and Notch pathways, where ErbB-2 is a negative regulator of Notch signaling in ErbB-2 positive breast cancer (Osipo et al., 2008). The evolutionary conserved Notch pathway is used iteratively during development and adulthood to regulate cell fates (Fortini, 2009; Kopan and Ilagan, 2009). Deregulation or loss of Notch signaling is associated with a wide range of human disorders from developmental syndromes to cancer. In human cancers, both Notch ligands and receptors are expressed on cancer cells and Notch acitivity is robustly regulated in a spatio-temporal manner. Thus, activation of Notch in cancer cells can transmit bidirectional signals to cancer cells, stroma, and endothelial cells. Therefore, it is not surprising that Notch signaling can crosstalk with many other oncogenic signaling pathways (Miele, 2006; Rizzo et al., 2008b). Notch and its crosstalk with ErbB-2 plays an important role in ErbB-2 positive breast cancer cell growth, survival, tumor recurrence, and drug resistance (Osipo et al., 2008; Pandya et al., 2011). Thus, significant attention has been given in recent years to understand how Notch activity is modulated in a spatio-temporal manner in presence of other active oncogenic kinases and to develop a clinically useful inhibitor of Notch signaling. A decade ago three seminal papers suggested a potential role for ubiquitylation, endocytosis and
trafficking of ligands as a mechanism to regulate Notch signaling (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). Based on what was known then, what is known now, we intend to extrapolate these findings in the context of ErbB-2 positive breast cancer and try to understand the role of ligand ubiquitylation in ErbB-2 mediated inhibition of Notch signaling.
CHAPTER III

MATERIALS AND METHODS

1. Cell Culture

SKBr3 and BT474 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA). BT474 trastuzumab resistant (BT474 HR) breast cancer cells were generated by treating BT474 trastuzumab sensitive (BT474 HS) cells with 10 μg/mL trastuzumab (Genentech, San Francisco, CA) for 6 months in vitro. SKBr3 cells were maintained in Iscove’s Minimal Essential Media (IMEM, Thermo Fischer Scientific, Waltham, MA) supplemented with 100 μM non-essential amino acid (Invitrogen, Carlsbad, CA), 1% L-glutamine (2 mM, Thermo Fischer Scientific, Waltham, MA) and 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA). BT474 HS and HR cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Scientific, Waltham, MA) supplemented with 100 μM non-essential amino acid, 1% L-glutamine (2 mM) and 10% FBS. Mouse fibroblast LTK-parental and LTK-Jagged-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 μM non-essential amino acid,
1% L-glutamine (2 mM) and 10% FBS. All cells were incubated at 37°C with 95% O₂ and 5% CO₂.

2. Drugs and Chemicals

Gamma-secretase inhibitors

The final proteolytic cleavage in the activation of Notch receptor is catalyzed by the γ-secretase complex (Kopan and Ilagan, 2004, 2009). This final step can be targeted using γ-secretase inhibitors (GSIs). The γ-secretase inhibitor LY 411,575, a reversible competitive inhibitor, was kindly provided by Drs. Abdul Fauq and Todd Golde from the Mayo Clinic in Jacksonville, FL. For xenograft studies, 5 mg/Kg LY 411,575 GSI was used for 3 days on, 4 days off. MRK-003 GSI was kindly provided by Merck Oncology & Co. (Whitehouse Station, NJ). MRK-003 GSI is small molecule inhibitor which binds to the binding pocket of presenilin, the catalytic subunit of the γ-secretase complex. For xenograft studies, 100 mg/Kg MRK-003 GSI dissolved in 0.2% methylcellulose was fed to mice by oral gavage in a volume of 200 µl per mouse for 3 days on, 4 days off.

Trastuzumab

Trastuzumab is a humanized monoclonal antibody targeted against the extracellular portion of ErbB-2 (Carter et al., 1992). Trastuzumab was resuspended in sterile PBS to obtain a stock concentration of 22 mg/mL. For xenograft studies, 10 mg/Kg trastuzumab in a total volume of 100 µl sterile PBS was intraperitoneally injected (i.p) into the mice once a week. For cell culture treatments, the working concentration of 10 or 20 µg/mL was used.
Lapatinib

Lapatinib is a dual tyrosine kinase inhibitor of EGFR and ErbB-2. Lapatinib was purchased from Selleck Chemicals, Houston, TX. For xenograft studies, 100 mg/Kg lapatinib was fed by oral gavage, twice daily for 5 days. For cell culture treatments, lapatinib was dissolved in the solvent dimethylsulfoxide (DMSO) to obtain a stock concentration of 2 mM and was stored at -20°C. The working concentration used for the cell treatment was 2 µM.

N-Ethylmaleimide

N-Ethylmaleimide (NEM) was purchased from Sigma Aldrich (St. Louis, MO). NEM is added fresh to lysis buffer at a concentration of 5 mM and allowed to rock for 20 minutes at 4°C. NEM is an irreversible inhibitor of cysteine proteinases (deubiquitinases) and is used to stabilize and detect ubiquitylation of proteins by western blot analysis.

3. Expression Vectors

pcMV10 and pcMV10-Flag-Ub expression vectors were kindly provided by Dr. Adriano Marchese (Department of Pharmacology, Loyola University Medical Center, Maywood, IL). A wild type Jagged-1 expression plasmid was kindly provided by Dr. Urban Lendahl (Medical Nobel Institute, Karolinska Institute, Stockholm, Sweden).

4. RNA Interference and Transfection Reagents

Jagged-1 siRNA was purchased from Ambion (Grand Island, NY). Mib-1 and DLL-1 ON-TARGETplus SMARTpool siRNA were purchased from Thermo Fischer Scientific (Waltham, MA). Notch-1 and scrambled control siRNAs were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Transfection reagents, Lipofectamine 2000 and Lipofectamine RNAi max, were purchased from Invitrogen (Grand Island, NY).
Protocols were performed according to the manufacturer’s instructions. In a 6-well tissue culture dish, Jagged-1 (5 µL, 100 µM), DLL-1 (5 µL, 10 µM), and Notch-1 (5 µL, 10 µM) siRNA transfections were performed using the Lipofectamine RNAi max reagent. Transfections with Mib-1 (10 µL, 10 µM) siRNA alone or co-transfections with the Flag-Ub (1 µg) plasmid were performed using the Lipofectamine 2000 reagent. The siRNA sequences are shown in Table 2.

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<th>siRNA</th>
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<tr>
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<td>Scrambled control</td>
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Table 2: Sequences of siRNA(s).

5. *Antibodies*

Phosphorylated-AKT1 (Thr308, 244F9, 4056), total AKT1 (C67E7, 4691), phosphorylated-p44/42 ERK1/2 (Thr202/Tyr204, 197G2, 4377), total p44/42 ERK1/2 (137F5, 4695), PTEN (138G6, 9559), Flag (M2, 2368) and Jagged-1 (28H8, 2620) were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA). Notch-1 (C-20, sc-6014-R), Jagged-1 (C-20, sc-6011), Hes-1 (H-140, sc-25392), Ub (P4D1, sc-8017) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Mib-1 (EPR2762(2), ab124929) was purchased from Abcam (Cambridge, MA). Phosphorylated tyrosine-ErbB-2 (Y1248, 06-229) was purchased from Millipore,
Billerica, MA and total ErbB-2 (e2-4001 + 3B5, AB-17) from Thermo Fisher Scientific, Waltham, MA. PKCα (Y143, 1608-1) was purchased from Epitomics, Burlingame, CA. Beta-Actin (AC-15, A5441) was purchased from Sigma Aldrich (St. Louis, MO). Beta-Actin was used as an endogenous control for all Western blots. Secondary antibodies include donkey anti-mouse IgG-HRP (1:3000, sc-2314), donkey anti-rabbit IgG-HRP (1:2000, sc-2313), and donkey anti-goat IgG-HRP (1:2000, sc-2020) and were purchased from Santa cruz Biotechnologies (Santa cruz, CA).

6. Western Blot Analysis

Cultured cells

SKBr3 cells were plated in either a 6-well (5x10^5) or a 10 cm^2 (5x10^6) tissue culture treated dish. Twenty-four hours later, the cells were transfected with siRNA or plasmid DNA, treated with 2 µM lapatinib or 10-20 µg/mL trastuzumab and maintained at 37°C with 95% O_2 and 5% CO_2. After 48 hours, medium was aspirated while on ice and the cells were washed once with ice-cold PBS. Cells were then lysed using 300 µL per well or 500 µL per 10 cm^2 dish Triton X-100 lysis buffer (50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM o-vanadate, 10 mM NaF, 1 protease cocktail tablet), scraped, and collected in labeled eppendorf tubes. The collected samples were allowed to incubate on ice for 10 minutes. Following incubation, the lysates were sonicated 10 seconds using the Sonic Dismembrator (Model 100, Thermo Fischer Scientific, Waltham, MA). Lysates were then centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was isolated and used to determine protein concentration using the
BCA protein assay (Thermo Fisher Scientific, Waltham, MA). The plate was incubated for 30 minutes at 37°C and read on a 96-well plate FPR fluorescent plate reader. Samples from protein lysates were made at 10 – 30 µg in 2x Laemmlli buffer (161-0737, BioRad, Hercules, CA) and beta-mercaptoethanol (BP-176-100, Thermo Fischer Scientific, Waltham, MA). Samples were then heated at 95°C for 5 minutes. Based on the molecular weight and manufacturer’s instructions, detection of each protein by Western blotting was performed as shown in Table 3. The denatured proteins were loaded into each lanes of a SDS-PAGE gel. The gel was run at 150V for 1 hour for Tris acetate gel or 200V for 1 hour for Bis-Tris gel. The separated proteins were transferred to PVDF membranes at 40V for 2.5 hours in 1x Transfer Buffer (NuPAGE® 20x Transfer buffer diluted to 1x in deionized water, 20% methanol) purchased from Invitrogen (Grand Island, NY). Following transfer, the PVDF membrane was re-wetted briefly in methanol followed by deionized water. The membrane was then blocked with 5% non-fat milk (Bio-Rad, Hercules, CA) in TBST buffer (5 mM Tris-HCl, 5 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, and 0.2% NP-40 at pH 8.0) or 5% Roche blocking reagent (Indianapolis, IN) in 1X TBS for one hour at room temperature while constantly shaking. Primary antibodies were added for overnight incubation at 4°C with constant shaking. The following day, blots were washed three times in TBST at intervals of 10 minutes at room temperature while shaking rigorously. Secondary antibodies conjugated to horseradish peroxidase were added to 5% non-fat milk in TBST or 5% Roche blocking reagent in 1X TBS for one hour at room temperature. Blots were then washed again three times in TBST at intervals of 10 minutes at room temperature. Proteins were detected with the enhanced chemiluminescence (ECL) Western blotting substrate (Pierce,
Rockford, IL) or SuperSignal® West Dura (Thermo Fischer Scientific, Waltham, MA) added in 1:1 volume, incubated for 1 or 5 minutes, respectively, at room temperature while rocking, and stained bands were visualized by exposing the membrane to X-ray film and developing in the dark room.

To re-probe Western blots, the membranes were rinsed briefly with TBST at room temperature. Restore Plus Western Blot Stripping buffer (Thermo Fischer Scientific, Waltham, MA) was added to cover the membrane and incubated for 15 minutes at room temperature while rocking. The blots were then subjected to three quick rinses with deionized water, and then washed in TBST 3 times at intervals of 10 minutes at room temperature. Membranes were then blocked for 1 hour and Western blotting would continue as described previously.

**Frozen Tumor Samples**

Frozen tumor samples from each treatment group were homogenized by grinding in liquid nitrogen and lysed in lysis buffer (50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 μg/ml PMSF, 10 μg/ml TLCK, 10 μg/ml TPCK, 1 mM o-vanadate, 10 mM NaF). The tumor extract was sonicated, followed by centrifugation at 10,000 x g for 5 minutes. The supernatant was collected and protein concentration was measured using the BCA protein assay. Tumor lysates were then subjected to SDS-PAGE and Western blotting was performed using the following antibodies: phosphorylated tyrosine (Y1248)-ErbB-2, total ErbB-2, phosphorylated-ERK1/2, total ERK1/2, phosphorylated-AKT1, total AKT1, PTEN, and β-actin as a loading control. Anti-mouse or anti-rabbit secondary antibodies
conjugated to horseradish peroxidase were used to detect the primary antibody. The blot was then developed using SuperSignal® West Dura and the stained bands were visualized using the Fujifilm LAS-3000 imager.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Gel</th>
<th>Blocking Reagent</th>
<th>Primary Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jag-1</td>
<td>7% Tris acetate</td>
<td>5% Roche reagent containing casein</td>
<td>1:500</td>
</tr>
<tr>
<td>Mib-1</td>
<td>7% Tris acetate</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>Hes-1</td>
<td>10% Bis-Tris</td>
<td>5% Roche reagent containing casein</td>
<td>1:500</td>
</tr>
<tr>
<td>Flag</td>
<td>7% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>Ub</td>
<td>7% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>PKCα</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>Pan-Tyrosine</td>
<td>7% Tris acetate</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>7% Tris acetate</td>
<td>5% milk</td>
<td>1:500</td>
</tr>
<tr>
<td>Phosphorylated Tyr1248-ErbB-2</td>
<td>7% Tris acetate</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-AKT</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>AKT</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>PTEN</td>
<td>4-12% Bis-Tris</td>
<td>5% milk</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td>5% milk</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 3: Western Blotting Specifications

7. **Co-Immunoprecipitation (Co-IP)**

SKBr3 cells (5x10⁶) were plated in 10 cm² cell culture dishes for 24 hours. The cells were transfected with 6 μg of Flag-Ub and 60 μL of 10 μM solution of either scrambled control siRNA (SCBi) or Mib-1 siRNA (Mib-1i) for 48 hours at 37°C with 95% O₂ and 5% CO₂. Sixty microliters of Lipofectamine 2000 transfection reagent was used per plate. The total volume in the plates was 11 mL (9 mL media and 2 mL Flag-Ub – siRNA
– Lipofectamine 2000 mix). Before the completion of 48 hours incubation, cells were treated with 2 µM solution of lapatinib for 30 minutes. Media was aspirated while on ice and the cells were washed once with ice-cold PBS. Cells were then lysed using 500 µL per plate Triton X-100 lysis buffer (50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM o-vanadate, 10 mM NaF, 1 protease cocktail tablet), scraped, and collected in respective 1.5 mL eppendorf tubes. The collected samples were allowed to incubate on ice for 10 minutes. Following incubation, the lysates were sonicated using the Sonic Dismembrator (Model 100, Thermo Fischer Scientific, Waltham, MA). The lysates were then centrifuged at 4000 rpm at 4°C for 15 minutes. The lysate concentration was ascertained using the protein BCA protein assay, incubated for 30 minutes at 37°C, and measured using a 96-well plate FPR fluorescent plate reader. Lysates at a concentration of 4 mg/mL were incubated with 4 µg of rabbit anti-human Jagged-1 or rabbit isotype control IgG overnight with gentle rocking at 4°C. Thirty microliters of protein A-plus beads (sc-2002, Santa Cruz, CA) were added to the immune complexes for 2 hours at 4°C while gently rocking. Protein-antibody-bead complexes were washed three times in Triton X-100 buffer. The pellet was resuspended in previously boiled Laemmlil sample buffer supplemented with beta-mercaptoethanol and heated for 5 minutes at 95°C while vigorously shaking. After boiling, the samples were centrifuged at 5000 rpm at room temperature for 5 minutes. Western blotting was used for detection of proteins in co-immunoprecipitated samples and total lysates as described in the Western Blot section of these methods. Antibodies used for detection were: Ub, Mib-1, PKCa Jagged-1, and β-actin.
8. Reverse Transcription Real-Time Polymerase Chain Reaction (RT-PCR)

Cultured cells

SKBr3 (5x10^5) cells were plated in a 6-well tissue culture treated dish. Twenty-four hours later, the cells were transfected using appropriate reagents 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 RiboPure Kit (Ambion, Grand Island, NY). Total RNA recovered was then quantified using the NanoDrop Spectrophotometer (Thermo Fischer Scientific, Waltham, MA). The cDNA was reverse transcribed (RT) from 1 μg total RNA 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). The parameters were as follows: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, 60 minutes at 25°C, and held at 4°C until use. Real-time PCR was performed using iTaq™ SYBR® Green Supermix with ROX (BioRad, Hercules, CA). In a 96-optical PCR plate, 2.5 μL of cDNA was added to 22.5 μL of mastermix (2X Syber green Universal Master Mix, and 50 μM forward and reverse primers). The quantitative PCR parameters were as follows: the initial denature temperature was for 10 minutes at 95°C; PCR cycling for 40 cycles was carried out for 10 seconds at 95°C, and for 45 seconds at 60°C. A melt curve was added after completion of the 40 cycles set by the StepOnePlus thermocycler manufacturer (Applied Biosystems, Foster City, CA). Cycle number at
threshold (Ct) values was subsequently determined. For analysis, first, the Ct value were normalized to human ribosomal protein L 13A (hRPL13A) or 18s, an endogenous control. The values obtained from this normalization were 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.

**Frozen Tumor Samples**

Frozen tumor samples from each treatment group were homogenized by grinding in liquid nitrogen and total RNA was extracted, reverse transcribed to total cDNA, and real-time PCR was performed as previously described. The PCR primers that were used for detection of human specific transcripts are shown in Table 4.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jagged-1</td>
<td>5’-GACTCATCAGCGTGTTCTCA-3’</td>
<td>5’-TGGGAACAATCACTCACTCAA-3’</td>
</tr>
<tr>
<td>Notch-1</td>
<td>5’-GTCAACGGCGCTAGATGACC-3’</td>
<td>5’-TTTAGCCCGTTCTTTCAG-3’</td>
</tr>
<tr>
<td>Deltex-1</td>
<td>5’-CGTTTCCAGGCCAGACACAG-3’</td>
<td>5’-GCAGATCCATATCCTAGGC-3’</td>
</tr>
<tr>
<td>Hes-1</td>
<td>5’-CAGATTTTCTCGAATGACA-3’</td>
<td>5’-CAATGTCTGGGCATGCA-3’</td>
</tr>
<tr>
<td>Hey-1</td>
<td>5’-AGCTTCCTCGGACAGCGAGCTG-3’</td>
<td>5’-TACCAGCCTTCTAGCTGACA-3’</td>
</tr>
<tr>
<td>hRPL13A</td>
<td>5’-CATAGGAAAGCTGGAGGCAA-3’</td>
<td>5’-ACAAGATAGGGCCCTCAAT-3’</td>
</tr>
<tr>
<td>18s</td>
<td>5’-ATGAACCAGGTTATGACCTGA-3’</td>
<td>5’-CATGTGGAAGAGCTGAGTCA-3’</td>
</tr>
</tbody>
</table>

Table 4: Sequences of PCR primers

9. **Cell Cycle Analysis**

SKBr3 (3x10⁵), BT474 HS (3X10⁵), and BT474 HR (3X10⁵) cells were seeded in a 6-well plate. After 24 hours, the cells were transfected with 5 µL of 100 µM solution of scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) using 5 µL of Lipofectamine RNAi max transfection reagent. Six hours after transfection, the cells
were treated with PBS or 10 µg/ml trastuzumab for 48 hours. After 48 hours, the media was collected in a tube and mixed with the trypinized cells. Cells and media were centrifuged at 4°C, 1000 rpm for 5 minutes. The cell pellet was resuspended in 2 mL ice-cold PBS. From the 2 mL single cell suspension, cells were counted and 1x10⁶ cells were placed into FACS tubes. Cells were pelleted at 4°C, 1200 rpm for 5 minutes. The supernatant was removed by inverting and decanting and the pellet was washed by adding 2 mL of ice cold 5% bovine calf serum (BCS) made in PBS. Cells were again centrifuged at 4°C, 1200 rpm for 5 minutes and the supernatant was removed by decanting. Cells can be now fixed for later use by gently resuspending cells in 1 mL of 70% ethanol. The tubes can be stored at 4°C for a week after this step. However, the cells were resuspended in 100 µL of ice cold 5% BCS to which 600 µL of ice cold 100% ethanol was added slowly. The tubes were incubated on ice for 30 minutes and then washed twice by adding 2 mL of ice cold 5% BCS. Cells were then centrifuged at 4°C, 1200 rpm for 5 minutes and resuspended in 250 µL of 10 µg/mL RNase A. They were incubated at 37°C for 15 minutes followed by incubation at room temperature for 5 minutes. To the 250 µL volume, 250 µL of 100 µg/mL Propidium Iodide (P4170, Sigma Aldrich, St. Louis, MO) was added, gently mixed, covered with foil and incubated at room temperature for at least one hour in a dark area. The samples were measured by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA).

10. Annexin-V Apoptosis Assay

SKBr3 (3x10⁵), BT474 HS (3X10⁵), and BT474 HR (3X10⁵) cells were seeded in a 6-well plate. After 24 hours, the cells were transfected with 5 µL of 100 µM solution of scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) using 5 µL of
Lipofectamine RNAi max transfection reagent. Six hours after transfection, the cells were treated with PBS or 10 μg/ml trastuzumab for 48 hours. After 48 hours, the media was collected in a tube and mixed with the cells that were trypsinized, washed twice with ice-cold PBS, and resuspended in a 1X Annexin-V binding buffer (556454, BD biosciences, San Jose, CA). From that suspension, pipette 100 μL of the solution and place it in a FACS tube. To the tube, 5 μL of 1 μg/mL FITC-Annexin-V stain (556420, BD Biosciences, San Jose, CA) and 5 μL of 50 μg/mL propidium iodide (P4170, Sigma Aldrich, St. Louis, MO) were added, covered with aluminium foil and incubated for 15 minutes at room temperature in a dark area. 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, San Jose, CA). The following control tubes were used for compensation and background normalization: 1) a tube containing 100 μL of cells alone, 2) a tube containing 100 μL of cells plus FITC-conjugated Annexin-V antibody, 3) a tube containing 100 μL of cells with Propidium Iodide, and 4) a tube containing 100 μL cells, FITC-conjugated Annexin-V antibody, and Propidium Iodide.

11. Co-culture Assay

Figure 6 shows the schematics of the co-culture technique. SKBr3 cells were plated at a density of 3x10^6 cells per 10 cm^2 plate. The next day, cells were transfected with 30 μL of 100 μM scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) using 30 μL Lipofectamine RNAi max. Thirty hours after transfection, mouse fibroblast (LTK) cells expressing no ligand or rat Jagged-1 were added in equal parts (1:1) to plated, siRNA
transfected SKBr3 cells. The cells were treated with PBS or 20 µg/ml trastuzumab at the same time as adding the mouse fibroblast cells for 18 hours. After 48 hours of incubation, cells were washed with ice cold PBS, trypsinsed, and centrifuged at 4°C, 1200 rpm for 5 minutes. Decant the supernatant and wash the cells with 1 mL of 1:50 diluted sterile filtered Fetal Bovine Serum (FBS). The cells were centrifuged again at 4°C, 1200 rpm for 5 minutes. Then, 10 µL of PE-conjugated human-ErbB-2 antibody (340552, Becton Dickinson, Franklin Lakes, NJ) was added to the pellet, incubated at 4°C for 30 minutes, and washed twice with 1 mL of 1:50 diluted FBS. Samples were then sorted for ErbB-2 expression at the cell surface by FACS. Sorted samples were centrifuged at 4°C, 1200 rpm for 5 minutes and total RNA was extracted, reverse transcribed to total cDNA, and real-time PCR was performed as previously described in the reverse transcription real-time polymerase chain reaction – Cultured cells section of these methods. The PCR primers that were used for detection of specific transcripts are Hes-1, Deltex-1, and hRPL13A and their sequences are shown in Table 4.
SKBr3 cells were plated at a density of 3.5x 10^6 in 10 cm^2 plates. The following day, SKBr3 cells were transfected with 5 µL of either scrambled siRNA (SCBi) or Jagged-1 siRNA. After 30 hours, the cells were co-cultured with mouse fibroblast LTK cells expressing either no ligand (LTK-Parental) or ligand, Jag-1 (LTK-Jag-1). Concurrently, the co-culture was treated with PBS or trastuzumab (20 µg/mL). Eiteen hours later, the cells were trypsinized and stained using PE-conjugated human ErB-2 antibody. ErbB-2 positive cells were sorted by FACS and relative mRNA levels of Hes-1 and Deltex-1 were measured using Real-time PCR. Human RPL13A (hRPL13A) was measured as a loading control and used for normalization.
12. Development of BT474 Trastuzumab Sensitive, Trastuzumab Resistant, and Lapatinib Sensitive Xenografts

BT474 HS, BT474 HR and BT474 LS (sensitive to lapatinib) breast cancer cells were used to generate breast tumor xenografts. Five million cells were injected bilaterally into mammary fat pads of ovariectomized, FoxN1\(^{nu/nu}\) athymic nude mice (Harlan Sprague-Dawley, Madison, WI) followed by implantation of a 17β-estradiol-containing silastic capsule of 0.3cm in length with a constant release providing 83-100 pg/mL as described previously (O'Regan et al., 1998). The identity of each mouse and tumor was tracked by their ear tag. Once tumors grew to a mean cross sectional area (CRA) of 0.30-0.50 cm\(^2\), mice were sacrificed; tumors were extracted, and re-transplanted into a set of 56 mice as previously described (Osipo et al., 2005). Tumors were allowed to grow to a mean CRA of 0.20-0.30 cm\(^2\) and mice were randomized to four treatment groups with each group containing 14 mice: Vehicle control (0.2% methylcellulose in sterile PBS), trastuzumab (10 mg/Kg in a total volume of 100 µl sterile PBS, i.p. once a week) or lapatinib (100 mg/Kg; fed by oral gavage, twice daily for 5 days), LY 411,575 GSI(5 mg/Kg; 3 days on, 4 days off) or MRK-003(100 mg/Kg dissolved in 0.2% methylcellulose, 200 µl fed by oral gavage, 3 days on, 4 days off), or trastuzumab plus MRK-003 or LY 411,575 GSI or lapatinib plus MRK-003 GSI. Tumor area (l x w) was measured weekly using Vernier calipers and cross sectional area \([(l \times w)\pi]/4\) was calculated and graphed. Tumor recurrence was monitored after treatments with trastuzumab or trastuzumab plus GSI were stopped. Lack of tumor recurrence was confirmed by the Vevo 770 Ultrasound.
Imaging system. Tumor recurrence was monitored for approximately 100 days post treatment. Protocols that were used to study breast tumor xenografts in mice were approved by Loyola University’s Institutional Animal Care and Use Committee.

13. Immunohistochemistry

Tumor sections (4 µm) were sliced from formalin-fixed, paraffin-embedded tumor samples, incubated in 60°C oven for 15 minutes, and cooled down to room temperature. Tumor sections were then de-paraffinized and re-hydrated as follows: two washes of 5 minutes each with xylene, two washes of 4 minutes each with 100%, 95%, 80%, and 50% ethanol, and finally with deionized water for 4 minutes. De-paraffinizing and rehydration step, the slides containing tumor sections were put in coplin jars filled with 1x reveal buffer to unmask the epitope using a decloaker. After cooling the coplin jars, rinse the tumor sections 5-6 times with distilled water, once with PBS for 15 minutes, 3% hydrogen peroxide for 15 minutes, and again 3 times with PBS for 5 minutes each. Following washes incubate the tumor sections with 10% normal horse serum for 15 minutes. Tumor sections were stained by overnight incubation with an antibody against the proliferation marker, Ki67 (1:100, DAKO, Carpinteria, CA), for all four treatment groups. After 24 hours, biotinylated anti-mouse secondary antibody (VECTASTAIN Elite ABC Kit, Vector laboratories, Burlingame, CA) was applied to the slides to detect the primary antibody, followed by incubation with the avidin-horseradish peroxidase complex reagent (VECTASTAIN Elite ABC kit, Vector laboratories, Burlingame, CA). The three immunohistochemical staining steps described above were each followed by three 5 minutes washes with PBS. The staining was developed in the diaminobenzidine
chromogen substrate solution (Peroxidase Substrate Kit, Vector laboratories, Burlingame, CA). Mayer’s haematoxylin was used as a counter-stain. Immunohistochemical analysis of Ki67 was performed using at least 3-5 tumors per treatment group. The number of Ki67 positive cells as shown on the Y-axis was determined by taking an average number of Ki67 positive cells from 60 high power fields at 40x magnification per treatment group. H & E was performed to ensure the presence of tumor cells in the tumor sections.

14. TUNEL Assay

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) assay with the TMR detection kit (Roche Diagnostic, Indianapolis, IN) was used to detect 3’OH-associated DNA fragmentation resulting from apoptosis. Paraffin-embedded BT474 trastuzumab and lapatinib sensitive tumors extracted from xenografts were deparaffinized as follows: two times, 10 minutes each with citrisolve (Thermo Fischer Scientific, Waltham, MA); two times, 10 minutes each with 100% ethanol; two times, 5 minutes each with 95% ethanol; 5 minutes with 70% ethanol; 5 minutes with 50% ethanol, and 5 minutes with PBS at 25°C. Tumor sections were then permeabilized using 0.1% Triton X-100 and 0.1% sodium citrate prepared fresh for 8 minutes each at 25°C, followed by 5 washes with PBS at 5 minutes each. TUNEL mix was prepared by mixing the enzyme and label solution according to the manufacturer’s instructions and the tumor sections for each treatment group were incubated with the prepared TUNEL mix for 60 minutes at 37°C. After incubation, the slides were washed three times with PBS for 5 minutes each. One drop of vectashield mounting medium containing Dapi (H-1200, Vector laboratories, Burlingame, CA) is added to TUNEL stained tumor sections in
order to stain the nucleus. The TUNEL assay was performed using 3-5 tumors from each treatment group. The number of TUNEL positive cells shown on the Y-axis was the average number of TUNEL positive cells counted per 20 high power fields (HPF) per tumor for a total of 60 HPFs at 40x magnification per treatment group.

15. Statistical Analysis

Karyn Richlyk, a statistician at Loyola University Medical Center assisted in performing Power Analysis in order to determine the number of mice needed per treatment group for the in vivo study. Based on experience, we hypothesized the following average tumor size for the 4 groups in trastuzumab or lapatinib sensitive xenograft studies at the end of the experiment (all measurements are in cross-sectional area = cm$^2$): 1. Vehicle = 2.0 (SD = 0.3); 2. Trastuzumab or Lapatinib = 0.4 (SD = 0.1); 3. GSI = 1.5 (SD = 0.1); and 4. GSI + Trastuzumab or Lapatinib < 0.1 (SD = 0.01). For the trastuzumab resistant xenograft study, the average tumor size for Vehicle, GSI, and GSI + Trastuzumab should remain the same as above. However, since these are trastuzumab resistant tumors we would expect the average tumor size for the trastuzumab group as 1.5 cm$^2$. Calculations were conducted using PASS 2002 software (Kaysville, Utah, 2002). In a one-way ANOVA, sample sizes of 7, 7, 7, and 7, were obtained for the 4 groups whose means are to be compared, assuming 100% tumor take. The total sample of 28 mice achieves 95% power to detect differences among the means versus the alternative of equal means using an F test at a significance level of 0.05. The common standard deviation within a group is assumed to be between 1 and 0.01. However, experience suggests that tumor take will be 50-70%; therefore, in order to maximize the
likelihood that 7 subjects per group will present with tumors, we must assume that a sample of 7 represents 50-70% from a group of 14 mice, for a total of 56 mice per experiment of four groups. Each mouse was identifiable with a numbered-tag. Each tumor area on the left flank and right flank of the mouse was measured weekly with Vernier calipers. At the end of the study, tumor CRA was calculated and linear regression analysis was performed to determine the slope of the line for determination of the rate of growth for each tumor. Slopes of lines were used only if the correlation coefficients were greater than or equal to 0.85. A one-way ANOVA with Bonferroni correction for multiple comparisons and alpha = 0.05 was used to test statistical significance between groups for tumor growth rates, mRNA expression levels, and IHC assays. A non-paired Student’s T-test was used to test statistical significance between two groups.
CHAPTER IV

HYPOTHESIS AND SPECIFIC AIMS

The *ErbB-2* gene is amplified and the resulting protein product overexpressed in 15-30% of breast tumors, and associated with aggressive behavior and poor overall survival. Trastuzumab, a humanized monoclonal antibody is directed against the extracellular domain of ErbB-2. Unfortunately, trastuzumab resistance remains a major problem in metastatic breast cancer. Our data suggested that gene amplification or overexpression of ErbB-2 inhibits Notch-1 transcriptional activity and inhibition of ErbB-2 activity with trastuzumab or a dual EGFR/ErbB-2 TKI such as lapatinib increased Notch-1 transcriptional activity (Osipo et al., 2008). Furthermore, Notch-1 is a novel target for the treatment of trastuzumab resistant ErbB-2 positive breast cancer *in vitro* (Osipo et al., 2008). The Notch-1 receptor is another potent breast oncogene (Stylianou et al., 2006) that is overexpressed with its ligand Jagged-1 in breast cancers with the poorest overall survival (Reedijk et al., 2005). Notch signaling is thought to be necessary for breast tumor initiating cells (Grudzien et al., 2010; Harrison et al., 2010; Pannuti et al., 2010). We showed that ErbB-2 inhibition activates Notch-1 which results in a compensatory increase in Notch-1-mediated proliferation. However, we do not yet know the mechanism by which ErbB-2 overexpression suppresses Notch-1 activity and whether inhibition of
Notch-1 would reverse resistance to trastuzumab *in vivo*. Preliminary results demonstrated that trastuzumab or lapatinib treatment of SKBr3 cells increased the cell surface protein expression of Jagged-1 by flow cytometry (Figure 7A). Cell surface biotinylation assays showed that Jagged-1 was enriched on the cell surface in response to trastuzumab treatment (Figure 7B). Moreover, confocal studies indicated that Jagged-1 exited early endosomal antigen-1 (EEA-1) positive vesicles when SKBr3 cells were treated with trastuzumab (Figure 8). Taken together, these results suggest the following hypothesis (Figure 9):

**ErbB-2 inhibits Notch-1 by limiting the cell surface availability of Jagged-1.**

**Dual inhibition of Notch and ErbB-2 signaling will result in prevention and/or reversal of trastuzumab resistance *in vivo*.**

We will address this hypothesis with the following specific aims:

**Specific Aim 1: Determine the role of ErbB-2 in Jagged-1-mediated regulation of Notch activity.**

**Specific Aim 2: Establish the therapeutic efficacy of targeting Jagged-1 *in vitro* or Notch signaling *in vivo* in ErbB-2 positive breast cancer.**

Our studies will elucidate the mechanism by which ErbB-2 and Notch pathways crosstalk in ErbB-2 positive breast cancer cells. ErbB-2 overexpressing breast cancer cells exhibit low Notch-1 activity, which is then reversed when ErbB-2 activity is inhibited. However, we do not know yet the molecular mechanism by which ErbB-2 inhibits Notch activity.
Mechanisms underlying trans-activation and cis-inhibition of Notch by its ligand even though not well characterized yet are critical processes regulating Notch activity. We will demonstrate for the first time that Jagged-1 has a dual role in regulation of Notch activity in ErbB-2 positive breast cancer: Jagged-1 inhibits Notch in cis and activates Notch in trans. More interestingly, ErbB-2 prevents Jagged-1-mediated trans-activation of Notch signaling by limiting the association of Jagged-1 and Mib-1 and subsequent ubiquitylation of Jagged-1, a critical step in generating the pulling force required for Notch activation. Moreover, Mib-1 is the E3 ubiquitin ligase required for lapatinib-mediated ubiquitylation of Jagged-1 and induction of Notch activity. Additionally, ErbB-2 promotes an association between Jagged-1 and PKCa. Further, PKCa inhibits Notch transcriptional activity. These findings will provide a mechanism and functional relevance of Jagged-1–Notch interactions in ErbB-2 positive breast cancer cells. These studies will provide a mechanistic basis for the observation that activated Notch-1 promotes a survival advantage in response to ErbB-2 targeted therapies and contributes to resistance. Furthermore, these studies will identify Jagged-1 as a novel and better therapeutic target for the treatment of ErbB-2 positive breast cancer. Finally, these studies will provide a preclinical proof of concept for future clinical trials using combination of trastuzumab or lapatinib and a Notch pathway inhibitor (GSI or Jagged-1 targeted therapy) for the treatment of ErbB-2 positive breast cancer.
Figure 7: Cell Surface Localization of Jag-1, Notch-1, and ErbB-2.

(A.) SKBr3 breast cancer cells were plated at a density of 25x10⁴ cells per well in a 6-well plate. The following day, the adherent cells were treated with increasing concentrations of trastuzumab (0 to 20 μg/mL; upper panel) or lapatinib (0 to 5μM; bottom panel). Flow cytometry was performed 24 hours after treatment to specifically measure cell surface protein expression of Jag-1 (Jag-1). The panels showing flow cytometry results are representative of at least three independent experiments. (B.) SKBr3 cells were seeded in 10 cm² plates at a density of 3x10⁵ cells. After 24 hours, SKBr3 cells were treated with either isotype control IgG (20 μg/mL) or trastuzumab (20 μg/mL) for 3 hours. The cell surface proteins were labelled with 2 mM biotinylation reagent and immunoprecipitated using 30 μL Neutravidin Protein. 30 μL of the precipitated protein was analysed by western blot using an antibody for Jag-1, Notch-1, tyrosine phosphorylated ErbB-2, and total ErbB-2. No biotin negative control was included to ensure specificity. The blots shown are representative of three independent experiments.
Figure 8: Co-localization of Jag-1 and EEA-1.

SKBr3 cells were grown on glass slides for 48 hours and treated with 20 µg/mL IgG or 20 µg/mL trastuzumab for 3 hours. At the end of the treatment, cells were rinsed twice in PBS and fixed in cold methanol. Staining for Jagged-1 (Jag-1) was performed using 1 µg/mL of goat anti-human Jag-1 antibody and 20 µg/mL of AlexaFluor 488 labelled anti-goat IgG. Staining for Early Endosomal Antigen-1 (EEA-1) was performed using 1 µg/mL of EEA-1 and 20 µg/mL of AlexaFluor 555 labelled anti-rabbit IgG. Confocal immunofluorescence microscopy was used to detect Jag-1 (green) and EEA-1 (red). The figure is a representative of three similar independent experiments.
Figure 9: Hypothesis of the Investigation

ErbB-2 promotes PKCα and Jagged-1 (Jag-1) association while inhibiting Mib-1 and Jag-1 association. This results in suppression of Jag-1 mediated trans-activation of Notch in ErbB-2 positive breast cancer. Dual inhibition of ErbB-2 and Notch signaling will result in prevention of tumor recurrence and partial reversal of trastuzumab resistance in vivo.
CHAPTER V

RESULTS

SPECIFIC AIM 1

Determine the role of ErbB-2 in Jagged-1-mediated regulation of Notch-1 activity.

The goal of specific aim 1 is to test the following hypotheses:

Aim 1A. ErbB-2 promotes Jagged-1-mediated cis-inhibition of Notch.

Aim 1B. ErbB-2 limits the expression and/or association of Jagged-1 with Mib-1 to inhibit trans-activation of Notch.

Aim 1C. ErbB-2 through PKCa inhibits Jagged-1 – Mib-1 association and Notch activity.

Aim 1A. ErbB-2 Promotes Jagged-1-Mediated Cis-Inhibition of Notch.

Rationale

Our preliminary data demonstrated that trastuzumab or lapatinib treatment of SKBr3 cells increased the cell surface protein expression of Jagged-1 by flow cytometry (Figure 7A). Additionally, cell surface biotinylation assays showed that Jagged-1 and Notch-1 were enriched on the cell surface in response to trastuzumab treatment (Figure 7B). Finally, our data showed that Jagged-1 co-localized with early endosome antigen-1 (EEA-1) to possibly sub-membraneous endosomes in ErbB-2 positive breast cancer cells...
as determined by confocal immunofluorescence (Figure 8). However, Jagged-1 exited EEA-1 positive vesicles and localized to the cell surface in response to trastuzumab (Figure 8). Together, these data suggest that ErbB-2 overexpressing breast cancer cells trap Jagged-1 in sub-membranous compartments such as early endosomes. However, trastuzumab releases Jagged-1 to the cell surface, making Jagged-1 available to promote trans-activation of Notch signaling via ligand-receptor interaction. The goal of aim 1A is to determine if ErbB-2 promotes Jagged-1-mediated cis-inhibition of Notch.

1. Inhibition of ErbB-2 decreased Jagged-1 protein levels.

Our preliminary data from Figure 7A and 7B demonstrated that the cell surface level of Jagged-1 was increased in response to trastuzumab. In order to determine whether this effect was due to an increase in the total Jagged-1 mRNA or protein levels, we asked the question: Does ErbB-2 decreases the total Jagged-1 mRNA or protein levels? To study this question, we first measured the expression of Jagged-1 in SKBr3 cells in the absence or presence of treatment with increasing concentrations of trastuzumab (0, 10, and 20 
µg/mL) or lapatinib (0, 1, and 5 µM) by Real-time PCR and Western blot analysis. The results showed that trastuzumab treatment (10 and 20 µg/mL) had no significant effect on Jagged-1 mRNA expression compared to 0 µg/mL trastuzumab (Figure 10A; left panel). However, 1 and 5 µM lapatinib significantly increased Jagged-1 mRNA expression by 3.5 and 4.5 fold, respectively compared to 0 µM lapatinib (Figure 10A; right panel). In contrast, Jagged-1 and Notch-1™ protein was decreased in response to both trastuzumab and lapatinib (Figure 10B). Therefore, it appeared that ErbB-2 inhibitors decreased Jagged-1 protein levels, despite an up-regulation of Jagged-1 mRNA transcript levels.
This suggested that ErbB-2 may stabilize, rather than inhibit, Jagged-1 protein. To determine whether this decrease in Jagged-1 protein by anti-ErbB-2 inhibitors was reproducible in other breast cancer cells, MCF-7 cells stably expressing either empty vector (MCF-7/Neo), ErbB-2 (MCF-7/HER2), or heregulin-1 (MCF-7/HRG-1) were treated with trastuzumab (20 μg/mL) for 24 hours and Jagged-1, PY-ErbB-2, and ErbB-2 proteins were detected by Western blot. HRG-1 is a growth factor that mediates its effects through ErbB-3 receptor. Therefore, HRG-1 overexpressing cells served as a negative control to observe ErbB-2 specific effects. The results showed that while trastuzumab treatment decreased Jagged-1 protein in MCF-7/HER2 cells, the levels of Jagged-1 remained unchanged in ErbB-2 non-overexpressing MCF-7/Neo and MCF-7/HRG-1 cells (Figure 10C). These results taken together suggest that trastuzumab or lapatinib-mediated decrease in Jagged-1 protein is most likely ErbB-2 specific. Indeed, trastuzumab decreased the tyrosine phosphorylation of ErbB-2 with no change in total ErbB-2 levels (Figure 10C). These data suggest that ErbB-2 signaling could be stabilizing Jagged-1 protein levels, thus providing ample ligand to inhibit Notch in cis and restrict binding of Notch to the ligand expressed on adjacent cells.
Figure 10: Expression of Notch Ligand, Jag-1, in Breast Cancer cells

SKBr3 breast cancer cells were plated at a density of 25x10^4 (A.) or 5x10^5 (B.) and MCF-7 cells at a density of 5x10^5 (C.) cells per well in a 6-well plate. (A.) The following day, the cells were treated with increasing concentrations of trastuzumab (0 to 20 µg/mL; left panel) or lapatinib (0 to 5 µM; right panel). Relative mRNA levels of Jagged-1 (Jag-1) were measured 24 hours after each treatment using Real-time PCR. 18s rRNA was measured as a loading control and used for normalization. Real-time PCR results show means of fold differences between treatments compared to vehicle control plus or minus standard deviations of three independent experiments. *denotes statistically significant differences between lapatinib and vehicle control. Statistical significance was calculated using a two-sided, non-paired Student’s T-test. (B. and C.) After 24 hours, the cells were treated with trastuzumab (10 µg/mL) or lapatinib (2 µM) for 6 hours and total protein levels of Jag-1, Notch-1, PY-ErbB-2, ErbB-2, were measured by Western blotting. 30 µg of total protein lysate was loaded onto each lane of gel as described in the materials and methods. β-actin was measured as an endogenous control.
2. **Jagged-1 inhibits Notch activity via cis-inhibition.**

Notch is regulated in at least two ways by Notch ligands: 1. trans-activation of Notch by neighboring signal sending cells (Figure 11; top panel), and cis-inhibition of Notch within its own cell (Figure 11; bottom panel). This level of tightly controlled activation is critical for cell fate determination during development and differentiation of adult stem cells. However, little is known about how Notch activation is regulated in breast cancer. If ligand and receptor are expressed on the same cell, then the ligand-receptor interaction results in cis-inhibition of Notch activity in that same cell. If ligand and receptor are expressed on adjacent cells, then the ligand-receptor interaction results in trans-activation of Notch signaling in the Notch receptor expressing cell. As shown in figures 7, 8, and 10, ErbB-2 hyperactivity resulted in an increase in total Jagged-1 protein that also coincided with enhanced co-localization of Jagged-1 and EEA-1 and a decrease in cell surface Jagged-1 expression and Notch activity. To further investigate the role of Jagged-1 on Notch activity, we asked the question: Can Jagged-1 mediate cis-inhibition of Notch in ErbB-2 overexpressing breast cancer cells? To address this question, we used a co-culture technique which has been used widely to understand cis and trans functions of Notch ligands and Notch receptors. Figure 12 demonstrates Jagged-1 protein levels in LTK-Parental and LTK-Jagged-1 cells and down-regulation of Jagged-1 protein by RNA interference in SKBr3 cells.
Figure 11: Trans-Activation vs Cis-Inhibition of Notch via its Ligand Jag-1.

Notch ligand and receptor expressed on the adjacent cells results in trans-activation of Notch signaling in signal receiving cell (Top panel). Whereas, Notch ligand and receptor expressed on the same cell results in cis-inhibition of Notch signaling in signal sending cell (Bottom panel).
Figure 12: Expression of Jag-1 in LTK-P, LTK-Jag-1, and SKBr3 cells.

LTK-Parental (LTK-P) and LTK-Jagged-1 (LTK-Jag-1) fibroblast cells were harvested and protein levels of Jagged-1 (Jag-1) were detected by Western blot analysis (left panel). SKBr3 cells were transfected with 5 µL of either scrambled control siRNA (SCBi) or Jag-1 siRNA (Jag-1i). Protein levels of Jag-1 were detected by Western blot analysis after 48 hours (right panel). β-actin was used as an endogenous control.
To examine if Jagged-1 is cis-inhibiting Notch, we co-cultured mouse fibroblast cells expressing no ligand (LTK-Parental) with SKBr3 cells transfected with a scrambled siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) (Figure 13A). Knockdown of Jagged-1 by Jag-1i up-regulated mRNA levels of canonical Notch target genes, Hes-1 by 4-fold and Deltex-1 by 13-fold compared to SCBi suggesting that Jagged-1 may be inhibiting Notch activation through a cis mechanism in ErbB-2 positive breast cancer cells (Figure 14). Nevertheless, the up-regulation of Hes-1 and Deltex-1 mRNA may be in part due to compensatory up-regulation of other Notch ligands upon Jagged-1 down-regulation.

3. **Jagged-1 restores Notch activity via trans-activation.**

Notch ligands including Jagged-1 have the ability to activate Notch in trans. Therefore, we examined what happens to Notch activity when we force trans-activation in the co-culture experiment. SKBr3 cells transfected with SCBi or Jag-1i were co-cultured with mouse fibroblast cells overexpressing Jagged-1 (LTK-Jag-1). Notch activity was measured in SKBr3 cells after sorting for ErbB-2 positive breast cancer cells (Figure 13B) and performing Real-time PCR to measure Hes-1 and Deltex1 transcripts. Providing SKBr3 cells with abundant Jagged-1 in trans demonstrated an increase in mRNA levels of Hes-1 by 3-fold (Figure 14A) but had no change in Deltex-1 mRNA levels (Figure 14B). Moreover, Jag-1i did not further induce Hes-1 mRNA levels (Figure 14A) but relieved cis-inhibition of Deltex-1 and increased its mRNA levels by 16-fold (Figure 14B). These results indicate that Hes-1 is down-regulated by Jagged-1 mediated cis-inhibition of Notch which can be rescued by providing Jagged-1 in trans, suggesting that Jagged-1 is regulating Hes-1 gene expression.
Figure 13: Schematics of Co-culture Experiment.

(A.) Mouse fibroblast cells expressing no ligand (LTK-Parental or P) were co-cultured with SKBr3 cells transfected with a scrambled siRNA (SCBi) or Jagged-1 siRNA (Jag-1i). (B.) SKBr3 cells transfected with SCBi or Jag-1i were co-cultured with mouse fibroblast cells expressing Jagged-1 (LTK-Jag-1). Notch activity in sorted ErbB-2 positive breast cancer cells was measured using Real-time PCR.
Figure 14: Jag-1 Cis-Inhibits and Trans-Activates Notch.

SKBr3 cells were plated at a density of $3.5 \times 10^6$ in 10 cm$^2$ plates. The following day, SKBr3 cells were transfected with 5 µL of either scrambled siRNA (SCBi) or Jagged-1 siRNA (Jag-1i). After 30 hours, the cells were co-cultured with mouse fibroblast LTK cells expressing either no ligand (LTK-Parental) or Notch ligand, Jagged-1 (LTK-Jag-1). Eiteen hours later, the cells were trypsinized and ErbB-2 positive cells were sorted by FACS and relative mRNA levels of Hes-1 (A.) and Deltex-1 (B.) were measured using Real-time PCR. Human RPL13A (hRPL13A) was measured as a loading control and used for normalization. Real-time PCR results show means of fold changes between Jag-1i/PBS and SCBi/PBS in LTK-Parental and LTK-Jag-1 cells. Statistical significance was calculated using ANOVA for multiple comparisons using Prism Pad. * denotes statistically significant differences between SCBi/PBS treated LTK-Parental vs LTK-Jag-1. ** denotes statistically significant differences between Jag-1i/PBS vs SCBi/PBS in LTK-Parental cells. *** denotes statistically significant differences between Jag-1i/PBS vs SCBi/PBS in LTK-Jag-1 cells.
However, Deltex-1 is strongly cis-inhibited by Jagged-1 which was relieved only with Jag-1i, suggesting that Deltex-1 gene expression is not induced by Jagged-1. Finally, the results so far suggest that Jagged-1 is a cis-inhibitor of Notch activity.


The previous results suggest that Jagged-1 cis-inhibits Notch in ErbB-2 positive breast cancer cells (Figure 14). To address the hypothesis that ErbB-2 inhibits Notch activity by promoting Jagged-1-mediated cis-inhibition of Notch, we treated the co-cultured cells with trastuzumab and asked whether inhibiting ErbB-2 reverses Jagged-1-mediated Notch inhibition. In the presence of LTK-Parental cells, trastuzumab treatment alone induced Notch activity as demonstrated by an increase in mRNA levels of Hes-1 by 4-fold and Deltex-1 by 13-fold compared to SCBi/PBS control. Furthermore, Jagged-1 knockdown had little effect on trastuzumab-mediated increase in Hes-1 or Deltex1 (Figure 15). These results indicate that ErbB-2 is potentially limiting trans-activation of Notch.

To specifically address the question that ErbB-2 is promoting Jagged-1 mediated cis-inhibition, SKBr3 cells were co-cultured with LTK-Jagged-1 cells and treated with trastuzumab. If ErbB-2 is specifically promoting Jagged-1-mediated cis-inhibition of Notch, then inhibiting ErbB-2 should activate Notch targets to the same degree as Jagged-1 knockdown. However, the results do not support that hypothesis.
SKBr3 cells were plated at a density of 3.5x10^6 in 10 cm^2 plates. The following day, SKBr3 cells were transfected with 5 µL of either scrambled siRNA (SCBi) or Jagged-1 siRNA (Jag-1i). After 30 hours, the cells were co-cultured with mouse fibroblast LTK cells expressing either no ligand (LTK-Parental) or Jagged-1 (LTK-Jag-1). Concurrently, the co-culture was treated with PBS or trastuzumab (20 µg/mL). Eighteen hours later, the cells were trypsinized and stained using PE-conjugated human ErbB-2 antibody. ErbB-2 positive cells were sorted by FACS and relative mRNA levels of Hes-1 (A.) and Deltex-1 (B.) was measured using Real-time PCR. Human RPL13A (hRPL13A) was measured as a loading control and used for normalization. Real-time PCR results show means of fold changes between treatments compared to SCBi/PBS control in LTK-Parental and LTK-Jag-1 cells. Statistical significance was calculated using ANOVA for multiple comparisons using Prism Pad. # denotes statistically significant differences between SCBi/Trast vs SCBi/PBS in LTK-Parental and LTK-Jag-1 cells.
Regardless of whether ErbB-2 is inhibited or not, Jagged-1 knock down was sufficient to relieve the cis-inhibitory signal on Hes-1 and more significantly on Deltex-1 mRNA levels by 8-fold when co-cultured with LTK-Jagged-1 cells (Figure 15). These data together imply that Jagged-1 is a potent cis-inhibitor of Notch activity in ErbB-2 positive breast cancer. However, ErbB-2’s role in Jagged-1-mediated cis-inhibition of Notch is not clear. More importantly, the results do support the conclusion that ErbB-2 most probably inhibits Jagged-1–Notch trans-activation.

5. Cis-inhibition of Notch by Jagged-1 might be a general mechanism in breast cancer cells.

Findings from figure 14 showed for the first time that Jagged-1 could be a cis-inhibitor of Notch-1 in ErbB-2 positive breast cancer cells, SKBr3. To determine whether Jagged-1-mediated cis-inhibition of Notch activity is a common mechanism in other subtypes of breast cancer cells. MCF-7 (Luminal A), SKBr3 (ErbB-2+), BT474 HS (Luminal B), BT474 HR (Luminal B), and MDA-MB-231 (triple-negative) cells were lysed and Jagged-1, Notch-1, and its target gene, Hes-1, protein levels were measured by Western blot. MDA-MB-231 cells express the highest Jagged-1 protein levels followed by MCF-7, SKBr3, BT474 HS and then BT474 HR cells (Figure 16). More importantly, the data indicated an inverse correlation between levels of Jagged-1 protein and Notch activity as measured by Hes-1 protein levels. For example, MDA-MB-231, MCF-7, and SKBr3 cells expressing the highest levels of Jagged-1 protein had the lowest Hes-1 protein levels (Figure 16). Whereas, BT474 HS and HR cells expressing low levels of Jagged-1 protein showed the highest Hes-1 protein levels (Figure 16). This result showed
that the inverse correlation between Jagged-1 and Hes-1 was not limited to breast cancer cell lines harboring a gene amplification for ErbB-2 (Figure 16).

Taken together, these results suggest that ErbB-2 inhibits Jagged-1-mediated trans-activation of Notch in SKBr3 cells. However, Jagged-1-mediated cis-inhibition of Notch activity could be ErbB-2 independent.
MCF-7, SKBr3, BT474 HS, BT474 HR and MDA-MB-231 cells were plated and total protein levels of Jagged-1 (Jag-1) was detected by Western blot analysis. 30 µg of total protein lysate was loaded onto each lane of gel as described in the materials and methods. β-actin was measured as an endogenous control.

**Figure 16: Protein Levels of Jag-1 in Breast Cancer Subtypes.**
Aim 1B. ErbB-2 Limits the Expression and/or Association of Jagged-1 with Mib-1 to Inhibit Trans-Activation of Notch.

Our results showed that Jagged-1 and Notch-1 co-localized in ErbB-2 positive breast cancer cells (Figure 17). However, upon trastuzumab treatment, Notch-1 and Jagged-1 were completely separated with Notch-1 localized throughout the cell and Jagged-1 concentrated near cell-cell junctions (Figure 17). Together, these data suggest that ErbB-2 overexpressing breast cancer cells trap both Jagged-1 and Notch-1 in sub-membranous compartments such as early endosomes. However, trastuzumab releases Jagged-1 to the cell surface, making Jagged-1 available for trans-activation of Notch signaling via ligand-receptor interaction and possibly in the correct orientation to pull the N^EC into the ligand expressing cell. Osipo et al and data from Figure 15 demonstrated an increase in Notch activity in response to trastuzumab (Osipo et al., 2008) and Notch could be activated in trans. Two E3 ubiquitin ligases, Mindbomb-1 (Mib-1) and Neuralized-1 (Neur), have been shown to be essential for ligand-mediated trans-activation of Notch as discussed in chapter II. In order for Jagged-1 to be endocytosed, Mib-1 mono-ubiquitylates the C-terminal tail of Jagged-1 which then generates the pulling force necessary for endocytosis of ligand - N^EC into the signal sending cell (De Renzis et al., 2006; Deblandre et al., 2001; Lai et al., 2001; Le Borgne and Schweisguth, 2003a; Pavlopoulos et al., 2001). Knockout studies in vertebrates revealed that, deficiency of Mib-1, but not Neur, abrogates Notch signaling (Barsi et al., 2005; Koo et al., 2005). Thus, the goal of aim 1B is to determine if ErbB-2 by regulating expression and/or association of Mib-1 with Jagged-1 inhibits Notch activity.
SKBr3 cells were grown on glass slides for 48 hours and treated with 20 µg/mL IgG or trastuzumab for 3 hours. At the end of the treatment, cells were rinsed twice in PBS and fixed in cold methanol. Staining for Notch-1 was performed using 1 µg/mL of the rabbit anti-human Notch-1 antibody and 20 µg/mL of AlexaFluor 555 labelled anti-rabbit IgG. Staining for Jagged-1 (Jag-1) was performed using 1 µg/mL of goat anti-human Jag-1 antibody and 20 µg/mL of AlexaFluor 488 labelled anti-goat IgG. Confocal immunofluorescence microscopy was performed to detect Jag-1 (green) and Notch-1 (red). The figure is a representative of three similar independent experiments.
1. **ErbB-2 does not regulate total Mib-1 protein levels.**

If Mib-1 is critical for Jagged-1 mediated trans-activation of Notch-1, then ErbB-2 by decreasing Mib-1 protein levels could be inhibiting Notch. Thus, we asked the question: Does ErbB-2 decrease Mib-1 protein levels? To address this question, SKBr3 cells were treated with trastuzumab or lapatinib and MCF-7/Neo, MCF-7/HER-2, and MCF-7/HRG-1 cells with trastuzumab for 6 hours and Mib-1 protein levels were measured by Western blotting. Our results showed that expression levels of Mib-1 protein was relatively unaffected by ErbB-2 inhibitors (Figure 18). Further, Mib-1 is expressed in breast cancer cells, MCF-7 (Luminal A), SKBr3 (ErbB-2+), BT474 HS (Luminal B), BT474 HR (Luminal B), and MDA-MB-231 (triple-negative) (Figure 19). Therefore, downregulation of Mib-1 is not a mechanism by which ErbB-2 inhibits Notch activation.

2. **ErbB-2 limits the association of Mib-1 with Jagged-1.**

While our data demonstrated that ErbB-2 does not regulate Mib-1 protein, it was important to determine whether ErbB-2 might be limiting the association of Mib-1 with Jagged-1 to prevent endocytosis of Jagged-1. Therefore, we asked the question: Does ErbB-2 restrict the interaction of Mib-1 with Jagged-1? To address this question, Jagged-1 was immunoprecipitated from SKBr3 cells treated with trastuzumab or lapatinib and Western blotting was performed to detect Jagged-1 and Mib-1 proteins. Co-immunoprecipitation demonstrated that Jagged-1 was in complex with Mib-1 upon inhibition of ErbB-2 by either trastuzumab or lapatinib (Figure 20). From these results, we can conclude that ErbB-2 restricts a critical interaction between Jagged-1 and Mib-1.
Figure 18: Expression of E3 Ubiquitin Ligase, Mib-1, in Breast Cancer Cells.

SKBr3 (A.) and MCF-7 (B.) breast cancer cells were plated at a density of 5x10^5 per well of a 6-well plate. (A.) The following day, the cells were treated with trastuzumab (10 µg/mL in A. or 20 µg/mL in B.) or lapatinib (2 µM in A.) for 6 hours and total protein levels of Mib-1 was measured by Western blotting. 30 µg of total protein lysate was loaded onto each lane of gel. β-actin was measured as an endogenous control.
Figure 19: Protein Levels of Mib-1 Among Breast Cancer Subtypes.

MCF-7, SKBr3, BT474 HS, BT474 HR and MDA-MB-231 cells were plated and total protein levels of Mib-1 was detected by Western blot analysis. 30 µg of total protein lysate was loaded onto each lane of gel as described in the materials and methods. β-actin was measured as an endogenous control.
Figure 20: Co-Immunoprecipitation of Endogenous Jag-1.

SKBr3 cells were treated with PBS, trastuzumab (10 µg/mL; trast), DMSO or lapatinib (1 µm; Lap) for 6 hours. Lysates were subjected to immunoprecipitation using Jagged-1 (Jag-1) or its isotype IgG control. Subsequent Western blot analysis was performed and Jag-1 and Mib-1 protein levels were detected. The figure shows three similar independent experiments.
3. **ErbB-2 by limiting an association between Mib-1 and Jagged-1 decreases Jagged-1 ubiquitylation.**

Jagged-1 is a substrate for Mib-1’s ubiquitin ligase activity. To determine if the increased association between Jagged-1 and Mib-1 in response to ErbB-2 inhibitors resulted in an increase in Jagged-1 ubiquitylation, SKBr3 cells were transfected with a Flag-Ub expression construct and treated with DMSO or lapatinib for 30 minutes. A Jagged-1 IP to detect its ubiquitylation was performed. The data showed that Jagged-1 is ubiquitylated upon treatment with lapatinib (Figure 21).

4. **Mib-1 is the E3 ubiquitin ligase that ubiquitylates Jagged-1.**

Because Mib-1 is in complex with Jagged-1 and Jagged-1 is ubiquitylated upon inhibition of ErbB-2, we asked the question: Is Mib-1 the E3 ligase that ubiquitylates Jagged-1? To answer this question, SKBr3 cells were co-transfected with scrambled control siRNA (SCBi) or Mib-1 siRNA (Mib-1i) and the Flag-Ub expression construct for a total of 48 hours. Cells were then treated with DMSO or lapatinib for 30 minutes and Jagged-1 was immunoprecipitated and Western blotting was performed to detect Jagged-1, Ubiquitin, and Mib-1 proteins. Co-immunoprecipitation confirmed that Mib-1 is in complex with Jagged-1 and that Jagged-1 is ubiquitylated in response to lapatinib. More importantly, Mib-1i decreased lapatinib-induced ubiquitylation of Jagged-1 (Figure 22).
SKBr3 cells were treated with DMSO or lapatinib (1 µm; Lap) for 15, 30, or 60 minutes. Lysates were subjected to immunoprecipitation using Jagged-1 (Jag-1) or its isotype IgG control. Subsequent Western blot analysis detected immunoprecipitated Jag-1 and Ub levels. Total Jag-1 and Ub levels in protein lysates were also detected by Western blot analysis. β-actin was measured as an endogenous control. Figure 22A shows two similar independent experiments.

Figure 21: Co-Immunoprecipitation of Endogenous Jag-1 and Associated Ub.
Figure 22: Mib-1 is the E3 Ubiquitin Ligase for Jag-1.

SKBr3 cells were transfected with Flag-Ub and either scrambled siRNA (SCBi) or Mib-1 siRNA (Mib-1i). At the completion of 48 hours, cells were treated with DMSO or lapatinib (1 µm; Lap) for 30 minutes. Lysates were subjected to immunoprecipitation using Jagged-1 (Jag-1) or its isotype IgG control. Subsequent Western blot analysis detected immunoprecipitated Jag-1, Mib-1, and Ub levels. Total Jag-1, Mib-1, Flag levels in protein lysates were also detected by Western blot analysis. β-actin was measured as an endogenous control.
5. *Mib-1 knockdown abrogates lapatinib-induced increase in Notch activity.*

If lapatinib promotes trans-activation of Notch and Mib-1 is critical for Jagged-1 mediated trans-activation of Notch, then Mib-1 should be required for lapatinib induced activation of Notch. Hence, we asked the question: Is Mib-1 necessary for trastuzumab or lapatinib-induced activation of Notch? In order to investigate the role of Mib-1 on Notch activity, the effect of Mib-1 siRNA on the expression of Hes-1 protein levels was measured. SKBr3 cells were transfected with either scrambled control siRNA (SCBi) or Mib-1 siRNA (Mib-1i) and treated with DMSO or lapatinib for 6 hours. As measured by Western blot analysis, Mib-1 protein levels were decreased upon Mib-1 knocked down (Figure 23). Either trastuzumab or lapatinib treatment increased Hes-1 protein (Figure 23). More importantly, Mib-1i decreased the trastuzumab or lapatinib-induced increase in Hes-1 protein (Figure 23), suggesting that Mib-1 is necessary for Notch activation when ErbB-2 is inhibited.

Taken together, these results indicate that ErbB-2 limits Mib-1’s association and subsequent ubiquitylation of Jagged-1 and fails to generate a competent ligand required for trans-activation of Notch.
Figure 23: Mib-1 Abrogates Trast or Lap Mediated Activation of Notch.

SKBr3 cells were transfected with either scrambled siRNA (SCBi) or Mib-1 siRNA (Mib-1i). At the completion of 48 hours, cells were treated with DMSO or lapatinib (1 µm) for 6 hours. Total protein levels of Mib-1 and Hes-1 were detected by Western blot analysis. 30 µg of total protein lysate was loaded onto each lane of gel as described in the materials and methods. β-actin was measured as an endogenous control.
Aim 1C. Identify the Downstream Factors of ErbB-2 Signaling that Regulates Jagged-1 Activity.

ErbB-2 is a receptor tyrosine kinase which when active initiates a series of downstream phosphorylation events that regulate cell proliferation and survival (Figure 2). To determine the mechanism by which ErbB-2 regulates the Jagged-1 and Mib-1 association, the protein sequences of Jagged-1 and Mib-1 were scanned using Prosite and several putative PKCα binding sites (Figure 24) were identified. Moreover, it has been demonstrated that ErbB-2 up-regulates c-Src which in turn activates PKCα (Tan et al., 2006). Additionally, PKCα is necessary for ErbB-2 driven cancer cell invasion (Tan et al., 2006). The goal of this aim 1C is to elucidate whether ErbB-2 through PKCα inhibits Jagged-1 or Mib-1 to restrict the association and Notch activation (Figure 25).

1. ErbB-2 promotes an association between PKCα with Jagged-1.

PKCα is a critical downstream mediator of ErbB-2. In order to determine the mechanism by which ErbB-2 limits Jagged-1 and Mib-1 association, we asked the question: Does ErbB-2 promote an association of PKCα and Jagged-1? SKBr3 cells were treated with DMSO or lapatinib for 30 minutes and Jagged-1 was immunoprecipitated and Western blotting was performed to detect PKCα, Ubiquitin, Mib-1, and Jagged-1 proteins. Co-immunoprecipitation demonstrated that Jagged-1 was in complex with PKCα when ErbB-2 was active (Figure 26). The results confirmed that upon lapatinib treatment, Mib-1 is in complex with Jagged-1 which leads to Jagged-1 ubiquitylation (Figure 26).
Figure 24: Schematic Representation of Jag-1 and Mib-1 Protein.
Figure 25: Hypothesis of Specific Aim 1C.

ErbB-2 up-regulates c-Src which in turn activates PKCα. ErbB-2 limits association of Jagged-1 (Jag-1) and Mib-1 and inhibits Notch activity. Therefore, we hypothesize that ErbB-2 through PKCα inhibits Jag-1 or Mib-1 to restrict their association and Notch activation.
SKBr3 cells were treated with DMSO or lapatinib (1 µm; Lap) for 30 minutes. Lysates were subjected to immunoprecipitation using Jagged-1 (Jag-1) or its isotype IgG control. Subsequent Western blot analysis was performed and Ub, PKCα, Mib-1, and Jag-1 protein levels were detected. Heavy chain IgG was detected as an endogenous loading control for immunoprecipitation. A., B., and C. represent Western blot images from three independent but similar experiments.

Figure 26: ErbB-2 Promotes Association of PKCα and Jag-1.
2. **PKCα is required for inhibition of Notch activity.**

ErbB-2 promotes an association of PKCα with Jagged-1 and PKCα is necessary for ErbB-2 driven malignancies, thus, we asked the question: Is PKCα required for ErbB-2-mediated inhibition of Notch activity? To investigate the role of PKCα on Notch activity, the effect of PKCα siRNA on the expression of canonical Notch target genes such as Hes-1 and Hey-1 was examined. SKBr3 cells were transfected with either scrambled control siRNA (SCBi) or PKCα siRNA (PKCai) for 48 hours. As measured by Real-time PCR and Western blot analysis, PKCα mRNA (Figure 27A) and protein (Figure 27B) levels were decreased upon PKCα knockdown. With the decrease in PKCα transcript and protein, the data showed an increase in transcript (Figure 27A) and protein (Figure 27B) levels of Hes-1, respectively. In addition, another Notch target, Hey-1 protein levels were increased upon PKCα knockdown (Figure 27B).

Taken together, these results indicate that ErbB-2 facilitates a PKCα-Jagged-1 association to limit Mib-1-mediated ubiquitylation of Jagged-1 and subsequent trans-activation of Notch.
SKBr3 cells were transfected with either scrambled siRNA (SCBi) or PKCα siRNA (PKCαi) for 72 hours. (A.) Relative mRNA levels of Hes-1 and PKCα were measured using Real-time PCR. Human RPL13A (hRPL13A) was measured as a loading control and used for normalization. (B.) Protein levels of PKCα, Hes-1, and Hey-1 were detected by Western blot analysis after 48 hours. β-actin was used as an endogenous control.

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**Figure 27: Knockdown of PKCα Activates Notch-1 Target Genes.**
SPECIFIC AIM 2

*Establish the therapeutic efficacy of targeting Jagged-1* in vitro *and Notch signaling in vivo in ErbB-2 positive breast cancer.*

**Rationale**

Notch signaling is critical for cell fate determination (Politi et al., 2004) and ErbB-2 is critical for proliferation and survival (Citri et al., 2003). Notch and ErbB-2 pathways when deregulated independently can lead to breast cancer and poor survival (Slamon et al., 1987; Slamon et al., 1989; Stylianou et al., 2006). We have identified a novel crosstalk between ErbB-2 and the Notch pathway, where ErbB-2 inhibits Notch activity, and trastuzumab increases Notch activity. Moreover, preliminary data from our lab showed that either Notch-1 siRNA or a pan-Notch inhibitor, GSI, enhanced sensitivity to trastuzumab in sensitive cells or reversed trastuzumab resistance *in vitro* (Osipo et al., 2008). In addition, results from specific aim 1 suggested that ErbB-2 mediates its inhibitory effects on Notch activity by limiting Jagged-1 and Mib-1 association. Thus, targeting of Notch receptors by a GSI or Jagged-1 by siRNA could provide a novel therapeutic strategy to enhance the efficacy of anti-ErbB-2 therapy or possibly reverse trastuzumab resistance. The goal of specific aim 2 is to address the following hypotheses:

**Aim 2A. Establish whether targeting Jagged-1 by siRNA increases the efficacy of trastuzumab in SKBr3 and BT474 cells.**
Aim 2B. Establish whether a combination of GSI plus trastuzumab prevents tumor growth.

Aim 2C. Establish whether a GSI in combination with trastuzumab reverses drug resistance.

**Aim 2A. Establish whether targeting Jagged-1 by siRNA increase the efficacy of trastuzumab in inhibiting the growth of SKBr3 and BT474 trastuzumab sensitive cells and restored sensitivity to trastuzumab in BT474 trastuzumab resistant cells.**

To understand the biological significance of the crosstalk between ErbB-2 and Notch signaling on growth of ErbB-2 positive breast cancer cells, we asked the question: Does knockdown of Jagged-1 enhances the growth inhibitory effects of trastuzumab on SKBr3, BT474 HS, and BT474 HR cells? Cell cycle analysis using Propidium Iodide was performed to determine where in the cell cycle the combination of trastuzumab plus a Jagged-1 siRNA inhibits growth of SKBr3 and BT474 cells.

1. **Dual blockade of Jagged-1 and ErbB-2 induces a growth arrest in SKBr3 cells.**

Trastuzumab treatment alone showed a 17% increase in the number of SKBr3 cells in the G1 phase of the cell cycle compared to IgG control. Consequently, trastuzumab decreased the number of cells in S phase by 33% (Figure 28). This result demonstrates that trastuzumab is functioning as expected to inhibit growth of ErbB-2 positive SKBr3 cells. Interestingly, Jagged-1 knockdown alone showed a similar increase in the G1 phase and a decrease in the S phase of the cell cycle compared to trastuzumab (Figure 28).
Figure 28: Trastuzumab plus Jagged-1 siRNA Induces a Growth Arrest.

SKBr3 cells were transfected with scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) alone or were treated with IgG control or Trastuzumab (Trast) for 48 hours. Cell cycle analysis using Propidium Iodide was performed by flow cytometry. Mean percentage of cells in each experiment were plotted. Statistical significance was determined by performing ANOVA analysis. The error bars represent standard deviations of the mean for three independent experiments. * denotes statistically significant differences between trastuzumab alone and trastuzumab plus Jagged-1 siRNA.
However, the combination of Jagged-1 knockdown and trastuzumab treatment resulted in a 29% increase in the G1 phase and 50% reduction in the S phase of the cell cycle compared to trastuzumab alone (Figure 28). These results indicate that both Jagged-1 and ErbB-2 are critical for the proliferation of SKBr3 cells.

2. **Blockade of Jagged-1 enhances sensitivity to trastuzumab or reverses trastuzumab resistance.**

To address the question whether Jagged-1 siRNA increases sensitivity to trastuzumab, we chose BT474 HS (sensitive to trastuzumab) cells. Trastuzumab treatment alone showed a 75% increase in the number of BT474 HS cells in the G1 phase of the cell cycle compared to IgG control. Consequently, trastuzumab decreased the number of cells in S phase by 50% (Figure 29A). Interestingly, Jagged-1 knockdown alone showed a 50% increase in the G1 phase and a 38% decrease in the S phase of the cell cycle compared to IgG control (Figure 29A). However, the combination of Jagged-1 knockdown and trastuzumab treatment resulted in a 29% increase in the G1 phase and 65% reduction in the S phase of the cell cycle compared to trastuzumab alone (Figure 29A). These results indicate that Jagged-1 knockdown further sensitized BT474 HS cells to trastuzumab.

To address the question whether Jagged-1 siRNA reverses trastuzumab resistance, BT474 HR (resistant to trastuzumab) were used. Trastuzumab did not change the cell cycle distribution of resistant cells (Figure 29B). As demonstrated previously, BT474 HR cells are resistant to trastuzumab.
Figure 29: Jagged-1 siRNA Sensitize BT474 HS Cells to Trastuzumab and Restore Sensitivity of BT474 HR Cells to Trastuzumab.

BT474 HS (A.) and BT474 HR (B.) cells were transfected with scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) alone or were treated with IgG control or Trastuzumab (Trast) for 48 hours. Cell cycle analysis using Propidium Iodide was performed by flow cytometry. Mean percentage of cells in each experiment were plotted. Statistical significance was determined by performing ANOVA for multiple comparisons. The error bars represent standard deviations of the mean for three independent experiments. * denotes statistically significant differences between trastuzumab alone and trastuzumab plus Jagged-1 siRNA. ** denotes statistically significant differences between trastuzumab alone and SCBi/IgG. *** denotes statistically significant differences between Jagged-1 siRNA alone and SCBi/IgG.
Interestingly, Jagged-1 knockdown alone showed a 50% increase in the G1 phase and a 33% decrease in the S phase of the cell cycle compared to IgG control (Figure 29B). However, the combination of Jagged-1 knockdown and trastuzumab treatment resulted in a 56% increase in the G1 phase and 50% reduction in the S phase of the cell cycle compared to trastuzumab alone (Figure 29B). These results indicate that Jagged-1 knockdown reverses resistance of BT474 HR cells to trastuzumab.

3. Dual inhibition of Jagged-1 and ErbB-2 induces apoptosis of SKBr3 and BT474 breast cancer cells.

Does dual inhibition using both Jagged-1 knockdown and trastuzumab induce apoptosis in SKBR3, BT474 HS, and BT474 HR cells? To address this question, flow cytometry using Annexin-V/Propidium Iodide dye was performed to detect early apoptotic cells.

In SKBr3 cells, trastuzumab treatment alone significantly increased the number of Annexin-V positive cells by 110% compared to SCBi/IgG (Figure 30). Jagged-1 siRNA alone increased the number of Annexin-V positive cells by 265% compared to SCBi/IgG (Figure 30). Importantly, the combination of Jagged-1 knockdown and trastuzumab treatment increased the number of Annexin-V positive cells by 133% compared to trastuzumab (Figure 30). These results, taken together with the cell cycle data, suggest that both Jagged-1 and ErbB-2 activities are critical for cell proliferation and survival of SKBr3 breast cancer cells.
Figure 30: Trastuzumab plus a Jagged-1 siRNA Induced Apoptosis in SKBr3 Cells.

SKBr3 cells were transfected with scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) alone or were treated with IgG or trastuzumab (Trast) for 48 hours. Annexin-V staining was performed by using flow cytometry. The mean percentage and standard deviation of cells in each experiment were plotted. Statistical significance was determined by performing ANOVA for multiple comparisons. The error bars represent standard deviations of the mean for three independent experiments. ** denotes statistically significant differences between trastuzumab alone and SCBi/IgG. *** denotes statistically significant differences between Jagged-1 siRNA alone and SCBi/IgG. **** denotes statistically significant differences between trastuzumab plus Jagged-1 siRNA and SCBi/IgG.
In BT474 HS cells, trastuzumab treatment alone increased the number of Annexin-V positive cells by 150% compared to SCBi/IgG (Figure 31A). Jagged-1 siRNA alone increased the number of Annexin-V positive cells by 175% compared to SCBi/IgG (Figure 31A). Importantly, the combination of Jagged-1 knockdown and trastuzumab treatment increased the number of Annexin-V positive cells by 40% compared to trastuzumab treatment alone (Figure 31A). These results, taken together with the cell cycle data, suggest that Jagged-1 decreases efficacy of trastuzumab and confers a protection from apoptosis in BT474 HS cells.

In BT474 HR cells, trastuzumab treatment alone showed a similar increase in the number of Annexin-V positive cells compared to SCBi/IgG (Figure 31B). This result further confirmed that BT474 HR cells are resistant to trastuzumab. A Jagged-1 siRNA alone was sufficient to increase apoptosis by 125% compared to SCBi/IgG (Figure 31B). Importantly, the combination of Jagged-1 knockdown and trastuzumab treatment increased the number of Annexin-V positive cells by 140% compared to trastuzumab control (Figure 31B).

These results, taken together suggested that inhibiting Jagged-1 could increase the sensitivity of ErbB-2 positive breast cancer cells to trastuzumab and reverse resistance.
BT474 HS (A.) and BT474 HR (B.) cells were transfected with scrambled control siRNA (SCBi) or Jagged-1 siRNA (Jag-1i) alone or were treated with IgG or trastuzumab (Trast) for 48 hours. Annexin-V staining was performed by using flow cytometry. The mean percentage and standard deviation of cells in each experiment were plotted. Statistical significance was determined by performing ANOVA for multiple comparisons. The error bars represent standard deviations of the mean for three independent experiments. * denotes statistically significant differences between trastuzumab alone and trastuzumab plus Jagged-1 siRNA. *** denotes statistically significant differences between Jagged-1 siRNA alone and SCBi/IgG.

Figure 31: Trastuzumab plus a Jagged-1 siRNA Induced Apoptosis in BT474 HS and HR Cells.
**Aim 2B. Establish whether a combination of GSI plus trastuzumab prevents tumor growth in vivo.**

The data from cell cycle and Annexin-V analyses suggested that Jagged-1 might play a novel role in resistance to trastuzumab, which could be prevented or reversed by inhibiting Jagged-1. Because of the lack of a pharmacological means to inhibit Jagged-1 in vivo, we inhibited Notch using γ-secretase inhibitors (GSIs) which block the final cleavage step of Notch preventing release of NICD. BT474 breast cancer cells contain an ErbB-2 gene amplification and are sensitive to trastuzumab. Osipo et al showed that ErbB-2 overexpression suppresses Notch-1 activity, thus BT474, which has an amplification and overexpression of ErbB-2, exhibit low Notch-1 activity (Osipo et al., 2008).

1. **Trastuzumab plus a γ-secretase inhibitor (GSI) prevents or reduces tumor recurrence.**

Breast tumor xenografts were generated using BT474 trastuzumab sensitive cells using athymic, nude mice. Growth of tumors was measured in response to Vehicle, trastuzumab, LY 411,575 or MRK-003 GSI, or trastuzumab plus GSI. The results from two independent studies showed that trastuzumab treatment almost completely inhibited the tumor growth of BT474 breast tumor xenografts with approximately 90-100% of tumors regressing to undetectable levels (Figure 32A and 32B). GSI treatment of tumors alone using LY 411,575 (Figure 32A) or MRK-003 (Figure 32B) had no statistically significant effect on tumor growth during the treatment phase of the study compared to Vehicle control. Moreover, GSI treatment of tumors using LY 411,575 (Figure 32A) or
MRK-003 (Figure 32B) in combination with trastuzumab had no significant effect on tumor growth compared to trastuzumab alone.

There is significant evidence of enhanced Notch signaling in tumor initiating or putative breast cancer stem cells (Grudzien et al., 2010; Harrison et al., 2010; Pannuti et al., 2010). As these cells are suggested to be responsible for tumor recurrence, we discontinued treatment and measured tumor recurrence in mice where tumors were no longer detectable using the Vevo 770 Ultrasound Imager. During the recurrence phase of the study, we detected significant tumor re-growth at week 14 (Figure 32A) and 25 (Figure 32B) for the previously trastuzumab-treated mice only. In contrast, mice previously treated with the combination of trastuzumab plus LY 411,575 GSI did not display tumor re-growth until week 25 and their tumors were significantly smaller (0.12 cm² versus 0.87 cm² for the trastuzumab alone arm, p<.001) (Figure 32A). Furthermore, no recurrent tumors were detectable in mice previously treated with the combination of trastuzumab plus MRK-003 GSI (p<.0001) (Figure 32B). Figure 33 is a Kaplan-Meier analysis of log-rank (Mantel-Cox) test for the rate of tumor recurrence post-treatment. In both studies, approximately 40% of mice previously-treated with trastuzumab alone displayed tumor recurrence at 40 weeks. In contrast, only 10% of mice displayed tumor recurrence in the trastuzumab plus LY 411,575 GSI group and 0% of mice displayed tumor recurrence in the trastuzumab plus MRK-003 group (Figure 33). All mice were sacrificed at the end of 40 weeks and it was confirmed via surgery that tumors were completely absent in the trastuzumab plus MRK-003 group.
Figure 32: Trastuzumab plus a γ-Secretase Inhibitor Prevents or Reduces Tumor Recurrence.

Fifty six ovariectomized, athymic nude mice were injected with 5 x 10^6 ErbB-2 gene amplified BT474 cells into both mammary fat pads. Once tumors grew to a mean tumor cross sectional area of 0.20 cm^2, mice were randomized and treated with vehicle (100μL sterile PBS injected i.p. 1 day/week and 200μL 2% carboxymethylcellulose), 10mg/Kg trastuzumab in 100μL PBS injected i.p. once weekly, 5mg/Kg LY 411,575 GSI (A) or 100mg/Kg MRK-003 GSI (B) in 200μL 2% carboxymethylcellulose, fed by oral gavage; three days on, 4 days off, or trastuzumab plus LY 411,575 GSI or MRK-003 GSI. Tumor area was measured weekly for up to 12 or 19 weeks using Vernier calipers. After the treatments were stopped, tumor recurrence was monitored up to 105 days or 98 days in mice that specifically showed complete tumor regression (A and B). The graphs from A and B show mean tumor cross sectional area [(area x π)/4] on the Y-axis and time in
weeks on the X-axis. Error bars are standard deviations of the mean for 12 and 8 mice-bearing tumors in the response phase and in the recurrent phase of the study, respectively. *denotes statistically significant differences between mean slopes of the curve for trastuzumab plus GSI versus GSI alone. **denotes statistically significant differences between mean slopes of the curve for trastuzumab versus trastuzumab plus GSI in recurrent tumors. Linear regression analyses were performed for tumor growth curves in panels A and B.
These data suggest that the main benefit of using a GSI (MRK-003) in trastuzumab sensitive, ErbB-2 positive breast tumors is prevention of tumor recurrence.

Tumors from Figure 33 were excised at week 10, which is prior to trastuzumab induced regression, to perform H/E staining, Ki67 staining, TUNEL assay, and Western blotting.

2. **Lack or Delay of Tumor Recurrence was due to Prevention of Trastuzumab Induced Increase in Notch.**

Real-time RT-PCR was performed to detect canonical Notch target gene transcripts which include Hey-1 and Deltex-1 (Kopan and Ilagan, 2009) as measures of Notch signaling and efficacy of the LY 411,575 (Figure 34A) or MRK-003 GSI (Figure 34B) on the Notch pathway. Figure 34A and 34B demonstrated that LY 411,575 or MRK-003 GSI alone significantly inhibited Hey-1 and Deltex-1 transcripts compared to Vehicle control. Trastuzumab treatment alone significantly increased Hey-1 by 2-4 fold and Deltex-1 by 20 fold compared to Vehicle control (Figure 34A and 34B). LY 411,575 or MRK-003 GSI significantly decreased the trastuzumab-induced increase in Hey-1 and Deltex-1 (Figures 34A and 34B). In addition, recurrent tumors that grew post-trastuzumab treatment showed increased baseline expression of Hey-1 by 2 fold and Deltex-1 by 4 fold as compared to trastuzumab treated tumors (Figure 34A). In contrast, recurrent tumors post-trastuzumab plus LY 411,575 GSI treatment showed decreased expression of Hey-1 and Deltex-1 transcripts compared to trastuzumab treated recurrent tumors (Figure 34A). These results would indicate that the lack or delay of breast tumor
recurrence observed for the combination of trastuzumab plus GSI treatment could be due to prevention of trastuzumab-induced increase in Notch signaling.

3. **BT474 xenograft histology and signaling pathways.**

Haematoxylin and eosin (H/E) staining was used to confirm the presence of tumors. H/E staining of tumors from Figure 35B showed that tumors treated with Vehicle, trastuzumab, or MRK-003 GSI alone appeared similar in histology (Figure 35A, upper panel). However, tumors treated with trastuzumab plus MRK-003 GSI contained vast numbers of pyknotic nuclei, a possible indication of cell death (Figure 35A, upper panel). Ki67 staining of tumors was used to detect proliferation. The Vehicle control, trastuzumab, or MRK-003 GSI alone treatments had similar numbers of Ki67 positive nuclei (200) (Figure 35A, middle panel) which was quantified using at least 3-5 tumors and 60 high powered fields (Figure 35B). In contrast, Ki67 staining was almost undetectable in trastuzumab plus MRK-003 GSI treated tumors (Figure 35A, middle panel and 35B). Furthermore, TUNEL assay was performed to detect early apoptotic cells. TUNEL assay showed a 10 fold increase in TUNEL positive cells from trastuzumab plus MRK-003 GSI treated tumors compared with vehicle, consistent with nuclear pyknosis observed by H/E (Figure 35A, lower panel and 35B). These results indicate that the lack of tumor recurrence from trastuzumab plus MRK-003 GSI treatment is probably due to simultaneous induction of apoptosis and down-regulation of proliferation.
Figure 33: Kaplan-Meier Curve.

Kaplan-Meier curve of percentage tumor-free mice generated from BT474 breast cancer cells among Trast1, Trast1+MRK-003 GSI, Trast2, and Trast2+LY 411,575 GSI treatment groups using a log rank (Mantel-Cox) test. *denotes statistically significant differences between Trast1 and Trast1 + MRK-003 GSI. **denotes statistically significant differences between Trast2 and Trast2 + LY 411,575 GSI.
Figure 34 Notch-1 Gene Target Expression in BT474 Tumors.

(A and B) In a separate experiment, 1mg of snap-frozen tumors were homogenized, lysed in TRI reagent solution, total RNA was extracted, and reverse transcribed to total cDNA as described in the methods section. Real-time PCR was performed using human-specific primers to detect transcripts from Notch target genes: human HEY1 and human Deltex1. Human-specific 18s rRNA was detected for normalization. Results are mean relative transcripts levels compared to Vehicle (Control) after normalization to 18s rRNA. Error bars are standard deviations of the mean for five independent tumor samples. * denotes statistically significant differences between MRK-003 or LY 411,575 GSI and Vehicle (control). ** denotes statistically significant differences between trastuzumab (Trast) and Vehicle (control). *** denotes statistically significant differences between GSI + Trast and Trast alone. # denotes statistically significant differences between Trast-treated tumors and recurrent tumors previously treated with Trast. ## denotes statistically significant differences between GSI + Trast and Trast alone in recurrent tumors.
Five Mice were euthanized and tumors excised at week 12 as shown in Figure 32B. One half of the tumors were immediately fixed in formalin and the remaining half snap-frozen in liquid nitrogen for future study. (A) Fixed tumors were paraffin-embedded and sectioned for H/E staining (upper panel), Ki67 (middle panel), and TUNEL (lower panel) assays. All sections were photographed at 40X magnification using a light microscope. Four panels are shown: BT474 tumors-treated with Vehicle, trastuzumab (Trast), MRK-003 GSI, or Trast plus GSI. Shown are representative photographs based on at least 3 tumors. (B) Quantification of Ki67 and TUNEL positive cells of 3 tumors using 60 high powered fields (HPF) at 40X magnification. The Y-axis represents number of Ki67 or TUNEL positive cells for 60 HPF. The bar graphs are means plus or minus standard deviations. *denotes statistical significance between trastuzumab and trastuzumab plus MRK-003 GSI (Trast + MRK-003). **denotes statistical significance between MRK-003 GSI and Trast + MRK-003 GSI. (C) Expression of ErbB-2 and downstream signaling pathways in BT474 tumors. Bits of tumors (1mg) that were snap-frozen in liquid nitrogen were homogenized and lysed in RIPA buffer containing protease and phosphatase inhibitors. Cellular debris was removed by centrifugation at 1000g for 5
minutes at 4°C. Supernatants were collected and 25μg of total protein loaded onto a 7% SDS-PAGE gel followed by Western blotting to detect tyrosine phosphorylated ErbB-2 or HER2 (PY1248-HER2), total HER2, P-ERK1/2, total ERK1/2, P-AKT1, total AKT1, PTEN, and actin proteins. The Western blot shown is a representative of 3 independent tumor samples with similar results. Density of bands corresponding to PY-HER2 and total HER2 for each treatment groups was quantified by the use of Image J program and expressed as a PY-HER2/HER2 ratio of area of the peak.
The ERK and AKT pathway are activated downstream of overexpressed ErbB-2 (Yarden, 2001; Yarden and Sliwkowski, 2001). Thus, we asked the question: What is the status of ERK and AKT activation in treated tumors? BT474 tumors excised at week 12 from Figure 33B were analyzed by Western blotting to detect tyrosine-phosphorylated ErbB-2 (PY1248), total ErbB-2, phosphorylated ERK1/2, total ERK1/2, phosphorylated AKT1, total AKT1, and PTEN proteins. Figure 35C demonstrated that MRK-003 GSI treatment of BT474 tumors increased PY-ErbB-2 protein compared to Vehicle control. Trastuzumab alone showed 80% decrease in PY-ErbB-2 protein versus Vehicle control (Figure 35C). Interestingly, PY-ErbB-2 protein was reduced by 60% with trastuzumab plus MRK-003 GSI compared to Vehicle control (Figure 35C). Furthermore, while either MRK-003 GSI or trastuzumab alone decreased P-ERK1/2 and P-AKT1 compared to Vehicle, only trastuzumab plus MRK-003 GSI decreased both P-ERK1/2 and P-AKT1 to almost undetectable levels (Figure 35C). This decrease in P-AKT1 was associated with increased PTEN protein levels (Figure 35C). These results would suggest that down-regulation of proliferation and induction of apoptosis by trastuzumab plus MRK-003 GSI could be due at least in part to synergistic or additive inhibition of ERK1/2 and AKT1 activities, which are critical signaling pathways for proliferation and anti-apoptosis, respectively.

4. **Lapatinib plus MRK-003 GSI reduces tumor growth, proliferation, and ERK1/2 and AKT1 activity and induces apoptosis.**

Lapatinib is a more potent drug *in vitro* compared to trastuzumab. Therefore, we chose BT474 lapatinib sensitive cells and generated breast tumor xenografts in athymic
nude mice. Growth of tumors was measured in response to Vehicle, lapatinib, MRK-003 GSI, or lapatinib plus GSI. The results showed that lapatinib treatment decreased tumor growth by only 40% compared to Vehicle control (Figure 36A). Treatment of tumors using MRK-003 GSI alone had no statistically significant effect on tumor growth compared to Vehicle control (Figure 36A). However, lapatinib plus GSI showed significant reduction in tumor growth at week 13 compared to GSI alone, lapatinib alone, or Vehicle (Figure 36A). The study was stopped at week 13 due to onset of diarrhea in lapatinib-treated mice. Haematoxylin and eosin (H/E) staining showed that tumors treated with Vehicle, lapatinib, MRK-003 GSI or lapatinib plus GSI appeared similar in histology and confirmed the presence of tumor in the samples excised (Figure 36B, upper panel). Ki67 staining of tumors from lapatinib, MRK-003 GSI, or lapatinib plus GSI treatments demonstrated 75-90% reduction in the Ki67 positive nuclei compared to the vehicle control (Figure 36B, middle panel and 36C). Furthermore, apoptosis as measured by TUNEL assay showed a 10 fold increase in TUNEL positive cells from lapatinib plus MRK-003 GSI treated tumors compared to Vehicle (Figure 36B, third panel, and 36C). These results indicate that induction of tumor regression from lapatinib plus MRK-003 GSI treatment is probably also due to simultaneous induction of apoptosis and down-regulation of proliferation. Moreover, Western blotting was performed to detect tyrosine phosphorylated ErbB-2 (PY1248), total ErbB-2, phosphorylated ERK1/2, total ERK1/2, phosphorylated AKT1 and total AKT1 proteins. Figure 36D demonstrates that lapatinib or MRK-003 GSI treatment alone decreased PY-ErbB-2 protein by 40% compared to Vehicle control. Interestingly, lapatinib plus GSI treatment, unlike trastuzumab, had little effect on PY-ErbB-2 protein. However, both phosphorylated ERK-1/2 and AKT1
proteins were reduced in tumors treated with lapatinib plus GSI compared to all other treatments (Figure 36D). These results indicate that induction of tumor regression by lapatinib plus MRK-003 GSI could also be due to simultaneous inhibition of both ERK1/2 and AKT1 activities.
B. Vehicle Lap MRK-003 GSI Lap + GSI

H&E (40x)

Ki67 (40x)

TUNEL (40x)

C.

# Ki67 positive cells

# TUNEL positive cells

* p<0.0001
** p=0.02
*** p<0.01
**** p<0.01
Figure 36: Lapatinib plus MRK-003 GSI Reduces Tumor Growth.

(A) ErbB-2 overexpressing BT474 xenografts were generated in 40 ovariectomized, athymic nude mice by injecting $5 \times 10^6$ cells into both mammary fat pads. Once tumors reached a mean tumor cross sectional area of 0.25 cm$^2$, mice were randomized and treated with Vehicle, lapatinib (LAP), MRK-003 GSI, or LAP plus GSI. Tumor area (length X width) was measured weekly using Vernier calipers. The measurements were performed up to 13 weeks. Results show mean tumor cross sectional area $((\text{area} \times \pi)/4)$ on the Y-axis and time in weeks on the X-axis. Error bars are standard deviations of the mean for 10 mice-bearing tumors. (B) Fixed tumors were paraffin-embedded and sectioned for H/E staining (upper panel), Ki67 (middle panel), and TUNEL (lower panel) assays. All sections were photographed at 40X using a light microscope. Four panels are shown: BT474 tumor-treated with Vehicle, lapatinib (LAP), MRK-003 GSI, and LAP + GSI. The photographs of a single tumor sample are representative of 3 tumors with similar results. (C) Quantification of Ki67 and TUNEL positive cells of 3 tumors using 60 high powered fields (HPF) at 40X magnification. The Y-axis represents number of Ki67 or TUNEL positive cells per 60 HPF. The bar graphs are means plus or minus standard deviations. *denotes statistical differences compared to Vehicle control. **denotes statistical differences between LAP and LAP + GSI. ***denotes statistical difference between MRK-003 GSI and LAP + GSI. Statistical analysis was performed using a two-sided, non-paired Student’s T-test. (D) Bits of tumors (1mg) that were snap-frozen in
liquid nitrogen were homogenized and lysed in RIPA buffer containing protease and phosphatase inhibitors. Cellular debris was removed by centrifugation at 1000g for 5 minutes at 4°C. Supernatants were collected and 25µg of total protein loaded onto a 7% SDS-PAGE gel followed by Western blotting to detect tyrosine phosphorylated ErbB-2 or HER2 (PY1248-HER2), total HER2, P-ERK1/2, total ERK1/2, P-AKT1, total AKT1, and actin proteins. Densitometry was performed on PY-HER2 and total HER2 bands for each treatment groups using Image J program and expressed as a PY-HER2/HER2 ratio of area of the peak. Western blotting was performed on at least 3 independent tumor samples. Representative Western blots are shown with similar results. *denotes statistically significant differences between mean slopes of the curve for LAP plus GSI and GSI alone. **denotes statistically significant differences between mean slopes of the curve for LAP and Vehicle control. ***denotes statistically significant differences between mean slopes of the curve for LAP plus GSI and LAP alone. Linear regression analyses were performed for tumor growth curve.
Aim 2C. Establish whether a GSI in combination with trastuzumab reverses resistance in vivo.

1. GSI partially restores trastuzumab sensitivity in resistant tumors.

Trastuzumab resistant, ErbB-2 positive, BT474 breast cancer cells were generated by treating cells with 10 μg/mL trastuzumab for 6 months in vitro as described previously (Osipo et al., 2008). These resistant cells were injected into athymic, nude mice to generate trastuzumab resistant breast tumor xenografts in vivo. Figure 37A and 37B show that xenograft tumors generated from trastuzumab resistant cells were resistant to trastuzumab in two independent studies. Treatment with LY 411,575 (Figure 37A) or MRK-003 (Figure 37B) GSI alone did not inhibit tumor growth when compared to Vehicle or trastuzumab alone (Figure 37A and 37B). However, trastuzumab plus LY 411,575 or MRK-003 GSI decreased the rate of tumor growth by almost 50% compared to GSI or trastuzumab alone (Figure 37A and 37B). While the reduction of tumor growth by trastuzumab plus LY 411,575 GSI reached significance, the reduced tumor growth for trastuzumab plus MRK-003 GSI did not reach statistical significance.

These data taken together suggest that the benefit of using a combination of trastuzumab plus GSI is prevention of tumor recurrence. However, once trastuzumab resistance occurs, a GSI can only partially restore trastuzumab sensitivity.
Figure 37: GSI Partially Restores Trastuzumab Sensitivity in Resistant Tumors.

(A and B) The exact same protocol as described in Figure 1 was used to generate trastuzumab resistant tumors in athymic, nude mice using BT474 trastuzumab resistant cells. (A) LY 411,575 GSI was used. (B) MRK-003 GSI was used. Tumor area (length X width) was measured weekly using Vernier calipers. The measurements were performed up to 15 or 10 weeks, respectively. Results show mean tumor cross sectional area \([(\text{area} \times \pi)/4]\) on the Y-axis and time in weeks on the X-axis. Error bars are standard deviations of the mean for 10 mice-bearing tumors. *denotes statistically significant differences between GSI and Trast + GSI. **denotes statistically significant differences between Trast and Trast + GSI.
CHAPTER VI

DISCUSSION

ErbB-2 positive breast cancer is currently treated with therapeutic agents, trastuzumab (Carter et al., 1992) or lapatinib (Nahta et al., 2007). Although trastuzumab or lapatinib plus chemotherapy have been successful in the treatment of ErbB-2 positive breast cancer, some patients will not respond to these drugs and, among responders, 15-25% will have disease recurrence and ultimately progression (Cobleigh et al., 1999; Vogel et al., 2002). Thus, anti-ErbB-2 therapy-associated resistance remains a serious clinical problem. One possible reason for this problem could be alterations in signaling pathways that are downstream or parallel to ErbB-2 upon chronic trastuzumab or lapatinib treatment. We showed that ErbB-2 inhibition activates Notch-1 which results in a compensatory increase in Notch-1-mediated proliferation (Osipo et al., 2008). Notch-1, a breast oncogene, is a modulator of cell-fate decisions (Politi et al., 2004). Notch-1 and Notch-4 has been implicated in the self-renewal and survival of tumor-initiating cells (Harrison et al., 2010; Magnifico et al., 2009). Overexpression of constitutively active forms of Notch-1, Notch-3, and Notch-4 develop spontaneous murine mammary tumors \textit{in vivo} (Callahan and Raafat, 2001). In addition, Notch-1 has been recently suggested as a novel marker of trastuzumab resistance from human breast cancer tissue (Huober et al., 2010). We have identified Notch-1 as a novel target in trastuzumab-resistant breast
cancer in vitro (Osipo et al., 2008). Flow cytometry (Figure 7A) and biotinylation assays (Figure 7B) showed an increase in Jagged-1 cell surface expression. In addition, preliminary results demonstrated that Jagged-1 and Notch-1 co-expressed in SKBr3 cells, by confocal microscopy studies (Figure 17). However, Notch-1 and Jagged-1 were completely separated at the sub-cellular level: Notch-1 localized throughout the cell and Jagged-1 was concentrated at the cell surface near cell-cell contacts, when SKBr3 cells were treated with trastuzumab (Figure 17). Together, these data suggest that ErbB-2 overexpressing breast cancer cells trap Jagged-1 and Notch-1 in sub-membranous compartments such as early endosomes. However, trastuzumab released Jagged-1 to the cell surface, making Jagged-1 available to interact with and trans-activate Notch signaling. These results, taken together suggest a dual role for ErbB-2 in inhibition of Notch activity: 1, ErbB-2 promotes Jagged-1 mediated cis-inhibition of Notch and 2, ErbB-2 inhibits Jagged-1 mediated trans-activation of Notch.

Our studies have provide insight into a mechanism by which ErbB-2 and Notch pathways crosstalk in ErbB-2 positive breast cancer cells. Results from specific aim 1 show that ErbB-2 might stabilize total Jagged-1 protein (Figure 10). More interestingly, ErbB-2 via PKCα possibly limits the interaction between Jagged-1 and Mib-1 and subsequent ubiquitylation of Jagged-1 (Figures 20, 21, and 26). Moreover, Mib-1 is the E3 ubiquitin ligase required for lapatinib-mediated ubiquitylation of Jagged-1 (Figure 22) and induction of Notch activity (Figure 23). Furthermore, PKCα, possibly downstream of ErbB-2, inhibits Notch transcriptional activity (Figure 27). These results, taken together imply that overexpression of ErbB-2 restricts the critical association between
Jagged-1 and Mib-1 to inhibit Notch activity and to drive breast tumor proliferation, survival which consequently decreases the efficacy of anti-ErBb-2 targeted agents. Results from specific aim 2 identified Jagged-1 as a novel target in ErbB-2 positive breast cancer and more importantly in trastuzumab resistance (Figures 28, 29, 30, and 31). Moreover, our data provide, to our knowledge, the first preclinical proof of concept in mice for future clinical trials of combination regimens including trastuzumab and a Notch inhibitor, MRK-003 GSI, for the prevention of tumor recurrence (Figure 32) and possibly reversal of trastuzumab resistance (Figure 37) in ErbB-2 positive breast cancer.

Based on the confocal immunofluorescence microscopy results, we hypothesized that ErbB-2 overexpressing breast cancer cells express Jagged-1 to inhibit Notch in cis. Results from the co-culture studies revealed that Jagged-1 could be a potent cis-inhibitor of Notch activity in ErbB-2 positive breast cancer cells (Figure 14) and possibly other breast cancer cell subtypes (Figure 16). However, the results did not demonstrate that ErbB-2 overexpression specifically promoted Jagged-1 mediated cis-inhibition of Notch (Figure 15). What the co-culture data revealed are that trastuzumab most likely promotes trans-activation of Notch by increasing the cell surface expression of Jagged-1. As shown in Figure 10, ErbB-2 seems to be important in stabilizing the Jagged-1 protein and this result coincided with inhibition of Notch activity. One idea is that the E3 ligase Neur ubiquitylates and degrades the cis-inhibiting ligand to allow Notch to bind another or the same ligand in trans on adjacent cells, leading to receptor activation (Glittenberg et al., 2006). Although, Mib-1 plays a critical role in ubiquitylation and generation of a signaling competent ligand, it has never been shown so far to play a role in regulation of
levels of the cis-inhibiting ligand for Notch activation (Deblandre et al., 2001; Lai et al., 2001). Because the function of Mib-1 and its role in the mammalian system, and more importantly in cancer, is beginning to be revealed, we aim to explore in the near future a role for Mib-1 in regulating Jagged-1 protein stability in the presence or absence of ErbB-2. It is possible that ErbB-2 by limiting Mib-1 binding to Jagged-1 confers a protection on Jagged-1 from Mib-1 or another E3 ligase dependent degradative pathway.

Jagged-1 knockdown showed an up-regulation of Notch target genes (Figure 14), suggesting the possible role for other Notch ligands or receptors in the transcriptional induction of Hes-1 and Deltex-1 mRNAs. Preliminary data suggested that Jagged-1 is a positive regulator of DLL-1, DLL-4, and Notch-2, and hence these components of the Notch pathway might not be responsible for the Jagged-1 siRNA mediated increase in Notch activity. We could not detect Jagged-2, DLL-3, and Notch-3 due to lack of availability of good antibodies. Alternatively, it could be the dosage of Jagged-1 that is critical to regulate Notch activation by cis or trans mechanisms. Sprinzak et al used a titration based approach to demonstrate how levels of cis and trans Delta are integrated by the Notch pathway (Sprinzak et al., 2010). Notch activity gradually increased with increasing concentrations of Delta in trans, independent of Delta levels in cis (Sprinzak et al., 2010). Moreover, upon decreasing the concentration of Delta in cis, a Notch response to Delta in trans was observed (Sprinzak et al., 2010). Most importantly, the threshold level of Delta in cis below which Notch is activated, was independent of Delta levels in trans (Sprinzak et al., 2010). Whether the same molecule can simultaneously interact in cis and/or in trans with Notch is not known. As shown in Figure 10, ErbB-2 clearly
regulates the amount of Jagged-1 protein. Therefore, ErbB-2 may play a role in Jagged-1 mediated cis-inhibition of Notch by stabilizing Jagged-1 levels in cis.

Our data for the first time identified ErbB-2 as a negative regulator of Jagged-1-mediated trans-activation of Notch (Figure 15). As shown in Figures 20 and 21, ErbB-2 limits Mib-1 association and subsequent ubiquitylation of Jagged-1. However, the mechanism regulating the critical association between Mib-1 and Jagged-1 is unknown. ErbB-2 is a RTK which activates a variety of kinases and related downstream phosphorylation events. Therefore, downstream kinase expression and/or activity could play a role in regulation of the Jagged-1 and Mib-1 association in ErbB-2 positive breast cancer. Using a proteomic approach, we can identify kinase(s) downstream of ErbB-2 whose expression and/or activity changes with anti-ErbB-2 therapy. It has been shown in the literature that ErbB-2 via Src positively regulates PKCα expression and activity (Tan et al., 2006). Therefore, using a candidate approach, we further identified PKCα as a downstream effector of ErbB-2. Our data demonstrate that high ErbB-2 activity promotes the formation of PKCα-Jagged-1 complex but limits Mib-1-Jagged-1 association (Figure 26). However, trastuzumab or lapatinib treatment decreases the PKCα-Jagged-1 interaction and conversely promotes the Mib-1 association with Jagged-1 (Figure 26). These results indicate that ErbB-2 by activating PKCα protein is facilitating its interaction with, and possibly phosphorylation, of Jagged-1. In contrast, anti-ErbB-2 therapy decreases PKCα protein and phosphorylation, and favors Mib-1 association with Jagged-1, generating a signaling competent ligand leading to Notch activation.
A single phosphorylation signal may directly alter the function and activity of proteins, e.g., by promoting a conformational change, protein-protein interactions, protein degradation, protein stability, and protein localization. PKCα is a kinase that phosphorylates many substrates. Scanning for putative PKC binding sites using the NCBI Prosite website in both Jagged-1 and Mib-1 proteins revealed several putative PKCα binding sites (Figure 24). In addition, four putative phosphorylation sites (T1197, S1207, S1210, and Y1216) along with a PDZ protein-protein interaction motif were identified in the intracellular domain of Jagged-1 (Popovic et al., 2011). This could indicate that PKCα, downstream of ErbB-2, is phosphorylating and modulating either Mib-1 or Jagged-1 to modulate their functions.

PKCα could physically bind and phosphorylate the C-terminal Jagged-1 tail. Phosphorylation of Jagged-1 may change its conformation, retain Jagged-1 in a cis-inhibitory endocytic compartment or regulate the amount of Jagged-1 protein expression. This could then disrupt Mib-1 binding and prevent generation of a competent ligand needed to activate Notch.

In addition to the PKCα’s ability to regulate Jagged-1, it may also regulate Mib-1’s function. Ossipova et al has demonstrated that PAR-1 kinase-mediated phosphorylation of Mib-1 targets Mib-1 for degradation by the proteasome. Because Mib-1 protein levels are regulated by phosphorylation events and PKCα is a kinase, ErbB-2 via PKCα could be phosphorylating Mib-1 and targeting it for degradation. However, Figure 19 showed that Mib-1 protein levels remain relatively unaffected upon trastuzumab or lapatinib treatment. Therefore, PKCα demonstrates no regulation on Mib-1 stability.
Overexpression of ErbB-2 drives proliferation and growth of ErbB-2 positive breast cancer cells. This was further confirmed when trastuzumab treatment reduced growth and induced apoptosis in 40% of SKBr3 cells (Figure 28 and 30). Jagged-1 siRNA reduced proliferation and increased apoptosis in 70% of SKBr3 cells (Figure 28 and 30) and that coincided with an aberrant increase in Notch activity (Figure 14). The enhanced apoptosis of ErbB-2 positive breast cancer cells in the presence of Jagged-1 siRNA could be a result of an increase in the differentiation signal in a context of ErbB-2 driven proliferation. Interestingly, it was demonstrated that dual inhibition of Jagged-1 and ErbB-2, further reduced proliferation (Figure 28) and increased apoptosis in 90% (Figure 30) of SKBr3 cells. Notch signaling is critical for cell fate determination and ErbB-2 is critical for proliferation and survival. Therefore, the combination inhibiting the proliferation signal and promoting the differentiation signal in a cancer cell further sensitized ErbB-2 positive breast cancer cells to trastuzumab and lead to catastrophic cell death. Similar results were obtained in other cell lines, BT474 HS (trastuzumab sensitive) and HR (trastuzumab resistant) cells (Figures 29A, 29B, 31A, and 31B). These data suggested that inhibiting Jagged-1 could increase the sensitivity of ErbB-2 positive breast cancer cells to trastuzumab and reverse resistance. To confirm that this is not an off target effect of Jagged-1 siRNA, similar experiments should be performed targeting Jagged-1 using the same siRNA and re-expressing either full length Jagged-1 or an interaction dead Jagged-1 mutant to investigate whether Jagged-1 expression rescued the cell death-mediated by Jagged-1 siRNA. In addition, to demonstrate that the Jagged-1-Mib-1-Notch-1-Hes-1 axis was critical for survival of ErbB-2 positive breast cancer cells, each component would need to be knocked down and apoptosis studies performed. This
would further test if the Jagged-1 siRNA mediated induction of apoptosis is due to the modulation of the canonical Jagged-1–Mib-1-Notch-1–Hes-1 pathway.

Co-overexpression of Jagged-1 and Notch-1 predicts for the poorest outcome in women with breast cancer. We observed that when Jagged-1 is abundant it acts as an inhibitor of Notch activity whereas when limiting it can orient itself in trans and now bind and activate Notch. Therefore, it is possible that a cell exhibiting increased expression of Jagged-1 sends a cis-inhibitory signal to limit aberrant activation of Notch which induced cell death in ErbB-2 positive breast cancer cells as shown. Thus, ErbB-2 promoting cis-inhibition of Notch is selected because it generates a stable cellular state driving growth and survival of ErbB-2 positive breast cancer cells. Although, inhibition of ErbB-2 decreases the total Jagged-1 protein, the remaining Jagged-1 may be sufficient to trans-activate Notch receptors. At a multicellular level, this low line level of activated Notch can amplify and provide a survival advantage to ErbB-2 positive breast cancer cells and could potentially cause resistance to anti-ErbB-2 treatment.

One of the major problems with anti-ErbB-2 treatments is resistance, particularly in metastatic disease. To circumvent these problems, we designed and evaluated for the first time a combination therapeutic strategy that can prevent and/or reverse trastuzumab resistance in vivo. Our findings, along with evidence from the literature, indicate that Notch could be an important target in trastuzumab-resistant, ErbB-2 positive breast cancer (Osipo et al., 2008). One class of compounds that are being used to inhibit the Notch pathway are GSIs that are currently in clinical trials for the treatment of breast cancer and other solid tumors (Pannuti et al, 2010). Recently, it was reported that MRK-003 GSI treatment of Balb/c-neuT female mice reduced tumor onset, tumor burden, and
AKT1/mTOR activities associated with ErbB-2 positive, murine breast tumors (Efferson et al, 2010). Our results demonstrate that although trastuzumab treatment caused virtually complete tumor regression (Figure 32A and 32B), which mimics what is observed in the clinic, it could not prevent tumor recurrence in 40% of the mice (Figure 32A and 32B, and 33). However, adding a GSI to trastuzumab treatment either completely abolished (MRK-003) or significantly reduced (LY 411,575) tumor recurrence (Figure 32A and 32B, and 33). Interestingly, recurrent tumors post-trastuzumab plus the LY 411,575 GSI treatment showed a significant decrease in Notch transcriptional activity compared with trastuzumab treatment alone (Figure 34), suggesting that Notch signaling could be responsible for ErbB-2 positive breast tumor recurrence post-trastuzumab treatment. Consistent with the literature, our data also suggest that Notch plays a critical role in the survival of tumor-initiating cells as demonstrated by the lack of tumor recurrence when Notch was inhibited in combination with ErbB-2 inhibition.

Treatment with the combination of trastuzumab plus MRK-003 GSI simultaneously decreased proliferation and induced tumor cell death (Figure 35A and 35B). These antitumor effects of the combination therapy may be because of near-complete blockade of two critical signaling pathways downstream of ErbB-2: ERK1/2 and AKT1 (Figure 35C). Thus, a combination of trastuzumab plus MRK-003 GSI could benefit those women with recurrent, or possibly resistant, ErbB-2-positive breast cancer ultimately to reduce or eliminate disease progression and deaths by simultaneously inactivating two critical prosurvival and antiapoptotic pathways such as ERK1/2 and AKT1.
Lapatinib is a very potent inhibitor of ErbB-2 activity *in vitro*; however, lapatinib alone reduced tumor growth by only 40% in our BT474 model of ErbB-2 positive breast cancer (Figure 36A). A combination of lapatinib plus MRK-003 GSI showed significant reduction in the tumor growth (Figure 36A). This is likely because of inhibition of ERK1/2 and AKT1 activities (Figure 36D) that resulted in increased apoptosis and decreased proliferation (Figure 36B and 36C). However, the onset of diarrhea-associated toxicity with lapatinib or lapatinib plus GSI treatment at week 13 caused the study to end prematurely and, therefore, complete tumor regression was not reached.

Our results also showed that a GSI could partially restore sensitivity to trastuzumab in resistant tumors (Figure 37). The mechanism by which a GSI only partially reverses trastuzumab resistance *in vivo* is being actively studied. It is possible that targeting all four Notch receptors with a pan-Notch inhibitor such as a GSI might not effectively target Notch-1, which we have shown to be necessary for trastuzumab resistance *in vitro* (Osipo et al, 2008). Moreover, Notch-2 has been implicated as a tumor suppressor. Thus, targeting all four Notch receptors using a GSI may inhibit a tumor suppressor which is why we observed only a partial reversal of resistance. Work from Han *et al*, however, suggested that GSI induced cytotoxic effects on the xenograft tumors and was due to inhibition of proteasome function (Han et al., 2009). Therefore, a more specific Notch-1 or possibly other Notch signaling pathway inhibitors could prove to be more effective and potent.

Our *in vitro* data identified Jagged-1 as a novel target for the treatment of ErbB-2 positive breast cancer. Jagged-1 inhibition using siRNA induced aberrant Notch activation which in turn leads to cell death in ErbB-2 positive breast cancer cells. Thus,
targeting Jagged-1 specifically in combination with current therapies (trastuzumab or lapatinib) would increase Notch activity and induce apoptosis in vivo. Differential effect of Notch has been observed between tissues and within a tissue between normal and disease states and is influenced by the number of receptors activated. Our data for the first time demonstrated the role of increased Notch activity in inducing apoptosis in SKBr3 and BT474 cells. On one hand, targeting Jagged-1 seems to be promising as it induced cell death in cancer cells but that coincided with aberrant increase in Notch activity. Since the dosage dependent effects of Notch activity on the proliferation and survival of the cancer cell are unclear, targeting Jagged-1 specifically seems to be a dicey approach to target Notch pathway. Until more light is shed on the dosage dependent functions of Notch activity, the best approach to target Notch pathway would be to inhibit trans interactions between Jagged-1 and Notch. This way we will inhibit Notch activation completely.

Over recent years many approaches have been taken to inhibit the Notch pathway. The first Notch pathway inhibitors, used both experimentally and clinically, were GSIs which prevent cleavage of NEXT and therefore the release of NICD from the plasma membrane. Agents that compete with MAML for binding to the RBPjk/NICD transcriptional activator complex have also been generated. These agents include SAHM1 and TR4 (Moellering et al., 2009). However, GSIs, SAHM1 and TR4 can disrupt signaling by all four Notch receptors. More recently, monoclonal antibodies directed against the EGF repeats 11 – 15 within Notch receptors that prevent ligand/receptor interaction have been generated. In addition, monoclonal antibodies that prevent the conformational change within the extracellular domain required to expose the
S2 cleavage site have been generated. Monoclonal antibodies that recognize specific ligands (Dll4) (Hoey et al., 2009) or receptors (Notch1-3) (Aste-Amezaga et al., 2010; Wu et al., 2010) have been developed. Pan-inhibition of Notch signaling with GSIs has been associated with goblet cell dysplasia in the gut (Pannuti et al., 2010; Wu et al., 2010). Not only that but long-term treatment with Notch pathway inhibitors is associated with development of vascular tumours (Liu et al., 2011b). Thus, targeting individual Notch pathway receptors and ligands would be more effective and potent.

Furthermore, the Notch pathway is complicated in tumors because of multiple modes of action. For example, it is known that Delta-like 4 (DLL-4) on endothelial cells engages and activates the Notch-1 receptor on cancer epithelial cells to promote angiogenesis (Yan et al, 2010). In addition, Notch signaling has been recently implicated to play a role in survival and differentiation of tumor stroma (Orr et al, 2009). The level of complexity for the role of Notch signaling in the tumor microenvironment requires a thorough investigation of the Notch pathway in breast cancer, and most notably in anti-ErbB-2-targeted drug resistance, with the goal of identifying novel and specific targets to treat or reverse resistance.

1. Conclusion

We demonstrated for the first time that high levels of Jagged-1 could possibly act as potent cis-inhibitor of Notch transcriptional activity. ErbB-2 prevents Jagged-1-mediated trans-activation of Notch. Jagged-1 is a novel and potentially better therapeutic target for the treatment of ErbB-2 positive breast cancer and trastuzumab resistance. Our findings suggest for the first time that the benefit of using a combination of trastuzumab plus a GSI is prevention of ErbB-2 positive breast tumor recurrence. Because Notch is a breast
oncogene that is critical for survival and proliferation of breast cancer cells, our findings strongly suggest that combined treatment with a Notch inhibitor could be an effective therapeutic strategy to prevent tumor recurrence and possibly disease progression and death in ErbB-2 positive breast cancer. The combination of trastuzumab plus MRK-003 GSI could benefit women with recurrent, or possibly resistant, ErbB-2-positive breast cancer to prevent disease progression, increasing percent disease free survival.
2. Model

**ErbB-2 is hyperactive**

When ErbB-2 is hyperactive, there is no signal sending or receiving cell. ErbB-2 positive breast cancer cells express equal number of Notch receptors and ligands. When ErbB-2 is active, Jagged-1 and Notch-1 co-localize to EEA-1 positive vesicles and there is a decrease in the cell surface expression of Jagged-1 levels. Moreover, PKCα is in complex with Jagged-1 and Jagged-1 protein is stabilized by ErbB-2’s activity. These events result in cis-inhibition of Notch activity.

![Diagram showing ErbB-2 Hyperactive (No signal sending or receiving cell), “Cis-Inhibition”](image-url)
ErbB-2 is Inactive

When ErbB-2 is inactive, there occurs a small stoichiometric change in the expression of Notch receptors and ligands resulting in two distinct cell types signal sending cell (High Jagged-1, low Notch) or signal receiving cell (High Notch, low Jagged-1). Upon anti-ErbB-2 therapy, we observed a decrease in total Jagged-1 protein and an increase in cell surface expression of Jagged-1. Notch-1 was distributed throughout the cell. Jagged-1 at the plasma membrane could engage with Notch expressed on adjacent cell. The pulling force required to pull ligand-N^EC and trans-activate Notch could be provided by an increase in Mib-1 binding and ubiquitylation of Jagged-1. These events ultimately result in trans-activation of Notch in signal receiving cell.

Anti-ErbB-2 Therapy
(Signal sending and receiving cell)
“Trans-Activation“
3. **Future Investigations**

Our studies demonstrated that ErbB-2 inhibitors decreased total Jagged-1 protein levels and that coincided with an increase in the cell surface levels of Jagged-1 and Notch activity. It would be important to investigate whether ErbB-2 activity regulates the amount of total and cell surface Jagged-1 to determine a dosage dependent effect of Jagged-1 on Notch activity. A time course of lapatinib treatment on SKBr3 cells could be performed to sort for cells exhibiting high levels of Jagged-1 at the cell surface. Cells expressing low-levels of Jagged-1 at the cell surface will be sorted from vehicle treatment group. The sorted cells could be analyzed using Jagged-1 and Hes-1 antibody by Western blot to determine the total amount of Jagged-1 and how the levels and localization of Jagged-1 regulates Notch activity in two distinct populations of cells.

Identification of the molecular mechanism underlying this regulation of Jagged-1 dose by ErbB-2 would be an important future study. ErbB-2 is a receptor tyrosine kinase which regulates a series of downstream phosphorylation events. Future studies will be aimed at identifying a kinase(s) whose expression and/or activity is regulated by ErbB-2 using a proteomic approach. Our data demonstrated that when ErbB-2 is active, PKC\(\alpha\) is in complex with Jagged-1 (Figure 26). As described previously, phosphorylation of Jagged-1 may change its conformation, regulate trafficking or regulate the amount of Jagged-1. Any one of these events could disrupt Mib-1 binding to Jagged-1 to prevent generation of a competent ligand needed to activate Notch. Therefore, a thorough investigation of the phosphorylation status of Jagged-1 and whether it plays a role in regulating total and cell surface Jagged-1 levels, Jagged-1 trafficking, and limiting the association between Mib-1 and Jagged-1 would be important future studies.
ErbB-2 inhibition decreased Jagged-1 protein levels, which in turn activated Notch in trans. To examine the role of Jagged-1 stability in ErbB-2-mediated regulation of Notch activity, inhibition of lysosomal entry using siRNA against Rab7, a protein localized in late endosome, or internalization to the lysosomal lumen using siRNA to LAMP, lysosomal-associated membrane glycoprotein, in SKBr3 cells in presence or absence of anti-ErbB-2 therapy could be performed. Notch activity will be measured by Real-time PCR. Inhibiting the Jagged-1 lysosomal degradation route will direct Jagged-1 protein to the recycling route and stabilize the Jagged-1 protein. Stabilizing Jagged-1 protein levels using siRNA against either Rab7 or LAMP should block the anti-ErbB-2 inhibitor-mediated decrease in Jagged-1 protein levels and possibly Notch activation.

To test the hypothesis that the kinase activity of ErbB-2 is required for Jagged-1-mediated regulation of Notch activity COS cells will be used. COS is a fibroblast-like cell line derived from monkey kidney tissue and lacks expression of ErbB-2, Jagged-1, and Notch-1. We will overexpress ErbB-2 wild type and its kinase dead mutant, as well as Jagged-1 and Notch-1 in COS cells. We will also transfect the cells with a Notch luciferase reporter construct to measure Notch activity. If ErbB-2 has a direct role in regulating Notch activity via Jagged-1, then using a kinase dead mutant of ErbB-2 should decrease protein levels of Jagged-1 and activate Notch as measured by an increase in luciferase activity.

Confocal microscopy studies showed that Jagged-1 co-localized with early endosome antigen-1 (EEA-1) to possibly submembraneous endosomes in ErbB-2 positive breast cancer cells (Figure 8). Jagged-1 exited EEA-1 positive vesicles and localized to the cell membrane in response to trastuzumab (Figure 8). We could examine the possibility that
ErbB-2 decreases Jagged-1 cell surface availability by either increasing the rate of endocytosis and/or decreasing the rate of recycling to the plasma membrane. To identify the role of endocytosis, down-regulation via siRNA of AP-2 (clathrin-mediated endocytosis), Dynamin (Dynamin mediated endocytosis) or caveolin (lipin raft mediated endocytosis) in SKBr3 cells in presence or absence of anti-ErbB-2 therapy could be performed. Jagged-1 distribution will be monitored with respect to markers (AP-2, Dynamin, and caveolin) that represent various types of endocytosis by confocal immunofluorescence. We could overexpress Rab11A/Rab4 in the presence or absence of trastuzumab or lapatinib to determine if Jagged-1 recycling to the plasma membrane is restored regardless of whether ErbB-2 is inhibited or not.

Our data showed that PKCα down-regulation via siRNA increases Notch activity (Figure 26). If PKCα is downstream of ErbB-2 and ErbB-2 inhibits Notch activity, it would be important to investigate whether PKCα or an unidentified kinase can abrogate lapatinib- or trastuzumab-mediated increase in Notch activity.

Additionally, Jagged-1 siRNA has been shown to up-regulate Notch activity. A thorough investigation of whether other Notch ligands or receptors regulate Notch activity in presence of Jagged-1 siRNA is thus an important future study. Inhibition of other Notch pathway components independently and in combination with Jagged-1 could explain the increase in Notch activity observed upon Jagged-1 down-regulation.

What these studies implied was that Jagged-1 mediated cis-inhibition of Notch could be dosage dependent and a general mechanism among different subtypes of breast cancer (Figure 16). After having a better understanding of the mechanism of this complex
regulation, we could determine the mechanism of Jagged-1 mediated cis-inhibition and trans-activation of Notch in other subtypes of breast cancer.

The effects of Jagged-1 down-regulation alone or in combination with trastuzumab on apoptosis were dramatic. To determine if the effects observed on cellular proliferation and apoptosis upon Jagged-1 knockdown were Jagged-1 mediated, we could perform a rescue experiment. If it is Jagged-1 mediated, then overexpression of wild type Jagged-1, but not J\textsuperscript{Ndr} mutant should rescue the effects of Jagged-1 siRNA and confer protection against apoptosis. If the results demonstrate Jagged-1 specific effect, then we could investigate if it is through the canonical Jagged-1–Mib-1–Notch-1–Hes-1 pathway. We could perform the exact same experiment but using siRNA to individual components and observe if it can recapitulate the Jagged-1 siRNA effects.

With the catastrophic effects observed on cell proliferation and apoptosis upon dual inhibition of Jagged-1 and ErbB-2 in ErbB-2 positive breast cancer cells, an \textit{in vivo} study using a doxycycline inducible Jagged-1 shRNA system could be designed. Xenografts of BT474 transfected with Jagged-1 shRNA whose expression is under the control of doxycycline could be injected into the mammary fat pads of nude mice. After the tumors grow, we would administer doxycycline and trastuzumab. Tumor burden and size could be measured to determine if dual inhibition has the same effects on tumor proliferation \textit{in vivo}. 
REFERENCES


VITA

The author, Kinnari Pandya, was born in Ahmedabad, Gujarat, India to Nina and Kirit Mehta. She currently resides in Chicago, Illinois with her husband Kunal Pandya.

Kinnari received her Bachelors of Science degree in Biochemistry and Post Graduate Diploma in Biotechnology from Gujarat University, Gujarat, India, in July of 2002. She continued her quest of gaining knowledge in the field of biochemistry by pursuing a Master of Science degree from Gujarat University where she graduated as a University Topper in June of 2004.

Due to further aspirations of gaining more knowledge in the field of science and research, she immigrated to the United States and received another Masters of Science degree in Molecular Biology from Illinois Institute of Technology (IIT), Chicago, Illinois. During her education at IIT, she pursued external research under the guidance of Dr. Jonna Frasor at University of Illinois at Chicago. Her thesis focused on understanding crosstalk between inflammation and estrogen that synergistically regulates a set of genes which promote the growth and progression of ERα positive breast tumors. She successfully completed her Master’s thesis in May of 2007.

In August of 2007, Kinnari joined the Ph.D. program in the Molecular Biology Department of Loyola University, Chicago. Shortly thereafter, she joined the laboratory
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Kinnari Pandya has accepted a position as adjunct professor in the Department of Biology at Elmhurst College. Also, following completion of her dissertation, she will continue her career as a Sr. Scientist at Abbott Molecular. She plans to use her knowledge of oncogenesis and expertise in troubleshooting Real Time PCR to design and evaluate diagnostic assays.