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The Role of Notch-1-Mediated Repression of PTEN on Growth and Cancer Stem Cell Survival in Trastuzumab Resistant, Her2+ Breast Cancer

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THE ROLE OF NOTCH-1-MEDIATED REPRESSION OF PTEN ON GROWTH AND CANCER STEM CELL SURVIVAL IN TRASTUZUMAB RESISTANT, HER2+ BREAST CANCER

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3’UTR	3’ Untranslated Region
4-OHT	4-hydroxytamoxifen
ACC	Adenoid Cystic Carcinoma
ACS	American Cancer Society
ADAM	A Disintegrin and Metalloprotease
ADCC	Antibody Dependent Cellular Cytotoxicity
AI	Aromatase Inhibitor
ALDH	Alcohol Dehydrogenase
ALKBH5	AlkB Homolog 5
ALTTO	Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization
ANK	Ankyrin Repeats
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
APH	Anterior Pharynx-defective
APP	Amyloid Precursor Protein
AREG	Amphiregulin
ATCC	American Type Culture Collection

ATP	Adenosine triphosphate
ATR	Ataxia Telangiectasia and Rad3-related protein
BC	Breast Cancer
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BCP	1-bromo-3-chloropropane
BCSC	Breast Cancer Stem Cell
bHLH	basic Helix-Loop-Helix
BIG	Breast International Group
BL	Basal-like
bp	Base Pairs
BRCA	Breast Cancer Susceptibility Gene
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
CAF	Cancer-Associated Fibroblasts
CBF-1	Core Binding Factor-1
CBNA	Cell Biology, Neurology, and Anatomy
CCL2	Chemokine (C-C motif) Ligand 2
CCNA2	Gene that encodes Cyclin A2
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
cDNA	complementary DNA

CENPA	Centromere Protein A
ChIP	Chromatin Immunoprecipitation
CK	Cytokeratin
c-Met	tyrosine-protein kinase receptor Met
CO_2	Carbon Dioxide
CoA	Co-Activator
CSC	Cancer Stem Cell
CSL	CBF-1, Su(H), Lag-1
c-Src	Proto-oncogene tyrosine-protein kinase Src
Ct	Cycle threshold
CtBP	C-terminal-Binding Protein
CTF	Carboxyl Terminal Fragment
CtIP	CtBP-Interacting Protein
CXCR	C-X-C Chemokine Receptor
Da	Dalton
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
DCIS	Ductal Carcinoma *In Situ*
DD	Dimerization Domain
DFS	Disease Free Survival
DLL	Delta-Like Ligand
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DUSP / MKP1	Dual specificity phosphatase / MAPK phosphatases
E_2	Estrogen
E2A	E-proteins E12 and E47
EC	Endothelial Cell
ECD	Ectodomain
ECM	Extracellular Membrane
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR/ErbB1/HER1	Epidermal Growth Factor Receptor 1
eIF4F	eukaryotic Initiation Factor 4F complex
EMT	Epithelial to Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
EphA2	Ephrin type A receptor 2
ER/ERα	Estrogen Receptor/Estrogen Receptor alpha
ErbB/HER	Epidermal Growth Factor Receptor
ErbB2/Neu/HER2	Epidermal Growth Factor Receptor 2
ERK	Extracellular signal–Regulated Kinases (MAPK)
ERRFI1	ErbB Receptor Feedback Inhibitor 1

ETS	E26 transformation-specific
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FcR	Fragment crystallizable region Receptor
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FOX	Forkhead box
GAP	GTPase-Activating Proteins
GATA	Transcription factor with DNA sequence GATA
GDF15	Growth Differentiation Factor 15
GDP	Guanosine Diphosphate
GEF	Guanine-nucleotide Exchange Factor
GEP	Gene Expression Profile
GPCR	G-Protein Coupled Receptor
Grb-2	Growth factor receptor-bound protein 2
GSI	γ-Secretase Inhibitor
GTP	Guanosine-5'-Triphosphate
HAT	Histone Acetyltransferase
HB-EGF	Heparin Binding EGF
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HES	Hairy and Enhancer of Split
HEY	Hairy/Enhancer of Split related with YRPW motif
HGF	Hepatocyte Growth Factor
HIF1α	Hypoxic-Inducible Factor 1 alpha
HMLE	Human Mammary Epithelial Cell
HMLER	Ras transformed Human Mammary Epithelial Cell
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
Hrg/Nrg	Heregulin/Neuregulin
hTERT	human Telomerase Reverse Transcriptase
IAP	Inhibitor of Apoptosis
IGF-1	Insulin-like Growth Factor-1
IGF-1R	Insulin-like Growth Factor-1 Receptor
IgG	Immunoglobulin G
IgG	Immunoglobulin E
IKK	IκB kinase
IL	Interleukin
IM	Immunomodulatory
INPPB	Inositol Polyphosphate-4-Phosphatase type 2
IP	Immunoprecipitate

Jag	Jagged
JAK	Janus kinase
JNK	c-Jun N-terminal Kinase
kD	kiloDalton
LAR	Luminal Androgen Receptor
lnc RNA	long non-coding RNA
LTT	Long-term Trastuzumab Treatment
M	Mesenchymal
MAML	Mastermind-like protein
MAP3K1	Gene encoding Raf (MAPKKK)
MAPK	Mitogen Activated Protein Kinase
MaSC	Multiprogenitor Mammary Stem Cell
MBC	Metastatic Breast Cancer
M-CSF	Macrophage Colony Stimulating Factor
MEK	MAPK/ERK Kinase (MAPKK)
MET	Mesenchymal to Epithelial Transition
MFE	Mammosphere Forming Efficiency
MI	Mitotic Index
MIC-1	Macrophage Inhibitory Cytokine 1
MIG-6	Mitogen-Inducible Gene 6
miR	Micro RNA
MKI67	antigen identified by monoclonal antibody Ki-67

MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
MRCK	Myotonic Dystrophy kinase-related CDC42-binding Kinase
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cell
MSL	Mesenchymal Stem-like
MTDH	Metadherin
mTOR	Mammalian Target of Rapamycin
mTORC	Mammalian Target of Rapamycin Complex
MUC	Mucin
MVP	Major Vault Protein
Myr-Akt	Myristoylated-Akt
N-1i	Notch-1 siRNA
NCoR	Negative Co-Regulator
NCR	Cytokine Response Element
ncRNA	non-coding RNA
NDF	Neu Differentiation Factor
NECD	Notch Extracellular Domain
NEDD4-1	Neural precursor cell Expressed Developmentally Downregulated 4 -1 protein
NEXT	Notch Extracellular Truncation

NF1	Neurofibromin
NFκB	Nuclear Factor kappa B
NH_2	Amino group
NICD	Notch Intracellular Domain
NK cell	Natural Killer Cell
NLS	Nuclear Localization Signal
NRR	Negative Regulatory Region
NTC	Notch Transcriptional Complex
NTMICD	Notch Transmembrane / Intracellular Domain
O_2	Oxygen
OH	Hydroxyl Group
Opti-MEM	Reduced Serum Media
ORF	Open Reading Frame
$p27^{Kip1}$	Cyclin-dependent kinase inhibitor 1B
p34cdc2	Cyclin Dependent Kinase 1
p4EBP1	Phospho- 4E-Binding Protein 1
p53	Protein 53
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly-ADP ribose Polymerase
PBS	Phosphate-Buffered Saline
PC5/6	Proprotein Convertase 5 / 6
PCNA	Proliferating Cell Nuclear Antigen

pCR	pathological Complete Response
PDCD4	Programmed Cell Death 4
PDGF	Platelet-Derived Growth Factor
PDGFB	Platelet-Derived Growth Factor subunit B
PDK	Phosphoinositol Dependent Kinase
PDX	Patient Derived tumor Xenografts
PDZ	PSD-95, Discs Large, Zona Occludens 1 proteins
PEA3	Polymavirus Enhancer Activator 3
PEI	Polyethylenimine
PEN	Presenilin Enhancer
PEST	Proline/Glutamic acid/Serine/Threonine
PHD	Plekstrin Homology Domain
PHD3	Prolyl Hydroxylase 3
PI3K	Phosphatidylinositol 3 Kinase
PIC	Protease Inhibitor Cocktail
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha (p110)
PIP_2	Phosphotidylinositol (4, 5)-bisphosphate
PIP_3	Phosphotidylinositol (3, 4, 5)-trisphosphate
PKB/Akt	Activated Protein Kinase B
PMSF	Phenylmethylsulfonyl Fluoride

Poly(A)	Adenosine monophosphate tail
PP2A	Protein Phosphatase 2
PPM1H	Protein Phosphatase 1H
PR	Progesterone Receptor
Pres-1	Presenilin 1
Pro	Proline Rich Region
P-Ser	Phosphorylated Serine
PTB	Phosphotyrosine Binding
PTEN	Phosphatase and tensin homolog deleted on chromosome TEN
PTENi	PTEN siRNA
PTN	Pleiotrophin
P-Thr	Phosphorylated Threonine
PTN	Pleiotrophin
PVDF	Polyvinylidene Fluoride
PY-HER2	Phospho-Tyrosine HER2
qPCR	Quantitative Polymerase Chain Reaction
Raf	Rapidly Accelerated Fibrosarcoma kinase (MAPKKK)
RALT	Receptor-Associated Late Transducer
RAM	RBP-Jκ Associated Molecule
Rheb	Ras homolog enriched in brain

RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
ROCK	Rho-associated, Coiled-coil-containing protein kinase
RPM	Revolutions Per Minute
Rq	Relative quantification
rRNA	ribosomal RNA
RTA	Replication and Transcription Activator
RTK	Receptor Tyrosine Kinase
RT-PCR	Real Time-Polymerase Chain Reaction
S1, 2, 3, 4	Scissile bond 1, 2, 3, 4
S6K1	S6 Kinase beta-1
SALL	Sal-like Protein
SAP97	Synapse Associated Protein 97
SC	Stem Cell
SCBi	Scrambled control siRNA
SDS	Sodium Dodecyl Sulfate
SERM	Selective Estrogen Receptor Modulator
SGK1	Serum and Glucocorticoid-regulated Kinase 1
SH	Src Homology
SHARP	SMRT/HDAC-1 Associated Repressor Protein
shRNA	Short Hairpin RNA

siRNA	Small interfering RNA
SIRT1	Silent mating type Information Regulation 2 homolog 1
SMAD	*SMA* gene for small body size / Mothers Against Decapentaplegic
SOS	Son of Sevenless
Src	Sarcoma, Proto-oncogene tyrosine protein kinase
STAT	Signal Transducer and Activator of Transcription
STR	Short Tandem Repeat
SV40	Simian Vacuolating virus 40
TAC	Transit Amplifying Cell
TACE	Tumor necrosis factor Alpha-Converting Enzyme
TAD	Transcription Activation Domain
T-ALL	T-cell Acute Lymphoblastic Leukemia
TAM	Tumor Associated Macrophages
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline plus Tween-20
Tbx3	T-box transcription factor 3
TCGA	The Cancer Genome Atlas

T-DM1	Trastuzumab-emtansine
TGF	Transforming Growth Factor
TH	T helper cell
TKI	Tyrosine Kinase Inhibitor
TMA	Tissue Microarray
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
TP53	Tumor Protein 53
TRB3	pseudokinase Tribble-3
tRNA	transfer RNA
TSC	tuberous sclerosis
Tyr	Tyrosine rich domain
Ub	Ubiquitin
US	United States
USNLM	United States National Library of Medicine
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
XIAP-XAF1	X-linked IAP-X-linked Association Factor-1 complex
YAP/TAZ	Yes-Associated Protein / Transcriptional coactivator with PDZ-binding motif

ABSTRACT

Trastuzumab targets the ErbB2 (HER2) receptor on breast cancer cells to attenuate HER2 driven tumor formation. Trastuzumab reduces both downstream PI3K/Akt and MAPK pathway signaling as well as the breast cancer stem cell (BCSC) population. BCSCs are hypothesized to be responsible for tumor recurrence, metastasis, as well as drug resistance. Today, resistance to trastuzumab remains a major clinical problem for women diagnosed with HER2+ breast cancer. Attenuation of PI3K/Akt and MAPK pathways may occur through the tumor suppressor, PTEN. Women with HER2+ breast tumors expressing less PTEN and increased PI3K/Akt or MAPK activity have worse overall outcome. Previously we have shown that trastuzumab resistant cells have increased expression of Notch-1 which drives cell proliferation *in vitro* as well as tumor recurrence *in vivo*. Here, we show, to our knowledge for the first time, that Notch-1 directly represses PTEN transcript RNA expression to promote trastuzumab resistant HER2+ breast cancer cell proliferation at least in part through activation of ERK1/2. Furthermore, we demonstrate that Notch-1 mediated inhibition of PTEN promotes BCSC survival and self-renewal both *in vitro* and *in vivo*.

CHAPTER 1

INTRODUCTION

Breast Development and Anatomy.

At birth, the mammary gland is identical in both males and females as it is comprised a series of ducts to form a tree-like structure (Moore and Persaud, 1998; Pandya and Moore, 2011). In females, the mammary gland develops into an exocrine gland that produces milk for the purpose of weening children. Thelarche is the onset of breast development which occurs primarily during puberty. At this time, an increase in ovarian estrogen and progesterone production in combination with somatotropin (human growth hormone) secretion from the pituitary gland promotes mammary gland formation inside the developing breast. Mammary gland development is completed upon the onset of pregnancy. During pregnancy, the breast enlarges due to estrogen, progesterone, prolactin, growth hormone, and placental hormones. This burst of female hormones drives proliferation of the glandular epithelium that replace breast tissue stromal elements. During the third trimester of pregnancy, the epithelium differentiates into secretory cells for the synthesis and secretion of milk. After birth, the release of oxytocin promotes myoepithelial cell proliferation and differentiation in preparation for the nursing of young (Beesley and Johnson, 2008). After delivery, estrogen and progesterone levels fall while prolactin, insulin, and growth hormones rise for milk production.

Milk secretion is induced reflexively by a suckling infant and regulated by oxytocin. As nursing of the infant decreases, the glandular, stromal, and ductal elements of the breast atrophy resulting in reduced breast size. Similarly, there is an overall reduction in mammary gland ducts and lobules as well as fat and stromal elements resulting in a reduction in breast size and volume during menopause (Macias and Hinck, 2012).

During embryogenesis, mammary glands develop from the primary epidermal ectoderm layer cells invading the underlying mesenchymal tissue (Drew et al., 2007). As the mammary gland develops, the primary bud of the mammary gland branches into 15 to 20 secondary buds that become lactiferous ducts. The breast tissue is comprised of 15 to 20 lobes that are divided into 4 lobules and each lobe is comprised of branched tubuloalveolar glands. The lobes drain into major lactiferous ducts that then drain into a lactiferous sinus which can be accessed through the nipple orifice. Similarly, the breast is divided into four quadrants: upper inner, upper outer, lower inner, and lower outer. The most common place for the development of breast tumors is in the upper outer quadrant where a majority of the breast volume exists (Clough et al., 2010).

Breast Cancer.

Breast cancer remains the leading cause of cancer-related deaths among women world-wide (ACS, 2015). In the United States, it is estimated that 246,660 women will be diagnosed with invasive breast cancer and 61,000 women will be diagnosed with breast cancer *in situ* in 2016, according to the American Cancer

Society. Additionally, it is estimated that 40,450 women in the U.S. will succumb to breast cancer. For men, it is estimated that there will be 2,600 new cases of invasive breast cancer as they tend to ignore breast cancer symptoms resulting in breast cancer diagnosis at a later stage compared to women. Women have a 1 in 8 risk of developing invasive breast cancer within their lifetime. This statistic has risen since 1975 (1 in 11 risk of breast cancer) mainly due to an increase in early detection and breast cancer awareness (breastcancer.org).

Cancer is not a single disease but a group of diseases that causes cells in the body to uncontrollably grow and proliferate compared to normal, healthy cells. Unregulated proliferation of cancer cells can form a tumor which is named after the tissue in which it originates; hence breast cancer is dubbed so due to the formation of tumors in the breast tissue. Breast cancer can have several different forms depending on the type of breast tissue that the tumor develops in as well as the expression of various biomarkers by the breast cancer cell. Breast cancer typically forms from cells of the lactiferous ducts (ductal carcinoma) or the lobules (lobular carcinoma) that supply the milk. There are over 18 sub-types of breast cancer which include tubular, medullary, mucinous, and papillary ductal carcinoma depending on the tumor origin. Using histopathology techniques, breast cancer grade as well as stage can help determine the progression of the disease. Cancer grade indicates the amount of disorganization occurring in the cancer cell. Cancer stage indicates the degree of invasiveness of the cancer. Both grade and stage of the cancer assist in determining the prognosis of the cancer and the treatment

course that will be taken. Several factors can affect breast cancer diagnosis including differences in race, diet, alcohol consumption, smoking, physical activity, hormone replacement therapy, and family history.

Breast Cancer Subtypes.

The intrinsic heterogeneity of breast cancer becomes apparent when determining how to organize the various subtypes of breast cancer that can be present in each woman. Beyond grading and staging of breast cancer, determination of the biological features of the breast cancer provides a better understanding of its cellular processes which in turn improves the treatment strategy for the particular breast cancer subtype. Today, there are three major classes of breast cancer: luminal, human epidermal receptor 2 (ErbB2, HER2), and triple negative (TNBC) which lacks detectable overexpression of pharmacologically targetable receptors. The first breast cancer “molecular portraits” were pioneered by Perou et al. and Sorlie et al. (Perou et al., 2000; Sørlie et al., 2001). These two research groups used microarray technology to establish the five intrinsic subtypes of breast cancer that persist today. The five subtypes of breast cancer are: Luminal A which overexpresses hormone receptors, estrogen and / or progesterone (ERα+/PR+); Luminal B, or triple positive, which overexpresses hormone receptors as well as the HER2 receptor (ER+/PR+/HER2+); HER2 positive (HER2+) which overexpresses the HER2 receptor; triple negative breast cancer (TNBC), or basal breast cancer, which lack overexpression of the hormone or HER2 receptors (ER-/PR-/HER2-); and normal-

like breast cancer which has gene expression patterns much like those displayed by cells in normal breast tissue. Many studies have been done to reinforce the predominance of these molecular subtypes as well as continue to define subtypes within these subtypes thereby further classifying breast cancer into specific categories. Tissue Microarrays (TMAs) by Abd El-Rehim et al. validate the categorization of breast cancer into five subtypes by further characterization of protein expression pertinent to cell differentiation, epithelial cell lineage, hormone and growth factor receptors, as well as proteins known to be altered in some breast cancer subtypes (Abd El-Rehim et al., 2005). Breast cancer can be further classified according to disease aggressiveness by comparing differences in: cell proliferation markers Ki-67 (*MKI67*) (Cheang et al., 2009), Mitotic Index (MI), Proliferating Cell Nuclear Antigen (PCNA) (Stuart-Harris et al., 2008); as well as relapse-free and overall survival (Hu et al., 2006).

Formation of different biological characteristics for each breast cancer subtype segregates these subtypes by distinct biological behaviors that lead to a variety of treatment responses. Continued analysis of the different breast cancer subtypes may complicate our understanding of the disease by diverging the subtypes into more refined groups as well as converging the major subtypes. Further assessment of breast cancer heterogeneity allows clinicians to gain insight into tumor progression in order to more accurately diagnose and treat breast cancer.

Luminal A. Luminal A is the most common breast cancer subtype, affecting nearly 70% of women (breastcancer.org). Luminal A breast cancer is categorized by it is overexpression of ERα and/or PR, low proliferation index (low Ki67), and low tumor grade. Oncogenic mutations have been identified in the luminal A subtype affecting multiple signaling pathways such as, PI3K (*PIK3CA*: 49%), p53 (*TP53*: 12%), MAPK (*MAP3K1*: 14%), as well as cell differentiation (*GATA3*: 14%) which can be used as predictors for the luminal A subtype (TCGA, 2012). The luminal A subtype has the best prognosis among the five breast cancer subtypes. Luminal A breast cancer is commonly treated with endocrine disrupting therapies such as aromatase inhibitors (AIs) or tamoxifen in conjunction with surgery such as a lumpectomy, quandrantectomy, partial mastectomy, mastectomy, or double mastectomy in an effort to reduce breast cancer recurrence.

The aromatase enzyme synthesizes forms of estrogen by converting testosterone to 17β-estradiol, or androstenedione to esterone (Santen and Harvey, 1999). There are two types of AIs, the irreversible (exemestane) and reversible (anastrozole or letrozole) that act as competitive inhibitors by binding to the aromatase enzyme. An AI is used to treat luminal A (ER+/PR+) breast cancer by selectively targeting the enzymatic activity of the aromatase enzyme thereby blocking estrogen synthesis. Tamoxifen is classified as a selective estrogen-receptor modulator (SERM) indicating that it acts directly on estrogen receptor α (ERα). Tamoxifen is a prodrug that is metabolized by the liver (Desta et al., 2004). Once metabolized, tamoxifen is converted to many metabolites. The most

abundant are 4-hydroxytamoxifen (4-OHT) and endoxifen which compete with 17β-estradiol for ERα resulting in reduced ERα-mediated transcription in the breast cancer cell. An aromatase inhibitor or tamoxifen is given as an adjuvant therapy to prevent potential breast cancer relapse after surgery. Aromatase inhibitors are primarily given to post-menopausal women who have hormone sensitive tumors while tamoxifen is given to premenopausal women as AIs can promote a compensational increase in estrogen production in younger women (Mathew and Davidson, 2015). Anti-hormonal therapies have been proven successful for the treatment of the luminal A subtype but current work shows that approximately 50% of women may not benefit from adjuvant endocrine therapy (Ejlertsen et al., 2010). Also, of the 50% of women that do show a response, about 40% will develop resistance to the AI or tamoxifen resulting in tumor recurrence or metastatic spread.

Luminal B. The luminal B breast cancer subtype affects 10 to 20% of women with breast cancer (ACS, 2015). Luminal A and B breast cancer cells are derived from the luminal epithelium of ducts in the breast tissue. Luminal B breast cancer is categorized by an increase in ERα/PR expression as well as a high Ki67 positivity (Ades et al., 2014). Increased HER2 receptor expression occurs in approximately 50% of women with the luminal B subtype causing this subtype to also be referred to as the triple positive subtype. Luminal B breast cancer is a more aggressive subtype compared to luminal A with similar DNA mutations except for *GATA3*. Luminal B has a high incidence of p53 mutation (*TP53*: 32%) as well as

chromatin hypermethylation compared to luminal A (TCGA, 2012). The luminal B subtype has the second best prognosis and can be treated with targeted therapies such as the anti-hormonal therapies used for luminal A as well as HER2 targeted therapies. The targeted therapies are often combined with chemo- and/or radiation therapy. Common chemotherapeutics used to treat breast cancer are tubulin-destabilizing taxanes (docetaxel, paclitaxel), DNA stabilizers (doxorubicin, camptothecin), DNA untangling (topoisomerase II), or DNA damaging alkylating / methylating agents (cyclophosphamide, temozolomide).

Triple negative breast cancer. Triple Negative Breast Cancer (TNBC) affects 15 to 20% of women with breast cancer (ACS, 2015). TNBC is categorized by its lack of ERα, PR, and HER2 receptor overexpression and thus cannot be treated with anti-hormonal or HER2-targeted agents. TNBCs have a high incidence of p53 mutations (*TP53*: 84%), loss of DNA repair protein expression (i.e. BRCA-1/2), as well as chromatin hypomethylation to further distinguish TNBC from the other BC subtypes (TCGA, 2012). TNBC includes basal-like and normal-like cancers which also lack targetable receptor overexpression (Perou et al., 2000). Basal-like tumor cells are similar to basal cells which surround the mammary duct and are also known as myoepithelial cells. Basal-like tumor cells are so named because of their expression of basal cell markers such as cytokeratin (CK) 5, 6, 14, and 17 (Perou et al., 2000; Sotiriou et al., 2003). While most TNBCs are basal-like and most basal-like are classified as TNBC, this is not true for all TNBC and basal-like BCs, hence the distinction between the two subtypes. Due to the lack of targeted

therapies for TNBC, it has the worst prognosis among the five BC subtypes. The first line therapy for TNBC is surgery combined with chemo- and/or radiation therapy.

Research continues to further classify TNBC in hopes of finding new treatable targets for the disease. Thus far, six new subtypes of TNBC have been established: Basal-like 1 and 2 (BL1, 2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) (Lehmann et al., 2011). The basal-like 1 and 2 subtypes are classified by increased cell cycle associated genes (*CENPA, CCNA2*) and DNA damage response (*BRCA/ATR*) gene expression. BL1 and 2 show sensitivity to platinum-based chemotherapeutics such as cisplatin as well as the single strand DNA repair protein, poly-ADP ribose polymerase (PARP) inhibitor. The immunomodulatory subtype displays increased signaling through the immune cell signaling pathways (TH1, TH2, B cell, NK cell) and has a similar GEP to medullary BC, which is associated with a favorable diagnosis. Mesenchymal and mesenchymal stem-like subtypes have increased epithelial to mesenchymal transition (EMT) markers (TWIST, SNAI2, ZEB1) and growth factor pathways (PDGF, EGFR, GPCR) as well as a claudin-low (Claudin 3, 4, 7) gene signature. Claudin low is similar to the M/MSL subtypes of TNBC as they both have high expression of EMT markers, growth factor pathways (EGFR), and resemble basal breast cells (Prat et al., 2010). Both mesenchymal and mesenchymal stem-like subtypes show increased sensitivity to the PI3K/mTOR inhibitor NVP-BEZ235. The luminal androgen

receptor (LAR) subtype is driven by increased androgen receptor signaling and is sensitive to an androgen receptor antagonist such as bicalutamide.

Normal-like breast cancer. Some reports fold normal-like breast cancer under the umbrella of TNBC as the normal-like breast cancer cells lack detectable overexpression of pharmacologically targetable receptors. Like its name suggests, normal-like breast cancer shares a GEP with normal cells in the breast tissue such as adipose or myoepithelial (basal) cells and dissimilarity to luminal cells (Perou et al., 2000). Normal-like breast cancer may be stratified by its overexpression of Alcohol Dehydrogenase 1B (ALDH1B) (Perou et al., 2000) as well as increased activation of the focal adhesion pathways (Smid et al., 2008). Normal-like breast cancers have a tendency to have a better prognosis than basal-like cancers (Fan et al., 2006), but typically do not respond to neoadjuvant chemotherapy (Rouzier et al., 2005). This subtype is consistently the smallest subtype among the five as it could be a luminal A subtype that is contaminated by normal breast cells during GEP analysis.

HER2+ Breast Cancer.

HER2 activity has been shown to promote ductal elongation, ductal branching, and modification of terminal end bud structures during pubertal mammary gland development (Andrechek et al., 2004; Jackson-Fisher et al., 2004). The role of HER2 during mammary gland development indicates that it is also plays a role in breast cancer. Approximately 15 to 25% of breast cancers overexpress the HER2 receptor. HER2 activity is a driving effector of signal

transduction networks that become dysregulated in cancer cells (Slamon et al., 1987). Increased HER2 expression in cancer cells is mostly due to amplification of the *ERBB2* gene located on chromosome 17. Overexpression of HER2 can drive oncogenesis making HER2 a bonafide proto-oncogene. The oncogenic activity of HER2 confers a strong proliferative advantage to tumor cells, which promotes an increase in tumor size (van de Vivjer et al., 1988), lymph node invasion (Berger et al., 1988), aneuploidy (Babiak et al., 1991) percentage of cells in S-phase (O'Reilly et al., 1991), and tumor grade (Tsuda et al., 1990). HER2 overexpression has been associated with aggressive disease and a worse, overall prognosis in women (Adair et al., 2008). Several types of human solid tumors have displayed HER2 overexpression, including breast cancer, in which a majority of HER2 oncogenic activity has been studied. Patient samples of the HER2+ subtype have undergone further evaluation to begin separating the HER2+ subtype into HER2 low, medium, and high expression profiles to better facilitate targeted treatment of HER2 expressing cancers (Wulfkuhle et al., 2012). ERα status has been shown to induce expression of different genes when coupled with HER2 overexpression creating two distinct subgroups: HER2+/ER+ and HER2+/ER- within the HER2+ breast cancer subtype (TCGA, 2012). Fortunately, HER2 overexpression primarily occurs in tumor cells and not in normal tissue making it an ideal target for breast cancer therapy.

EGFR/HER pathway. HER2 is a type 1 transmembrane protein receptor tyrosine kinase (RTK) and member of the Epidermal Growth Factor Receptor (EGFR)/HER family of receptors (Reviewed in Yarden and Sliwkowski, 2001). EGFR/HER receptors are expressed on the cell surface and activated by members of the Epidermal Growth Factor (EGF) family of extracellular ligands. The EGFR/HER family of receptors are comprised of four structurally related RTKs with EGFR as the original member of the family. In humans, these include: EGFR (EGFR, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4). The gene symbol for HER2, *ERBB2*, is derived from a homologous viral oncogene, Erythroblastic Leukemia Viral Oncogene, and has the official name: V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog or HER2 consequently (USNLM, 2011). The HER2 gene has been mapped on chromosome 17q21 which can be transcribed and translated into the 1225 amino acids to form the 185kD ($p185^{HER}$) transmembrane glycoprotein known as the HER2 receptor (Coussens et al., 1985).

The EGFR/HER family of receptors are activated through receptor homo- or hetero-dimerization induced by binding of a soluble, growth factor ligand. EGFR/HER receptor ligands are produced by ectodomain shedding of the membrane anchored ligand precursors from the surface of the cell by matrix metalloproteinases such as MMP9 or ADAM12 (Reviewed in Higashiyama et al., 2008). EGFR/HER receptor activation results in activation of downstream pathways to elicit an appropriate intracellular response to extracellular stimuli.

EGFR is activated by at least 11 different growth factor-ligands including EGF, Heparin binding EGF (HB-EGF), amphiregulin (AREG), or Transforming Growth Factor alpha (TGFα). In contrast, HER3 and HER4 are activated by the heregulin (neuregulin) family of growth factors (Hrg/Nrg 1, 2, 3, 4) (Figure 1). The EGFR/HER receptor ligands are classified by their epidermal growth factor-like domain which is comprised of three intramolecular loops formed by disulfide bonds (Harris et al., 2003). The EGFR/HER receptors contain an extracellular, transmembrane, and intracellular domain. The extracellular domain is made up of four subdomains, I-IV. The ligand binds to extracellular subdomains I-III which take on a “C” shape to receive the ligand (Ogiso et al., 2002; Garrett et al., 2003) (Figure 2). The ligand binds to subdomains I and III which result in the conformation of the receptor to “open” in preparation for dimerization. Dimerization of ligand bound receptors is facilitated by a β-hairpin structure from the cysteine rich subdomain II otherwise known as the “dimerization loop”. The dimerization loop holds the neighboring receptor by making specific contacts that stabilize dimerization of the two receptors. Dimerization is also stabilized by interactions between subdomain IV, the transmembrane domain, and the intracellular domain (Dawson et al., 2005). EGFR/HER receptors unbound by a ligand are in a “closed” or “autoinhibited” state in which subdomain II and IV interactions sequester the dimerization loop (Ferguson et al., 2003).

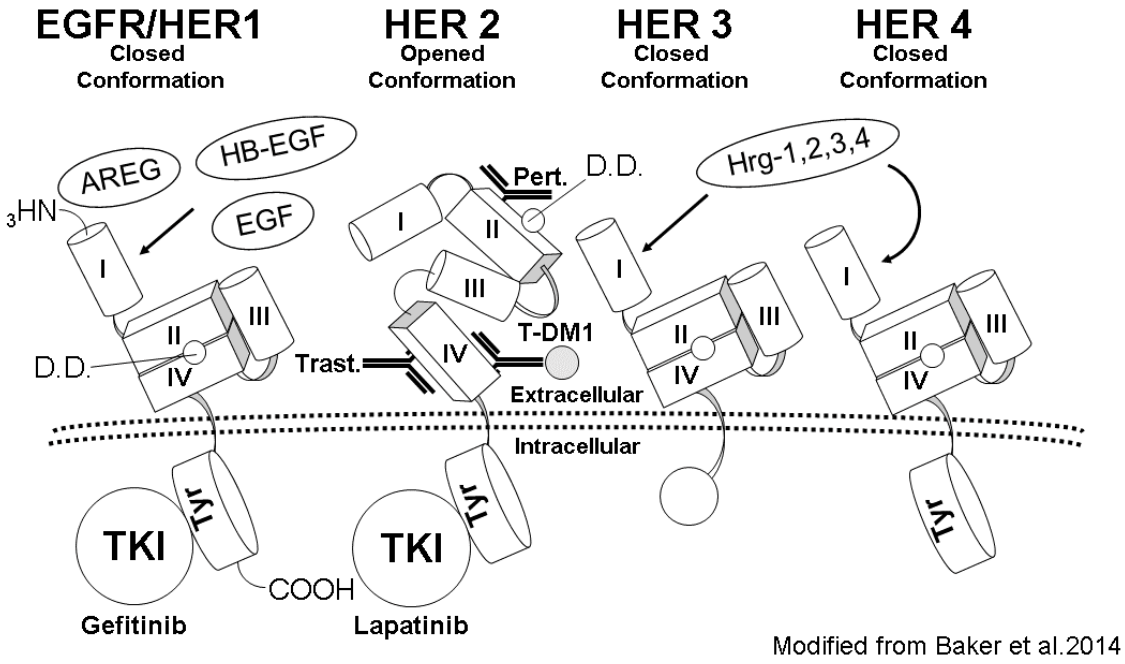

Figure 1: EGFR/HER Receptors

Members of the EGFR/HER family of receptors: EGFR/HER1, HER2, HER3, and HER4. Each receptor is composed of 4 extracellular domains (I-VI) and a tyrosine rich (Tyr) intracellular domain. Growth factor ligands such as amphiregulin (AREG), Epidermal Growth Factor (EGF), heparin binding EGF (HB-EGF), and heregulins 1-4 bind to and activate the EGFR/HER1 and HER3/4 receptors, respectively. Ligand binding causes rearrangement of the receptor exposing the dimerization domain (D.D.) to assume an open and active conformation. The HER2 receptor takes on a fixed active conformation while HER3 has a truncated, inactive intracellular domain. HER receptor activity can be blocked by small tyrosine kinase inhibitors [(TKIs) Gefitinib or Lapatinib], antibodies (pertuzumab or trastuzumab), or antibody-drug conjugates (T-DM1).

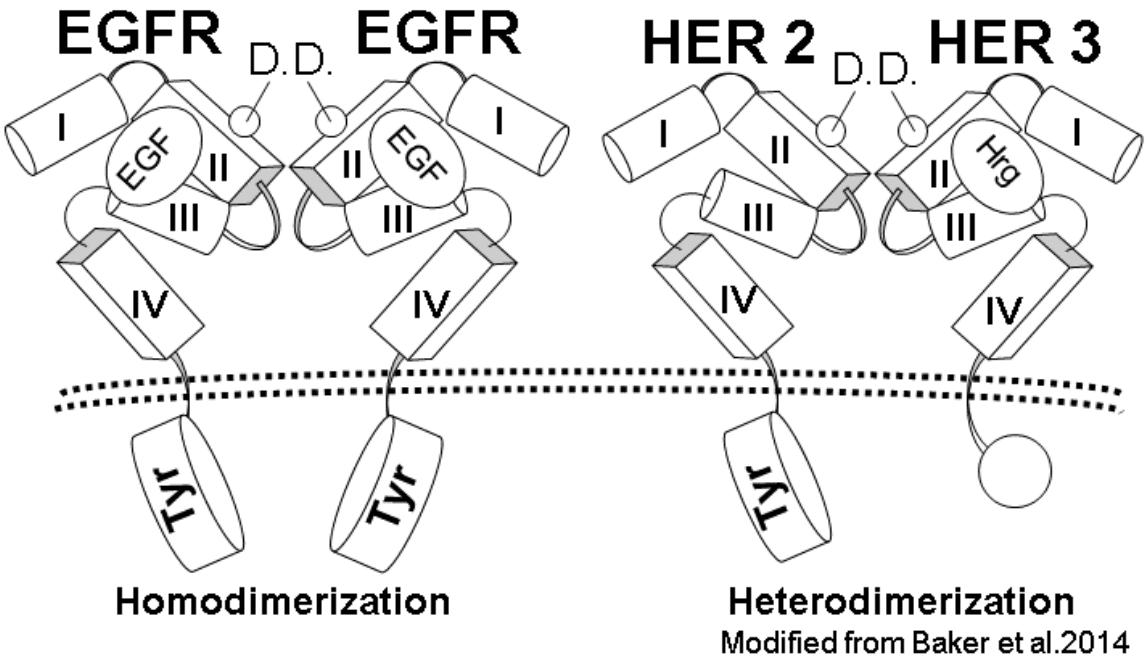

Figure 2: EGFR/HER Receptor Dimerization
EGFR/HER binding of a ligand exposes the dimerization domain (D.D.) of the receptor enabling the formation of homodimers (left: EGFR-EGFR) or heterodimers (right: HER2-HER3).

EGFR/HER receptors have a highly conserved tyrosine kinase domain in the intracellular domain of the receptor. After ligand-mediated dimerization of EGFR/HER receptors, the dimerized receptors autophosphorylate then transphosphorylate tyrosine residues in the tyrosine kinase domain (Figure 3). Tyrosine phosphorylation enables the activation of downstream pathways through the recruitment of adaptor proteins containing phosphotyrosine binding (PTB) or Src homology 2 (SH2) domains. Adaptor protein docking to the EGFR/HER receptors is able to elicit the activation of several downstream pathways including the Protein Kinase B (PKB/Akt) and the Mitogen Activated Protein Kinase (MAPK)

pathway (Figure 4). Activation of downstream pathways is dictated by the tyrosine phosphorylation pattern of the tyrosine kinase domain as 5 to 8 amino acids around the phosphorylated tyrosine residues confer specificity of PTB or SH2 binding (Songyang et al., 1995). Ligands can regulate RTK dimerization as well as the strength and duration of downstream signaling by influencing the tyrosine phosphorylation pattern (Olayioye et al., 1998; Sweeney et al., 2000). EGFR/HER-mediated activation of downstream pathways enables a ligand to elicit proliferation, survival, and/or migration signals to the cancer cell.

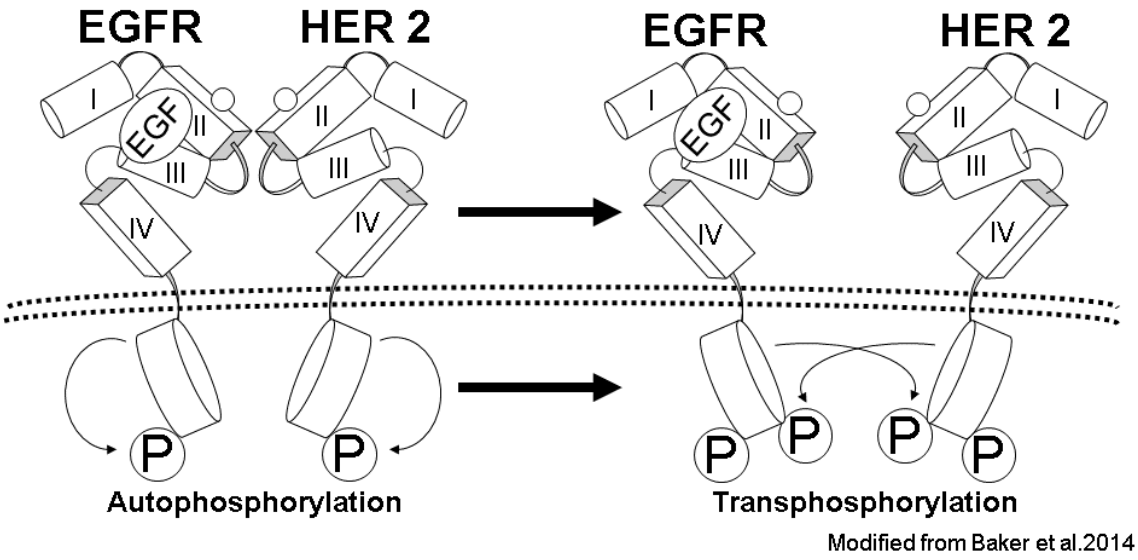

Figure 3: EGFR/HER Receptor Phosphorylation
EGFR/HER dimerization promotes auto-phosphorylation then transphosphorylation of the tyrosine residues in the receptor intracellular domains.

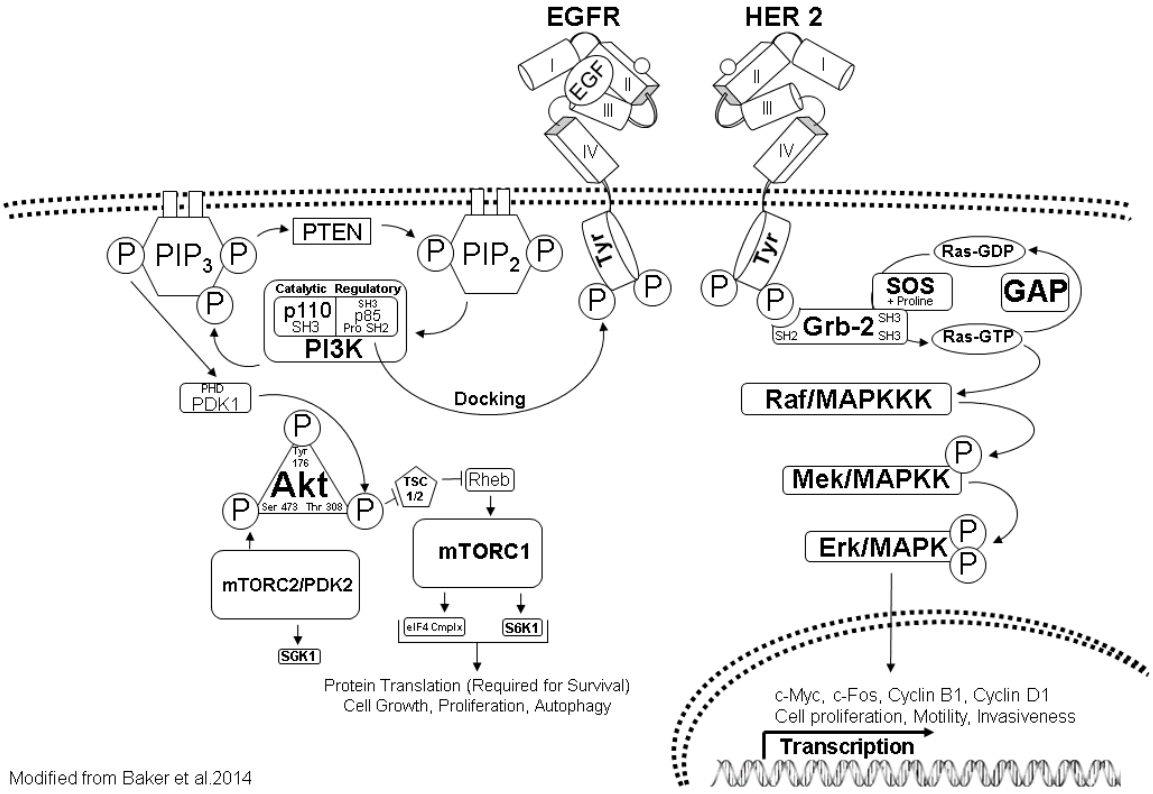

Figure 4: EGFR/HER Receptor Activation of Downstream PI3K/Akt and MAPK Pathways.
Activated EGFR/HER receptors are able to activate downstream PI3K/Akt (left) and/or MAPK (right) pathways.

All four members of the EGFR/HER family of RTKs share structural similarities, but they each have specific features that confer unique regulatory characteristics. Unlike EGFR, the extracellular region of HER2 resembles a ligand activated or “open” state, making HER2 an orphan receptor as there is no known ligand that binds to HER2 with high affinity. Thus the constitutively active state of HER2 does not necessitate ligand binding for its activation and is primed for dimerization with other RTKs (Cho et al., 2003; Garrett et al., 2003). HER3 is a kinase dead RTK meaning that its intracellular domain does not possess kinase

activity and can only potentiate downstream signaling when dimerized with another HER receptor. The fixed state of HER2 makes it the preferred dimerization partner of the HER receptors, particularly HER3. HER2-partnered heterodimers are more stable and display increased ligand binding and kinase activity compared to other heterodimers. In addition, HER2 has been shown to dimerize with other RTKs, such as Insulin-like Growth Factor-1 Receptor (IGF-1R) (Nahta et al., 2005). HER2 dimerization requires one ligand whereas dimers without HER2 require two ligands for downstream signaling. Therefore, dimerization with HER2 lowers the amount of ligands necessary for activation of downstream pathways (Graus-Porta et al., 1997). Together, EGFR/HER signaling is a complex mechanism capable of activating a number of downstream pathways. HER2 can act as a lateral signal transmission between other HER receptors (Graus-Porta, et al., 1997) as well as amplifying activation and strength of other RTKs (Karunagaran et al., 1996).

HER2 activation of downstream PI3K/Akt and MAPK pathways. An important trait of the HER family of receptors is their ability to activate protein kinase B (PKB/Akt) and MAPK pathways. Activation of downstream pathways is executed through a series phosphorylation events. HER2:HER3 dimerization preferentially activates the PI3K/Akt pathway (Graus-Porta et al., 1997). PI3K is a heterodimer comprised of the regulatory subunit, p85, bound to the catalytic subunit, p110. The p85 subunit uses its SH2 domain to dock to specific phosphotyrosine residues on the activated HER receptor [Figure 4 (on EGFR)]. Binding of p85 to EGFR recruits the p110 subunit through its Src homology domain

3 (SH3). PI3K is now fully activated and it phosphorylates the 3'OH of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP_3). PIP_3 can be dephosphorylated by the Phosphate and Tensin Homolog Deleted on chromosome TEN (PTEN) phosphatase back to PIP_2 thereby attenuating PI3K signaling. PIP_3 interacts with the Plekstrin homology domain (PHD) within the catalytic domain of phosphoinositol dependent kinase-1 (PDK1) thus activating the kinase. The lipid binding PHD recruits Akt to the intracellular surface of the plasma membrane where it docks to PIP_3. It is important to note that the physical interactions and activations of PI3K, PTEN, PDK1, and Akt occur at the outer surface of the plasma membrane inside the cell. PDK1 is able to phosphorylate Akt at the threonine308 residue and mTORC2/PDKs is able to phosphorylate Akt at serine473. Phosphorylation of Akt at both threonine308 and serine473 is necessary for full activation of Akt. Activated Akt can activate the mechanistic target of rapamycin (mTOR) which is made up of two protein complexes: mammalian target of rapamycin complex 1 (mTORC1) and 2 (mTORC2). Activated Akt is able to disrupt the formation of the tuberous sclerosis 1/2 (TSC1/2) dimer. The TSC1/2 dimer inhibits hydrolyzation of the GTP binding protein, Ras homolog enriched in brain (Rheb). Akt-mediated inhibition of the TSC1/2 complex allows Rheb to activate mTORC1. The mTORC1 complex is able to promote cell growth, proliferation, and autophagy by directing protein translation through eukaryotic initiation factor 4F complex [eIF4F (eIF4E, G, B, etc.)] and ribosomal protein S6 kinase beta-1 (S6K1 or p70S6K). Both eIF4F and S6K1

initiate transcription and ribosomal protein synthesis, respectively. The mTORC2 complex is involved in cellular metabolism but largely facilitates fluctuations in cytoskeletal formation and degradation throughout the cell via serum and glucocorticoid-regulated kinase 1 (SGK1).

Activation status of PI3K has been implicated in promoting tumorigenesis through the expression of *PIK3CA* (p110 gene) mutations. *PIK3CA* mutations constitutively activate PI3K which in turn increase oncogenic signaling by Akt. Additionally, increased PI3K activity can be promoted by loss of the PIP_3 phosphatase, PTEN. Due to the role of Akt in a variety of solid tumors and hematological malignancies, several therapies have been developed to target effectors of the Akt pathway in an attempt to diminish tumor survival. Akt pathway targeted therapies include: Wortmannin and LY294002, which are reversible and non-reversible inhibitors of PI3K, respectively; and Rapamycin which targets mTOR signaling downstream of Akt.

HER2 overexpression can result in homodimerization of HER2 which preferentially activates the MAPK pathway (Ghosh et al., 2011). Similar to p85, the SH2 domain of Grb-2 is able to dock to phosphorylated tyrosine residues on the intracellular domain of HER2 [Figure 4 (on HER2)]. Grb-2 is a scaffold protein that contains both SH2 and SH3 domains. Once Grb-2 is bound to phosphotyrosine residues on HER2, it can recruit the son of sevenless (SOS) protein. SOS contains a proline rich regions that binds to the SH3 domains of Grb-2. SOS is a guanine-nucleotide exchange factor (GEF) that acts on Ras-GDP. SOS facilitates the

exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) binding to form Ras-GTP, and initiating the MAPK phosphorylation cascade. GTPase Activating Protein (GAP) reduces Ras GTPase activity by hydrolyzing GTP bound to Ras thereby returning Ras to its inactive state (Ras-GDP), and thus limiting downstream phosphorylation of the MAPK pathway. Ras activation initiates the MAPK phosphorylation cascade, which is executed through a series of serine/threonine specific kinases. The rapidly accelerating fibrosarcoma/mitogen activated protein kinase kinase kinase (Raf/MAPKKK), mitogen activated protein kinase kinase (MEK/MAPKK), and extracellular signal-related kinase 1/2 / mitogen activated protein kinase (ERK/MAPK) are the main MAPK proteins. Ras-GTP binds Raf and initiates activation of Raf. Subsequently, Raf phosphorylates MEK and active MEK phosphorylates ERK. Activation of ERK1/2 facilitates phosphorylation and activation of transcription factors such as c-Fos and c-Myc. Activation of c-Myc and c-Fos initiate transcription of cyclin D1 and many other genes critical for coordination of cell motility, invasiveness, and proliferation (Reviewed in Hynes and Lane, 2005).

Both the MAPK and Akt pathways are known as central nodes in cancer whose increased activity have been shown to trigger tumorigenesis and metastasis. Several inhibitors have been designed to target the MAPK pathway such as: vemurafenib which inhibits Raf or U0126 which inhibits MEK1/2. Inhibition of Raf or MEK1/2 reduces downstream ERK1/2 phosphorylation and subsequent MAPK activity. EGFR or HER2-meidated stimulation of cell proliferation and

survival pathways can result in cancer cells to become addicted to these pathways in order to maintain tumor growth and survival (Sharma et al., 2006).

HER2 crosstalk. It has come to light that cancer cells are able to become resistant to targeted therapies by increasing compensatory pathways that continue downstream activation of protein synthesis, i.e., the Akt pathway, and/or transcriptional up-regulation of survival genes mediated by the MAPK pathway. Crosstalk, or bidirectional communication, between pathways to sustain cancer cell growth and survival is one mechanism by which cancer cells evade targeted therapy. Extensive research has been done on G-Protein Coupled Receptor (GPCR) crosstalk with EGFR/HER2 as some GPCR agonists such as Lysophosphatidic acid, carbachol (muscarinic acetylcholine inhibitor), and thrombin are known to increase HER activation by two independent mechanisms (Reviewed in Carpenter, 1999). The first consists of a GPCR-dependent surge in ectodomain shedding of HER ligands thus releasing them to bind and activate HER signaling. The second includes GPCR activation of c-Src to facilitate phosphorylation of tyrosine residues on HERs. HER activation via GPCR may be necessary for GPCR-mediated mitogenic activity via the MAPK pathway. Both HER-MAPK pathway initiation, as well as activation of a number of steroid hormone receptors, can initiate the transcription of HER ligands and trigger a positive feedback loop. HER2 has been shown to increase p53 expression through an unknown mechanism in skin squamous-cell carcinoma (Casalini et al., 2001). HER2 has been shown to induce p53 expression in HER2+ breast cancer cells

which may attribute to changes in the Cyclin Dependent Kinase (CDK)/Cyclin activity and regulation of cell division. Mechanisms of crosstalk between the HER family of RTKs and several other pathways including the: Wnt/β-catenin (Ayyanan et al., 2006), TNFa/IKK/NF-κB (Cao et al., 2007), and Notch pathways (Yamaguchi et al., 2008) have been under investigation for nearly a decade. Current data indicate that new mechanisms of crosstalk have been implicated in treatment resistance, Epithelial to Mesenchymal Transition (EMT), as well as enrichment and survival of the breast cancer stem cell (BCSC) population.

Anti-HER2 Therapies. ***Trastuzumab.*** HER2 is an ideal target for cancer cells as targeting of HER2 was found to be highly specific and have little off-target effects through the use of a murine anti-p185 antibody against HER2 which was shown to selectively inhibit growth of murine-derived *neu*-transformed cells and not Ras-transformed cells (Drebin et al., 1986). Consequently, a humanized, monoclonal antibody against HER2 in humans was shown to be highly effective in reducing proliferation of *HER2*-amplified cells *in vitro*, and promoted the antitumor effect of doxorubicin and paclitaxel in HER2+ breast cancer tumor xenograft models *in vivo* (Pegram et al., 1998). Anti-HER2 therapies have significantly improved the outcome of women with HER2+ breast cancer (Hicks et al., 2015; Michiels et al., 2016).

Trastuzumab is a first generation, FDA-approved, HER2 targeted therapy for women with HER2+ breast cancer. Primary therapy for HER2+ breast cancer includes the humanized, IgG1, monoclonal antibody, trastuzumab (Herceptin^{TM}),

which targets domain IV in the extracellular domain of the HER2 receptor. Trastuzumab as a monotherapy has shown poor efficacy as the first randomized trial was stopped early due to poor pathological complete response (pCR) in the trastuzumab group compared to the control group (Buzdar et al., 2005). The term pCR indicates an absence of residual invasive disease in the breast or lymph nodes upon completion of neoadjuvant treatment prior to definitive surgery. Trastuzumab gains significant efficacy when combined with a taxane-based therapy and/or the second generation of HER2 targeting antibodies, pertuzumab (Untch et al., 2011; Baselga et al., 2010). Trastuzumab is effective in early stage breast cancer, but approximately 15% of women treated with trastuzumab go on to develop metastatic disease within the first year of treatment (Piccart-Gebhart et al., 2005) and 20 to 50% of women develop trastuzumab resistance (Cobleigh et al., 1999; Slamon et al., 2001). So far, there are several potential mechanisms of HER inhibition by trastuzumab. The benefits of trastuzumab treatment are limited in some women due to intrinsic resistance to trastuzumab or the development of acquired resistance to trastuzumab treatment.

Trastuzumab and the immune response. The immune system response has proven to be important in the efficacy of antibody treatments such as trastuzumab. In the clinic, there is consistently a positive correlation between immune cell infiltration and positive patient prognosis in HER2+ breast cancer (Reviewed in Bianchini and Gianni, 2014). One such method of immune cell infiltration is mediated by ADCC. ADCC is activated by the Fc (Fragment crystallizable region)

receptors (FcR) on the surface of immune cells recognizing the IgG1 Fc heavy chain of a monoclonal antibody bound to the surface of the target cell. The BIG 02-98 (Loi et al., 2013) and FinHER (Loi et al., 2012) clinical trials have shown that a correlation exists between the benefits of trastuzumab-based therapy in early stage breast cancer and tumor infiltrating lymphocytes. Building from this work which demonstrates that both cytotoxic and targeted cancer therapies can improve immune function, researchers have begun manipulating these therapies to enhance immune function in tumors.

Researchers have been modifying trastuzumab to improve its ability to direct the immune system against HER2 overexpressing tumor cells. Removal of the fucose group from trastuzumab creates afucosylated trastuzumab which has increased FcγIIIRa binding to enhance ADCC activity resulting in reduced tumor growth in a HER2+ xenograft tumor (Junttila et al., 2010a). Trastuzumab-activated ADCC can be reduced in tumors with high matrix metalloproteinase expression. The lower hinge region of trastuzumab can be cleaved by matrix metalloproteinases thereby inhibiting its ability to activate ADCC resulting in reduced trastuzumab efficacy (Fan et al., 2012). An IgE homolog of trastuzumab can initiate monocyte-mediated ADCC as well as induce mast cell degranulation to promote an anti-tumor response *in vivo* (Karagiannis et al., 2008; Karagiannis et al., 2011). Natural Killer (NK) cells can induce ADCC to improve trastuzumab treatment through the upregulation of CD137, a member of the tumor necrosis factor (TNF) family of receptors, when exposed to HER2+ cells coated with

trastuzumab. To focus the lytic activity of NK cells on the trastuzumab coated tumor cells, an anti-CD137 antibody was used to enhance the killing function of the activated NK cells (Kohrt et al., 2012). The adaptive immune system plays a role in murine anti-HER2 treatment as tumor regression is dependent on cyctotoxic CD8+ T cell activation which is lowered when combined with chemotherapy. This finding suggests that trastuzumab may be more beneficial if administered after chemotherapy (Park et al., 2010a). Continued work is being done to develop vaccines against HER2 such as the peptide-based vaccine E75 which was shown to stimulate cytotoxic T lymphocytes against the HER2 peptide to improve disease free survival (DFS) and reduce tumor recurrence (Mittendorf et al., 2012).

Lapatinib. Lapatinib is a first generation small molecule tyrosine kinase inhibitor (TKI) that dually targets HER2 and EGFR. Lapatinib is administered to women with HER2+ breast cancer as a second line therapy. This is due to clinical evidence showing that lapatinib is effective against HER2+ breast cancer that has become resistant to trastuzumab (Scaltriti et al., 2010). Mechanistically, lapatinib is an ATP mimicking molecule that competitively blocks binding of ATP in the kinase pocket of HER2 or EGFR. Lapatinib is more effective than trastuzumab at attenuating both the PI3K/Akt and MAPK/ERK1/2 pathways *in vivo* (Xia et al., 2002). One important advantage of lapatinib over trastuzumab is its ability to cross the blood-brain barrier to inhibit or treat brain metastases in women with metastatic HER2+ breast cancer (Gril et al., 2008). Combined trastuzumab and lapatinib treatment improves overall survival of women with trastuzumab resistant,

metastatic HER2+ breast cancer compared to lapatinib alone (Blackwell et al., 2012) suggesting that trastuzumab and lapatinib have complementary mechanisms of action. Surprisingly, lapatinib alone (Piccart-Gebhart et al., 2014) or combined lapatinib and trastuzumab (Gelmon et al., 2012) was unable to extend DFS. One major problem with lapatinib is its poor bioavailability and thus lapatinib requires high doses to achieve optimal kinase inhibition. These high doses have been shown to increase skin rash, gastrointestinal toxicity, and other side effects making it difficult to achieve anti-tumor efficacy.

Pertuzumab. A new anti-HER2 antibody has been developed that targets extracellular domain II of HER2 to inhibit HER2 heterodimerization with EGFR or HER3 and therefore activation (Cho, et al., 2003). Pertuzumab is able to block HER2:HER3 dimerization thus attenuating HER2 activation and has proven to be effective in HER2+ breast cancer cell lines as well as in both high and low HER2 overexpressing breast tumor xenografts (Lee-Hoeflich et al., 2008). Combined trastuzumab and pertuzumab treatment resulted in significant regression of HER2+ breast tumor growth *in vivo* through blockade of HER2 dimerization and decreased formation of the truncated HER2 that evades trastuzumab treatment, p95HER2 (Scheuer et al., 2009). Currently, the combined trastuzumab plus pertuzumab therapy is standard of care for HER2+ breast cancer. Additionally, trastuzumab and pertuzumab combined with docetaxel for the treatment of metastatic HER2+ breast cancer significantly improved pCR and prolonged DFS in clinical trials (Baselga et al., 2014).

Trastuzumab-emtansine. Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate in which cytotoxic emtansine is covalently linked to the trastuzumab antibody. Emtansine (Mertansine, DM1) is a maytansinoid that disrupts microtubule function by binding to tubulin resulting in cell cycle arrest of dividing cells. T-DM1 binds to the HER2 receptor on the surface of the cell. HER2 bound TDM-1 is internalized into the cytoplasm where emtansine is released from trastuzumab via lysosomal enzymes (Erickson et al., 2006). Besides the site directed anti-mitotic action of emtansine, T-DM1 retains trastuzumab mechanisms of action such as induction of ADCC, inhibition of PI3K activation, and prevention of p95HER2 truncation along with inhibiting growth of lapatinib resistant HER2+ breast cancer cell lines and tumor xenografts (Junttila et al., 2010b). TDM-1 is now used in the clinic as a second line therapy for metastatic, HER2+ breast cancer due to extending overall and progression free survival with less adverse side effects in clinical studies (Verma et al., 2012).

Trastuzumab Resistance.

Unfortunately, cancers have become resistant to many types of therapies that specifically target HER2 or effectors of HER2-mediated downstream pathways. Resistance to targeted treatments leads to tumor recurrence and in some cases a more aggressive, metastatic cancer that invades vital organs and ultimately ends in death. Cancer cells can adapt to treatments using a variety of mutations that enable them to propagate under specific treatment conditions. Approximately 50% of women respond to the first course of trastuzumab treatment

suggesting that inherent resistance occurs in the other 50% that have no response to trastuzumab treatment. Roughly 40% of women with HER2+, metastatic breast cancer are inherently resistant to trastuzumab treatment and worst of all, most women who initially respond to trastuzumab treatment will develop acquired resistance to trastuzumab within a year (Marty et al., 2005; Slamon et al., 2001). Acquired resistance to trastuzumab leads to a more aggressive, recurrent tumor that is difficult to treat. The prevalence of resistance to trastuzumab underscores the need for more predictive biomarkers in addition to HER2 to accurately predict the course of resistance.

Mechanisms of trastuzumab resistance. ***Changes in HER2 status.*** Women can become insensitive to trastuzumab treatment due to changes in HER2 expression in tumors. Primary and metastatic tumors can lose HER2+ expression status which is associated with a decrease in recurrence-free survival (Mittendorf et al., 2009; Niikura et al., 2012). Conversely, an increase in HER2 expression suggests that the tumor may be becoming more dependent on HER2 activity. However, this is not always the case as resistant cells in culture can display elevated levels of HER2 receptor expression and activation as measured by increased HER2 phosphorylation and sustained proliferation under trastuzumab treatment conditions (Ginestier et al., 2007a). Trastuzumab may inhibit HER2 signaling through endocytosis or degradation of the receptor but some studies report that trastuzumab has no effect on HER2 receptor expression (Gennari et al., 2004). Fluctuations in tumor HER2 status may call for continued assessment

of HER2 expression throughout treatment in order to adjust and potentially improve therapeutic regimens.

Impaired HER2-Trastuzumab interaction. Resistance to trastuzumab can occur by masking of the HER2 receptor or disrupting trastuzumab binding. One such mechanism of HER2 alteration to evade trastuzumab occurs through expression of a HER2 splice variant, Δ16HER2. The Δ16HER2 splice variant is generated by exon 16 skipping of the *HER2* gene resulting in a conformational change of the HER2 receptor that promotes stable HER2 homodimerization. Ectopic expression of the Δ16HER2 variant *in vitro* increased cell invasion, receptor dimerization, and trastuzumab resistance-mediated through direct interaction of Δ16HER2 to Src kinase (Mitra et al., 2009). Increased expression of Δ16HER2 in an *in vivo* mouse model as well as in patient samples correlated with increased tumorigenesis. Interestingly, the expression of the Δ16HER2 variant increased sensitivity to trastuzumab treatment compared to wild type HER2 (Castagnoli et al., 2014). Given that expression of the Δ16HER2, as well as other HER2 splice variants such as Herstatin and p100, correlate to increased trastuzumab efficacy, detection of these variants could potentially change treatment options for women with HER2+ breast cancer.

An NH_2 terminally-truncated form of the HER2 receptor, p95HER2, lacks extracellular subdomain IV resulting in resistance to trastuzumab treatment (Molina et al., 2001). The p95HER2 protein can be generated two ways. One is by proteolytic cleavage of the HER2 ectodomain (HER2 ECD) by a matrix

metalloproteinase which is referred to as ectodomain shedding. HER2 shedding releases a portion of the HER2 ECD into the blood. HER2 ECD can be measured in patient serum and elevated HER2 ECD has been associated with poor prognosis and reduced response to both endocrine and chemotherapy in metastatic breast cancer (Colomer et al., 2000; Leitzel et al., 1995). Alternatively, p95HER2 can be expressed endogenously by an alternative translation initiation site on the HER2 mRNA which produces a 100-115 kDa size p95HER2 protein that is smaller than the wild type HER2 protein of 185 kDa. This p95HER2 protein is also referred to carboxyl terminal fragment (611CTF). The truncated form of the HER2 receptor is able to constitutively homodimerize and is considered a potent oncogene (Pedersen et al., 2009). Increased expression of p95HER2 is associated with worse overall patient outcome and importantly, trastuzumab resistance (Scaltriti et al., 2007). However, expression of p95HER2 in patient derived tumor xenografts and cell lines showed an increased sensitivity to chemotherapies such as doxorubicin. This increased sensitivity to chemotherapy also increased sensitivity to trastuzumab treatment (Parra-Palau et al., 2014). It is hypothesized that the doxorubicin treatment may have stabilized the HER2 protein in the cell membrane thereby increasing its sensitivity to trastuzumab treatment.

The HER2 receptor can be shielded from trastuzumab binding by interacting with other proteins to confer trastuzumab resistance. Mucin-4 (MUC4) is a high molecular weight membrane associated glycoprotein that has been shown to interact with and activate the HER2 receptor. MUC4 overexpression inhibits

trastuzumab binding to HER2 yet has no effect on the level of HER2 expression (Price-Schiavi et al., 2002). MUC4 levels were shown to be elevated in a trastuzumab resistant cell lines as well as reduce trastuzumab binding compared to a trastuzumab sensitive cell line, JIMT-1. The resistance phenotype was shown to be reversible by MUC4 inhibition (Nagy et al., 2005). MUC4 was also shown to disrupt HER2 interaction with other HER receptors, HER3 and EGFR. Upregulation of MUC4 in a ER+/HER2+ tumor xenograft model resulted in resistance to anti-HER2 therapies, trastuzumab and lapatinib, as well as anti-estrogen therapies, tamoxifen and estrogen deprivation (Chen et al., 2012). Interestingly, these tumors shifted their dependence from ER/PR expression to HER2 upon MUC4 upregulation. Trastuzumab resistance has also shown to be induced by increased expression of the MUC1 cleavage product, MUC1*. Both acquired and intrinsic trastuzumab resistance as well as chemotherapeutic resistance were reversed by MUC1* inhibition using a MUC1* antagonist (Fessler et al., 2009). These results suggest that the mucin family of glycoproteins may be heavily involved in trastuzumab resistance and could serve as potential markers of resistance.

Augmented signaling through the HER receptor family. HER2 can escape the effects of targeted trastuzumab treatment through activation of other members of the EGFR/HER family of receptors. Trastuzumab-mediated growth inhibition in BT474 and SkBr3 cells can be modulated by expression of the EGFR receptor as well as the heregulin or EGF ligand (Diermeier et al., 2005).

Trastuzumab resistant BT474 cells were shown to have increased EGFR expression as well as an elevated EGFR:HER2 heterodimerization suggesting that targeting of EGFR in HER2+ breast cancers may be a viable therapeutic option for some women (Ritter et al., 2007). Upregulation of EGFR expression was observed in 15% of HER2+, metastatic breast cancers and associated with worse overall survival in these women who received trastuzumab treatment (Gallardo et al., 2012). Targeting EGFR with the TKI gefitinib reduced HER2 activation and HER2-mediated cell proliferation *in vitro* (Moulder et al., 2001), yet trastuzumab combined with gefitinib failed to provide any clinical benefit to women with HER2+, metastatic breast cancer (Arteaga et al., 2008). Similar results were seen with the EGFR selective TKI, erlotinib (Dickler et al., 2008), yet the combination of gefitinib, trastuzumab, and docetaxel did improve progression free survival in some women (Somlo et al., 2012).

Trastuzumab has demonstrated the ability to inhibit the formation of HER2 homodimers as well as the potent, oncogenic HER2:HER3 heterodimers (Ghosh et al., 2011; Junttila et al., 2009). Yet, Akt signaling can be activated in a compensatory manner by ligand stimulated HER2:HER3 heterodimers allowing evasion of HER2 targeted trastuzumab treatment. Attenuation of Akt signaling through inhibition of PI3K resulted in an increase in HER3 expression which amplified ERK1/2 activation in a compensatory manner. These data suggest that compensatory signaling in HER2+ cells may be overcome by dual targeting of HER2 or MEK and PI3K/Akt (Serra et al., 2011). High HER3 expression in women

with HER2+, metastatic breast cancer who received trastuzumab treatment was associated with shorter overall survival (Lipton et al., 2013) as well as shorter progression-free survival post trastuzumab and taxane treatment (Park et al., 2014).

HER4 has been shown to play opposing roles in trastuzumab resistance. The fact that HER4 has 4 different splice variants may be the reason why its role in HER2+ breast cancer has been difficult to understand. Among the 4 variants, the JM-a/CYT1 and JM-a/CYT2 were associated with better event free survival in women with ER+ and HER2+ breast cancer (Machleidt et al., 2013). Localization of HER4 appears pertinent to its activity as cytoplasmic HER4 has been associated with increased survival while nuclear HER4 has been associated with trastuzumab resistance and may be a poor prognostic indicator for women with HER2+ breast cancer (Mohd Nafi et al., 2014). Interestingly, trastuzumab treatment was shown to promote upregulation of HER4, its cleavage, and nuclear translocation, suggesting that HER4 localization may be a potential therapeutic predictor of trastuzumab resistance.

Trastuzumab sensitivity can be affected by endogenous inhibitors of HER2 such as mitogen-inducible gene 6 (MIG-6) [also known as ERBB receptor feedback inhibitor 1 (ERRFI1) or receptor-associated late transducer (RALT)]. MIG-6 is a cytoplasmic protein that is upregulated during cell growth, specifically during the G1 phase of the cell cycle. MIG-6 has been shown to interact with and inhibit HER2 and EGFR activation enabling MIG-6 activity to counteract

EGFR/HER ligand-mediated trastuzumab resistance (Anastasi et al., 2005; Fiorentino et al., 2000).

Increased expression of EGFR/HER ligands such as heregulin-1 (Hrg-1) have been shown to confer resistance to cancer therapies. A subset of women with HER2^{low}/HER2- breast cancer have displayed clinical benefit from trastuzumab treatment (Paik et al., 2008). These clinical data spurred the work that demonstrated that HER2:HER3 activation through Hrg-1 promotes BCSC survival and self-renewal in HER2- low expressing cells *in vitro* and *in vivo* (Lee et al., 2013). BCSC from low HER2 expressing cells secreted Hrg-1 to form a positive feedback loop in which Hrg-1 activated HER3/HER4 dimers in BCSCs. Similar results were seen in head and neck cancer (Wilson et al., 2011) and melanoma in which Notch-1 was shown to directly activate transcription of Hrg-1 to perpetuate the autocrine feedback loop and progress cancer cell growth (Zhang et al., 2012).

HER2 interaction with other receptors. Trastuzumab resistance has been shown to be facilitated by the interaction of HER2 with other non-HER receptors. Trastuzumab resistant cell lines have displayed overexpression of the RTK, Ephrin type-A receptor 2 (EphA2). Inhibition of EphA2 *in vivo* increased sensitivity to trastuzumab treatment as elevated EphA2 expression correlated with worse overall survival in women with HER2+ breast cancer (Zhuang et al., 2010). Another RTK commonly overexpressed in HER2+ breast cancer is the c-Met receptor. Trastuzumab treatment *in vitro* resulted in a rapid upregulation of c-Met which contributes to trastuzumab resistance as inhibition of c-Met sensitizes cells to

trastuzumab treatment (Shattuck et al., 2008). Interestingly, the accelerated up-regulation of c-Met by trastuzumab treatment results in resistance to trastuzumab. Increased expression of c-Met and its ligand, HGF, in HER2+, metastatic breast cancer correlates with failure of trastuzumab treatment as well as shorter time to cancer progression (Minuti et al., 2012).

HER2 has been shown to interact with growth factor receptors such as the insulin-like growth factor 1 receptor (IGF-1R) to promote trastuzumab resistance (Nahta et al., 2005). IGF-1R activation by its ligand, IGF-1, results in increased HER2 phosphorylation and activation of Akt and MAPK pathways in trastuzumab resistant cells compared to sensitive. IGF-1-mediated stimulation of IGF-1R is more prevalent in trastuzumab resistant cells and can promote downregulation of $p27^{Kip1}$. The $p27^{Kip1}$ protein is a cyclin dependent kinase (CDK) inhibitor that binds to CDKs to prevent CDK/Cyclin formation that is necessary for cell division. An increase in $p27^{Kip1}$ activity stops cell cycle progression and results in the cell remaining in the G1 phase of the cell cycle. HER2 is able to heterodimerize with IGF-1R. Overexpression of IGF-1R amplifies trastuzumab resistance and correlates with shorter progression-free survival and increased probability of residual disease in women who received adjuvant trastuzumab treatment. Overexpression of IGF-1R was induced by epigenetic silencing of miR-375 expression, a known regulator of IGF-1R expression. The re-expression of miR-375 could be a potential method to re-sensitize HER2+ breast cancers to trastuzumab (Ye et al., 2014).

Adipose tissue is a major component of the breast. In adipose tissue, it has been shown that adipocytes can secrete macrophage inhibitory cytokine 1 (MIC-1), also known as growth differentiation factor 15 (GDF15), to reduce trastuzumab sensitivity (Ding et al., 2009). MIC-1 is related to TGF-β and can promote HER2 phosphorylation through Src as well as trastuzumab resistance. Inhibition of MIC-1, TGF-β, or Src restores trastuzumab sensitivity in resistant cells indicating that MIC-1 mediates TGF-β/Src crosstalk to evade trastuzumab treatment (Joshi et al., 2011).

PIK3CA mutations in trastuzumab resistance. The most common pathway attributed to promoting drug resistance is the PI3K/Akt pathway. Elevated PI3K/Akt pathway activity has been identified as the source of resistance to targeting the HER2 pathway (Berns et al., 2007). Akt activity can be increased by expression of PIK3CA mutations, downregulation of PTEN, HER3 and HER2 overexpression, Akt-1 mutations, and loss of inositol polyphosphate-4-phosphatase type 2 (INPPB) expression. Activation of the Akt pathway results in resistance to apoptosis as well as increased cell migration, invasion, and survival.

In approximately 50% of HER2+ breast cancer cases, Akt signaling is activated by expression of PIK3CA mutations or loss of PTEN expression (Jensen et al., 2011). PIK3CA mutations confer trastuzumab resistance *in vitro* and *in vivo* (Cizkova et al., 2012; Dave et al., 2010; Razis et al., 2011) and have proven to be key biomarkers, and potential predictors, of trastuzumab resistance. PIK3CA mutations were associated with lower rates of pCR in several clinical trials using

lapatinib, trastuzumab, or combined neoadjuvant trastuzumab and lapatinib treatment, yet no difference was observed in overall patient survival (Majewski et al., 2015). Assessment of PIK3CA mutations in primary tumors predicted a worse clinical outcome for women with the PIK3CA mutation in the CLEOPATRA study which assessed pertuzumab, trastuzumab, and docetaxel treatment of HER2+, metastatic breast cancer but failed to predict for resistance to trastuzumab treatment (Baselga et al., 2014). As a potential biomarker, PIK3CA mutations were found to be positively associated with survival but not with trastuzumab treatment response (Loi et al., 2013; Pogue-Geile et al., 2015). These data indicate that the role of the PIK3CA mutations in HER2+ breast cancer is not straightforward and continued investigation is warranted.

Role of apoptotic and cell cycle regulators in trastuzumab resistance. Cells can control their proliferation rate through cell cycle arrest leading to cell quiescence and potentially programmed cell death or apoptosis. Cell cycle arrest can be mediated by stabilization of CDK inhibitors such as $p27^{Kip1}$. Trastuzumab treatment of sensitive cells reduces Akt pathway activity and upregulates $p27^{Kip1}$ resulting in subsequent cell cycle arrest (Yakes et al., 2002). Conversely, trastuzumab resistant cells express less $p27^{Kip1}$ protein as well as CDK2 which promotes an increased rate of cell cycle progression and proliferation (Nahta et al., 2004). Similarly, trastuzumab resistant cells display higher expression of cyclin E compared to trastuzumab sensitive cells (Scaltriti et al., 2011). Elevated cyclin E expression is associated with shorter progression-free survival as well as

reduced clinical benefit from trastuzumab treatment compared to women whose tumors express low levels of cyclin E. Interestingly, inhibition of CDK4/6 has no effect on cyclin E overexpression, hence tumors with heightened cyclin E expression may be resistant to CDK4/6 and HER2 targeted therapies. The $p27^{Kip1}$ protein is positively regulated by phosphorylation. The protein phosphatase PPM1H, encoded by the *PPM1H* gene, dephosphorylates serine/threonine residues and is able to dephosphorylate $p27^{Kip1}$ leading to protein degradation and reversal of cell cycle inhibition. Inhibition of PPM1H resulted in increased proliferation of HER2+ cells in response to trastuzumab treatment. Importantly, low PPH1M expression correlates with worse patient outcome (Lee-Hoeflich et al., 2011). These data suggest that $p27^{Kip1}$ may be a marker of trastuzumab resistance and its re-expression may re-sensitive resistant cells to trastuzumab treatment.

Acquired trastuzumab resistant BT474 (BT474TR) cells display an increase in B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein. BT474TR cells have shown sensitivity to the Bcl-2 inhibitor, ABT-737, compared to trastuzumab sensitive (BT474TS) cells. Combined treatment of resistant cells with trastuzumab and ABT-737 reduced cell proliferation. Bcl-2 activity was found to be mediated through PI3K/Akt and IKK/NFκB activity (Crawford and Nahta, 2011). Inhibition of both PI3K and IKK promoted downregulation of Bcl-2 thereby increasing sensitivity to trastuzumab treatment in the BT474TR cell model.

In culture, trastuzumab resistant cells were found to overexpress cyclin D1 which activates cyclin dependent kinase 4 and 6 (CDK4/6) through MAPK and Akt

pathway activity. Elevated cyclin D1 results in an increase in CDK-mediated phosphorylation of cyclins to promote cell cycle progression and increase cell proliferation. Inhibition of cyclin D1 reduced HER2-driven breast tumorigenesis *in vivo* when MMTV-Neu (HER2) mice were crossed with cyclin $D1^{-/-}$ mice. (Choi et al., 2012; Yu et al., 2001). CDK4/6 inhibition was shown to reduce resistant cell proliferation *in vitro* (Roberts et al., 2012) and tumorigenesis *in vivo* (Witkiewicz et al., 2014). Lapatinib has been shown to inhibit CDK4/6 and this may be one of the reasons it has an additive effect on cell growth inhibition when combined with trastuzumab treatment in HER2+ breast cancer.

Role of hormone receptors in trastuzumab resistance. Resistance to trastuzumab treatment in ER+/HER2+ breast cancers may occur through an escape route offered by ER activity (Giuliano et al., 2015). Conversely, HER2 activity can reduce sensitivity to anti-hormonal treatments as ER+/HER2+ tumors have displayed resistance to endocrine therapy. The inverse relationship between ER and HER2 is mediated through a number of different bi-directional crosstalk mechanisms (Konecny et al., 2003). HER2 inhibition enables ER-mediated upregulation of HER signaling through increased expression of IGF-1 and TNFα ligands. ER can inhibit EGFR and HER2 as well as upregulate IGF-1R as a compensatory mechanism to evade the HER2 blockade. The inverse relationship between HER2 and ER suggests that targeting of both receptors may be necessary to suppress ER+/HER2+ tumorigenesis. The combined use of trastuzumab and anastrozole, an AI, with chemotherapy increased progression-

free survival compared to anastrozole alone (Kaufman et al., 2009). Increased ER activity has been associated with trastuzumab resistance in women with ER+/HER2+, metastatic breast cancer that received trastuzumab combined with chemotherapy. Interestingly, the addition of endocrine therapy post chemotherapy was shown to increase progression-free survival indicating that tumors can retain responsiveness to estrogen (Montemurro et al., 2012). These data suggest that the role of ER is to promote trastuzumab resistance in women with ER+/HER2+ tumors and careful consideration of treatment options must be made to avoid treatment resistance.

Role of microRNAs in trastuzumab resistance. MicroRNAs (miRNAs) are small, non-coding RNAs that regulate protein translation. Similar to silencing RNAs (siRNAs), microRNAs form base pairs with complementary mRNA to silence mRNA translation into a protein. Post transcriptional regulation by miRNAs can occur by: binding to the 3' untranslated region (3'UTR) of mRNA causing truncation of the poly(A) tail and destabilization of mRNA, cleavage of the mRNA, or blocking ribosome binding to inhibit mRNA translation. Continued analysis of breast cancers by microarray expression profiles attempts to better assess potential promoters of breast cancer as well as future treatment options. Trastuzumab resistant, HER2+ cell lines have displayed upregulation of miR-21 which has been shown to reduce PTEN protein expression in liver cancer as well as programmed cell death 4 (PDCD4) in colorectal and breast cancer cells (Talotta et al., 2008). Inhibition of miR-21 increased PTEN expression both *in vitro* and *in vivo* resulting in reduce

cell proliferation as well as increased sensitivity to trastuzumab treatment (Gong et al., 2011). In BT474TR cells, miR-210 expression was higher compared to BT474TS cells and increased circulating levels of miR-210 in women with HER2+ breast cancer were found to be associated with lower response to trastuzumab treatment and increased lymph node metastases (Jung et al., 2012). Trastuzumab treatment was found to induce miR-30b and miR-26a expression in HER2+ cell lines, SkBr3 and BT474. miR-30b promotes G1 cell cycle arrest, apoptosis, as well as reduce cyclin E2 protein expression (Ichikawa et al., 2012). These data suggest that miRNA expression could provide important clues as to why some HER2+ breast cancers become resistant to trastuzumab.

Biomarkers of Trastuzumab Resistance.

A number of clinical trials have been done to identify potential markers of anti-HER2 targeted resistance. Some of these trials include: NEO-ALTTO, CLEOPATRA, TRYPHAENA, Neo-Sphere, and EMILIA to name a few. These studies consistently show that HER2 expression is currently the only marker appropriate for selecting patients for anti-HER2 therapy (Baselga et al., 2014; Gianni et al., 2016; Schneeweiss et al., 2014). Nonetheless, previous markers such as: HER2/IGF-1R heterodimerization, Src activation, low PTEN, and increased p95HER2 expression were not consistently associated with overall or disease-free patient survival. Poor patient prognosis, and not necessarily trastuzumab resistance, was commonly associated with the presence of PIK3CA mutations (Berns et al., 2007). These data indicate that today, the only biomarker

suitable for determining anti-HER2 treatment is gene amplification and overexpression of HER2 in breast cancer.

Stem Cells and the Cancer Stem Cell Hypothesis.

Stem cells (SCs) are characterized by their ability to self-renew into undifferentiated stem cells and differentiate into specialized cells that can generate or regenerate tissue. There are two general types of stem cells: embryonic and adult stem cells. Embryonic SCs are retained in the inner mass of the blastocyst where they are able to differentiate into the three primordial germ layers: the ectoderm, endoderm, and mesoderm. Adult SCs are retained in various tissues throughout the body where they act as a repair system to maintain the body. Adult SCs are also critical for maintaining regenerative cells which can be found throughout the adult body in locations such as the bone marrow (periosteum), intestinal lining, or skin (epithelium) (Reviewed in Rumman et al., 2015). Adult SCs can be used for stem cell therapies such as bone marrow therapy in which donor adult SCs are extracted from the bone marrow or peripheral blood and used to rebuild a cancer patient's ablated immune system. SCs can be harvested from previously collected umbilical cord blood for stem cell therapies. The continued development of stem cell therapies has driven the creation of stem cells from terminally differentiated somatic cells (dedifferentiation) or by nuclear transfer of an oocyte nucleus into a terminally differentiated somatic cell. A majority of stem cell work has been done in mice in which it has been shown that one multipotent mammary stem cell is capable of regenerating the entire murine mammary gland

in vivo (Shackleton et al., 2006). Cancer cells with stem like characteristics were first discovered in human acute myeloid leukemia (Bonnet and Dick, 1997) then in breast cancer (Al-Hajj et al., 2003) which laid the ground work to propose the cancer stem cell hypothesis.

The cancer stem cell hypothesis challenges the traditional concept of cancer development. Traditionally, the clonal evolution, or the stochastic model, of cancer hypothesizes that cancer develops from normal, somatic cells through an accumulation of mutations throughout the life time of the cell. These mutations give the cell a selective growth advantage over healthy cells enabling them to grow uncontrollably, invade neighboring tissues, become metastatic, resistant to treatments, and contribute to cancer recurrence. The acquisition of mutations creates a clonal subset of cancer cells in which any cancer cell can self-renew to produce the number of different cells within a tumor. Support for the clonal evolution model in breast cancer is exemplified by the most common mutation in breast cancer, the PIK3CA^{H1047R} mutation (Koren et al., 2015). The PIK3CA^{H1047R} mutation is able to induce cell dedifferentiation into a multipotent stem-like state which may be involved in the formation of multi-lineage, heterogeneous breast tumors.

The cancer stem cell hypothesis considers a small subset of stem cell-like cancer cells [also referred to as tumor initiating cells (TICs)] that are at the top of the cancer cell hierarchy. The CSCs are able to self-renew to create more undifferentiated CSCs as well as differentiate into the various bulk cells that

comprise a tumor. The ability of a stem cell, or cancer stem cell, to differentiate into one or many different cell types is referred to as potency, such as pluripotent or multipotent cells. Hypothetically, a single cancer stem cell can create all the cells required for the construction of a complete, heterogeneous tumor, much like a single totipotent stem cell can create an entire organ. Similarly, to stem cells, CSCs have shown reliance on the microenvironment as interactions with stromal or inflammatory cells can regulate their self-renewal or differentiation capabilities. The origin of CSCs remains contested as it is hypothesized that CSCs arise from transformed adult SCs or by lineage committed cells acquiring mutations resulting in dedifferentiation into CSCs. Though the two cancer models are considered mutually exclusive, as each fails to sufficiently address tumor heterogeneity, it is possible that the genetic and cancer stem cell models coincide by CSCs acquiring driver mutations during tumor progression (Reviewed in Kreso and Dick, 2014). An increase in CSC mutational burden may enhance CSC properties and result in enrichment of the CSC population residing within a tumor. In a heavily CSC populated tumor, tumor functionality becomes homogenous as a majority of the CSCs self-renew and few differentiate into progenitor cells.

CSC properties and BCSCs. Upon the discovery of cancer cells with stem cell characteristics, a lot of work has gone into discerning the phenotype of these cells and their role in tumor invasiveness, metastasis, therapeutic resistance, and recurrence. There are two types of cancer stem cells, the epithelial-like and mesenchymal-like CSCs (Liu et al., 2014). The mesenchymal-like CSCs are

primarily quiescent and at the invasive tumor front. In the breast, these cells are similar to basal stem cells found in the normal breast tissue that exist in the basal layer (myoepithelial layer) of mammary ducts. The mesenchymal-like CSCs are highly invasive yet largely non-proliferative. On the other hand, the proliferative epithelial-like CSCs are primarily retained in the hypoxic zones at the center of a solid tumor. This SC type has the multilineage capacity to differentiate into a wide variety of cells to construct a heterogenic tumor. Epithelial-like BCSCs are similar to luminal stem cells and can be found below luminal cells of mammary lobules. It is important to note the plasticity of these two stem cell types as the mesenchymal-like CSCs can transition to epithelial-like CSCs by undergoing mesenchymal to epithelial transition (MET), Conversely epithelial-like CSCs can become mesenchymal-like CSCs via epithelial to mesenchymal transition (EMT). The ability of the CSCs to shift between epithelial and mesenchymal states perpetuates their ability to promote an aggressive cancer phenotype. Remarkably, both epithelial and mesenchymal-like BCSCs have similar GEPs across various breast cancer subtypes. CSCs have been shown to transdifferentiate into endothelial and vascular smooth muscle-like cells for improved tumor vascularization. Additionally, women whose tumors have high levels of CSC markers have poorer overall survival and these CSC markers may be used as prognostic factors for breast cancer progression in the clinic (Zhou et al., 2010).

Transit amplifying cells (TACs) differentiate from stem cells that have not completely transformed into specialized, terminally differentiated cells, hence the

state of TACs is between that of undifferentiated stem cells and terminally differentiated cells (Figure 5). TACs are highly proliferative yet short lived cells whose progeny differentiate into the various lineages of mature cells necessary to form a complete organ. Due to their high proliferation rate, TACs typically represent a greater portion of the cell population compared to stem cells. The limited proliferative nature of TACs can promote the accumulation of mutations, but these are usually repaired within the cell offspring. It is possible that some mutations remain in the TACs, resulting in dedifferentiation of this mutated TAC subset, thereby allowing these mutations to enter the SC population. Similarly, mutations retained by the TAC progeny may occur, in which they are more likely to transform into cancerous cells. Therefore, targeting TACs may be a viable option to reduce or prevent the initiation of tumorigenesis, in an effort to intercept the transition from the mutated TAC to mutated SC and eventually into a CSC.

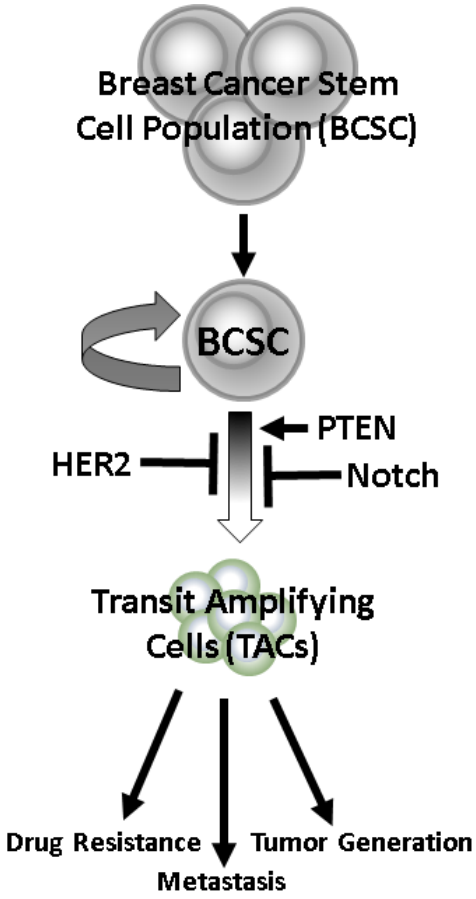

Figure 5: Breast Cancer Stem Cell Self-Renewal and Differentiation Pathways
The tightly regulated process of BCSC self-renewal and differentiation into transit amplifying cells (TACs) then terminally differentiated cells can become dysregulated in breast cancer. Loss of PTEN or upregulation of Notch or HER2 receptors can result in an increase in the BCSC population which is hypothesized to promote drug resistance, metastasis, and the generation of new, heterogeneous tumors.

Breast cancer cells with stem cell characteristics were first found in breast cancer patient samples by Al-Hajj et al. (Al-Hajj et al., 2003). Breast cancer cells with similar surface markers as multiprogenitor mammary stem cells [MaSCs ($CD44^{+}/CD24^{-/Low}/Lin^{-}$)] may originate from BCSCs. As few as 100 breast cancer stem-like cells were able to form tumors in immune compromised mice while up to

50,000 non cancer stem-like cells ($CD44^+/CD24^+/Lin^-$) failed to form tumors. The discovery of BCSCs opens the door to further investigate the role of BCSCs in breast cancer.

BCSC markers. The first BCSCs were defined by their tumorigenicity *in vivo* using models of mouse BCSC tumor xenografts and *in vitro* by colony formation, sphere formation, migration, and invasion assays (Al-Hajj et al., 2003). These assays allow serial dilution of BCSCs to determine the ability of a logarithmically increasing amount of BCSCs, or non-BCSCs, to form tumors or display BCSC properties. Initially, BCSCs were isolated from patient samples by flow cytometry. Cell surface expression of $CD44^+/CD24^{-/low}$ was the most common BCSC marker within patient samples. This subset of cells was used in BCSC colony forming assays and in an immunodeficient mouse model to determine tumorigenicity of serial diluted (100 to 50,000) BCSC and non-BCSC populations. CD44 (Cluster of differentiation 44) is a receptor for hyaluronic acid and is involved in cell to cell and cell to extracellular Membrane (ECM) interactions. CD24 is a small glycoprotein that negatively regulates the chemokine receptor CXCR4, which has been shown to regulate metastasis of breast cancer cells (Schabath et al., 2006). To confirm that tumor cells were being selected, $Lineage^-$ (Lin^-) enabled selection of neoplastic tumor cells from normal cell type lineage markers (CD2, 3, 10, 16, 18, 31, 64, and 104b). Cells were also selected by epithelial specific antigen (ESA^+) expression, or epithelial cell adhesion molecule (EpCAM), which is expressed on the surface of most epithelial cells and is used to distinguish epithelial cancer cells from

mesothelial cells (Al-Hajj et al., 2003). Establishment of the $CD44^{+}/CD24^{-/low}$ surface expression is now a common marker for epithelial BCSC as well as a poor prognostic factor for women with early stage breast cancer (Buess et al., 2009). Further research into the $CD44^{+}/CD24^{-/low}$ BCSC marker found that its expression does not always correlate with tumorigenicity (Fillmore and Kuperwasser, 2008) and women with the opposite $CD44^{-}/CD24^{+}$ status had a worse breast cancer prognosis (Ahmed et al., 2012).

Infrequencies with the use of $CD44^{+}/CD24^{-/low}$ as a marker of BCSCs has initiated the search for other BCSC markers. BCSCs have been shown to express aldehyde dehydrogenase 1 (ALDH1), an enzyme that catalyzes the oxidation of toxic aldehydes into inert carboxylic acids. ALDH1 can be fluorescently tagged and used as a stem cell marker in breast cancer (Ginestier et al., 2007b). ALDH expression is a good marker of epithelial BCSCs as they have been shown to express ALDH (Liu et al., 2014). CD133 (Prominin 1) expression was found to be similar to $CD44^{+}/CD24^{-/low}$ expression in cells derived from BRCA-1 knockout mice as a marker of BCSCs (Wright et al., 2008). $CD44^{+}/CD24^{-/low}$ as well as increased vimentin (Borgna et al., 2012), and IL-1/prostaglandin E_2 (Li et al., 2012) expression have been developed as markers of mesenchymal stem cells in breast cancer. BCSC markers continue to be found which include integrin markers: CD29, CD61 (Vaillant et al., 2008), CD49f (Cariati et al., 2008); a mucin family marker, MUC1 (Engelmann et al., 2008); as well as the Delta ligand from the Notch family of receptors, DLL1 (Pece et al., 2010). Other markers of BCSCs continue to be

found and verified both *in vitro* and *in vivo* and can vary greatly between breast cancer subtypes. The discovery of multiple markers of BCSCs strongly suggests that BCSCs are heterogeneous and their diversity may be the main reason that breast cancers are intrinsically heterogeneous. None the less, the most relevant markers of BCSC still need to be identified for incorporation into the breast cancer treatment strategy.

Role of BCSCs in metastasis. Metastasis of breast cancer to vital organs is one of the major contributors to breast cancer-associated mortality. There remains no targeted therapeutic approach for the treatment of metastatic breast cancer outside of chemotherapy. Metastasis may be mediated by a small set of BCSCs from the primary tumor that can invade and migrate to distal vital organs to form secondary tumors. BCSC-mediated metastasis has been demonstrated in several studies. Genetic expression profiling of $CD44^{+}/CD24^{-/Low}$ isolated cells from primary tumors was found to be similar to metastatic breast cancer cells isolated from the distant organs and in tumors from women with worse overall outcome (Liu et al., 2007). Early metastatic cancer cells found in the bone marrow of breast cancer patients expressed a $CD44^{+}/CD24^{-/Low}$ profile (Balic et al., 2006). Additionally, ALDH positive cells displayed greater metastatic capacity compared to ALDH negative cells in a xenograft mouse model (Charafe-Jauffret et al., 2009). Combined, these studies underline the importance of BCSCs in promoting metastasis in breast cancer.

The role of BCSCs in EMT / MET. Epithelial to mesenchymal as well as mesenchymal to epithelial transition are fundamental processes in early embryogenic morphogenesis and are proposed to be critical regulators of tumor metastasis. Epithelial cells convert to mesenchymal cells (EMT) and mesenchymal cells re-convert to epithelial cells (MET) to promote secondary tumor growth at distal sites away from the primary tumor. EMT occurs by epithelial cells losing their epithelial characteristics: cell to cell adhesion and apical-basolateral polarity, to take on mesenchymal cell characteristics: spindle shape and motility (Mani et al., 2008; Morel et al., 2008). Cells undergoing EMT downregulate epithelial cell markers such as E-cadherin and lose occludins, adherins, and/or tight junctions. During EMT, the epithelial cell upregulates mesenchymal cell markers: N-Cadherin, vimentin, and fibronectin, as well as transcription factors: SNAIL, SLUG, ZEB1, ZEB2, TWIST 1 and TWIST2 (Reviewed in Thiery et al., 2002). The transcription factors inhibit E-Cadherin, specifically claudins, and Zonula Occludens 1 [(ZO1) a tight junction protein], Platelet-Derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), Transforming Growth Factor β (TGF-β), or matrix metalloproteinase expression. EMT can be induced *in vitro* by over expressing TWIST or SNAIL, or exposing the cells to growth factors such as EGF or TGF-β (Morel et al., 2008).

Continued research on BCSCs makes it clear that this small subset of cells can be generated through EMT to promote cancer progression. It is hypothesized that BCSCs can become metastatic by undergoing EMT as it has been shown that

$CD44^{+}/CD24^{-}$ cells have an increase in EMT markers and the potential to invade and migrate to distal organs. The constitutively active protein, AXL, in BCSCs induces EMT by inhibiting E-Cadherin while upregulating N-Cadherin, Snail, Slug, Zeb1, and fibronectin expression (Asiedu et al., 2013). AXL has also been shown to regulate a number of pathways including STAT, Akt, NF-κB, and MAPK, which may be pertinent to EMT. EMT can be induced *in vitro* by the increased activation of the transcription factor, FOXC2, which has been shown to promote a more aggressive cancer phenotype (Mani et al., 2007). EMT can be activated through metadherin (MTDH)-mediated activation of Twist to induce the BCSC phenotype (Liang et al., 2015). The more aggressive breast cancer subtypes (TNBC and HER2+) have increased markers for BCSCs and EMT compared to less aggressive subtypes (Luminal A) (Park et al., 2010b).

The two types of BCSCs, epithelial and mesenchymal, can be further characterized by their expression of mesenchymal or epithelial cell markers (Liu et al., 2014). Mesenchymal-like BCSCs are identified as having low E-cadherin and high vimentin at the invasive edge of a tumor. The mesenchymal BCSCs are ready to enter the blood or lymphatic circulation to form new, small tumors (micrometastasis) at distant organ sites. The invasive mesenchymal BCSCs can transition to more proliferative epithelial BCSCs (METs) to generate a secondary tumor. The more proliferative epithelial BCSCs express high levels of E-cadherin and low vimentin in the center of the tumor. The central epithelial BCSCs can transition to the more invasive mesenchymal state on the outside of the tumor to

promote metastatic formation of secondary tumors. The two BCSC states can switch between invasive mesenchymal and proliferative epithelial to promote tumor spread and growth, respectively. The transition between these two states may be regulated by chemokine and cytokine signaling as the plasticity of the BCSCs enables them to readily metastasize or adapt to changing microenvironment conditions.

BCSC maintenance and non-coding RNAs. BCSCs are able to transition between stem-like and non-stem-like states. BCSC plasticity indicates that there are regulatory mechanisms that are able to induce or maintain the stemness of BCSCs. Stemness of BCSCs can be regulated through interactions with non-coding RNAs (ncRNAs), the tumor microenvironment, or fluctuations in cell signaling pathways within the cell.

There are many types of ncRNAs including: long non-coding RNAs (lnc RNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and miRNAs that are not translated into a protein. ncRNAs have a wide variety of functions but here we will focus on a set of ncRNAs associated with BCSC plasticity (Reviewed in Liu et al., 2013). Expression of miRNAs are altered in BCSCs compared to bulk tumor cells. Some miRNAs altered in BCSCs are the: *miR-183 cluster, miR-200 cluster, miR-221-222 cluster, miR-142, miR-214,* and *let-7*. These miRNAs may target a variety of pathways that are responsible for maintaining the stemness of BCSCs. Assessment of the BCSC population can be tested *in vitro* by using mammosphere forming assays in which BCSC survival and self-renewal are determined through

primary and secondary mammosphere formation, respectively. It has been shown that *let-7a* reduces both cell proliferation and mammosphere formation in a k-Ras-dependent manner both *in vitro* and *in vivo* (Ma et al., 2015). The lncRNA, *Linc00617*, induces stem cell properties in breast cancer cells by upregulating the stem cell marker, Sox2 (Li et al., 2017). BCSC properties may be inhibited by targeting ncRNAs as a possible therapeutic strategy.

Role of BCSCs in the tumor microenvironment. The tumor microenvironment creates a “niche” that supports and maintains survival and self-renewal capacity of CSCs. CSCs interact with a variety of cells in the tumor microenvironment such as: Tumor-associated Macrophages (TAMs), Mesenchymal Stem Cells (MSCs), Cancer-Associated Fibroblasts (CAFs), Extracellular Matrix (ECM), endothelial cells (ECs), and immune cells. CSC interaction with other cells in the tumor microenvironment occurs through a complex network of cytokine and growth factor expression. These ligands stimulate CSC self-renewal, angiogenesis, as well as stromal and immune cell recruitment into the CSC niche to perpetuate the production of additional factors in order to promote tumor cell invasion and migration.

Hypoxic conditions can induce the CSC phenotype as well as enrich the CSC population in various types of cancer. The BCSC population of TNBC cells, MDA-MB-231, is enriched by hypoxia (Xie et al., 2016). Hypoxia has been shown to enrich the BCSC population in primary epithelial and tumor cells (Iriondo et al., 2015). $ALDH^+$ BCSCs were shown to be dependent on Hypoxic-Inducible Factor

1 alpha (HIF1α). Downregulation of prolyl hydroxylase 3 (PHD3) mimics hypoxic conditions and was found to increase the $CD44^{+}/CD24^{-/low}$ population of BCSCs. Hypoxic activation of HIF1α has been shown to upregulate embryonic stem cell markers *OCT4, SOX2, KLF4, NANOG, c-MYC,* and *microRNA-302* resulting in expansion of the BCSC population (Mathieu et al., 2011). Hypoxia-mediated enrichment of the BCSC population has been shown to be dependent on HIF1α-mediated expression of AlkB Homolog 5 (ALKBH5) which stabilizes *NANOG* mRNA thereby increasing NANOG protein expression (Zhang et al., 2016). The hypoxic environment and hypoxia-induced pathways may be potential therapeutic targets to prevent BCSC expansion.

Tumor cells can increase cytokine and growth factor production which has been shown to stimulate CSC proliferation and survival as well as promote recruitment of stromal and immune cells into the CSC niche. Enrichment of stromal and immune cells into the CSC niche results in the secretion of additional growth factors creating a positive feedback loop. This positive feedback loop between CSCs and immune/stromal cells has been shown to increase tumor cell metastasis. Autocrine or paracrine signals between BCSCs and stromal cells can regulate BCSC phenotypes. In HER2-negative MCF-7 breast cancer cells, human umbilical cord-derived MSCs stimulate Interleukin-6 (IL-6) and IL-8 autocrine signaling which promotes the $CD44^{+}/CD24^{-}$ BCSC population to migrate *in vitro* and metastasize *in vivo* (Ma et al., 2015). TNBC cell lines, MDA-MB-231 and MDA-MB-453, secrete IL-6 to activate *OCT4* gene expression through an IL-6-JAK1-

STAT3 signal transduction pathway in non-CSCs (Kim et al., 2013). The OCT4 transcription factor has been shown to promote dedifferentiation of non-CSCs into CSCs. Primary breast CAFs and fibroblasts produce high levels of the chemokine (C-C motif) ligand 2 (CCL2) to induce CSC self-renewal through upregulation of Notch-1 (Tsuyada et al., 2012). BCSCs can be regulated though interactions between endothelial cells and $CD44^{+}/CD24^{-}$ cells. Tumor-endothelial cell interactions can regulate BCSCs by establishing a feedback loop in which tumor cells secrete endothelial stimulatory signals such as FGF12, VEGF, NF1, and PTN causing endothelial cells to secrete PDGFB to promote cancer cell proliferation (Buess et al., 2009). Cytokines and growth factors are critical to maintaining the CSC population and require careful consideration when assessing the BCSC environment.

A major cellular component of the breast tumor microenvironment is Tumor Associated Macrophages (TAMs) which have been hypothesized to originate from resident tissue macrophages or circulating monocytes to establish leukocyte accumulation within the stroma of a tumor. The function of TAMs is debatable as evidence has shown their involvement in tumor growth and metastasis by stimulating angiogenesis as well as tumorostatic or tumoricidal processes (Bingle et al., 2002). TAMs have been shown to promote a CSC-like phenotype through paracrine EGFR/Stat3/Sox-2 signaling in murine breast cancer cells (Yang et al., 2013). TAMs may play a critical role in BCSC maintenance as previous work has demonstrated that an injection of TAMs with CSCs significantly amplifies tumor

growth compared to the injection of CSCs cell alone. Increased TAM infiltration around breast tumors correlates with increased tumor vascularization and a worse overall prognosis for women with breast cancer (Leek et al., 1996). The accumulation of CAFs, TAMs, stromal cells, and freshly produced blood vessels at the tumor invasive front allow CAFs to secrete macrophage colony stimulating factor (M-CSF) to activate TAM's pro-angiogenic switch. The cellular milieu of the CSC niche plays an important role in supporting the BCSC population.

T-cells can promote breast cancer when recruited into the tumor microenvironment. TAMs and CD4+ T helper cells are able to secrete TNFα in order to upregulate NF-κB signaling. This increase in NF-κB signaling stimulates transcription of Snail, Slug, and Twist as well as increased crosstalk with the TGF-β pathway resulting in CSC maintenance and self-renewal (Reviewed in Smith et al., 2012). Infiltration of FOXP3+ regulatory T cells and CD8+ cytotoxic T cells was associated with high histological grade and steroid receptor negative status (Liu et al., 2011). Elevated levels of CD4+ and CD8+ T cell infiltration are closely correlated with EMT and the BCSC phenotype. Interestingly, infiltration of both T cell types was also associated with the absence of residual invasive disease in the breast or lymph nodes upon completion of neoadjuvant treatment (Seo et al., 2013). Therefore, the presence of T cells in the tumor could be an early response to therapy where the non-CSCs are targeted by the immune system but not necessarily the CSCs.

The ECM is critical in the niche of both normal and cancer stem cells as it plays a multitude of roles in maintaining normal cell and stem cell properties. ECM anchorage retains stem cells in the niche allowing them to be available to receive critical paracrine signals necessary for maintaining stem cell properties. Cellular differentiation processes can be obstructed by irregular changes in the ECM resulting in enrichment of the stem cell population and a reduction in cell differentiation (Reviewed in Lu et al., 2012). ECM stiffness in breast cancer can promote transcriptional coactivator with PDZ-binding motif (TAZ) activity, resulting in an increase in the BCSC population (Cordenonsi et al., 2011; Dupont et al., 2011). These data suggest a possible role for the ECM in promoting BCSC self-renewal resulting in breast cancer disease progression.

Role of BCSCs in HER2+ breast cancer. Treatment of HER2+ breast cancer by biologics such as trastuzumab has greatly improved the clinical outcome of women with tumors overexpressing the HER2 protein. Evidence has been presented that shows a positive correlation between HER2 amplification and BCSC frequency as assessed by ALDH1 expression (Korkaya et al., 2008) (Figure 5). Although the precise mechanism of trastuzumab's actions remain unknown, trastuzumab has been shown to reduce BCSC enrichment in trastuzumab sensitive cells but not trastuzumab resistant cells *in vitro* (Magnifico et al., 2009). Previous work has demonstrated that HER2 cooperates with c-Myc to promote a stem-like phenotype and BCSC self-renewal (Nair et al., 2013a). The BCSC population is enriched in cells that have developed acquired resistance to

trastuzumab. The Akt pathway has been shown to be required for this enrichment (Korkaya et al., 2008). Several markers for BCSCs have been found in HER2+ breast cancer cell lines and patient samples including: $MUC1^+$ (Engelmann et al., 2008), $Vimentin^+$, $CK18^+$, and $GATA3^+$ (Park et al., 2010b) combined with $CD24^+$ or $CD44^+$ expression. It has been shown that inhibition of CXCR1 and 2 (CXCR1/2), which are regulated by IL-8, significantly reduces BCSC activity in patient-derived HER2+ breast cancers (Singh et al., 2013). Trastuzumab may not be inhibiting the BCSC population specifically but acting on the emerging TAC population. Trastuzumab-mediated TAC inhibition may occur in resistant cells in which trastuzumab inhibits CSC differentiation into TACs thereby increasing the BCSC population. Another possibility is the occurrence of a mutation in the TAC population promoted by prolonged trastuzumab treatment that results in TAC dedifferentiation into BCSCs.

Role of BCSCs in treatment resistance. BCSCs have been shown to play a role in drug resistance both *in vitro* and *in vivo*. TNBC BCSCs are enriched by chemotherapy such as paclitaxel and thus are inherently resistant to this type of therapy (Samanta et al., 2014). Therapy was shown to increase the BCSC population in HER2+ breast cancer from patient biopsies post chemotherapy and/or lapatinib treatment (Li et al., 2008). A number of BCSCs from different breast cancer cell lines demonstrated resistance to radiotherapy (Phillips et al., 2006). Additionally, the Wnt pathway has been shown to drive *SOX2* gene expression and increase the BCSC population to promote resistance to tamoxifen

(Piva et al., 2013). Similarly, either letrozole or docetaxel treatment has been shown to increase the BCSC population in patient tumor samples post therapy (Creighton et al., 2009). Together, these data suggest that BCSCs have an intrinsic resistance to some breast cancer treatments or their ability to be quiescence enables the BCSC population to evade chemotherapy and/or radiotherapy. Therefore, investigating novel therapies to target the BCSC population may prove to be an effective strategy for the treatment of breast cancer.

The Notch Pathway.

The Notch receptor is a type 1, single pass, transmembrane protein (Kopan, 2009). The *NOTCH1* gene is transcribed by the E26 transformation-specific (ETS) family member, polymavirus enhancer activator 3 (PEA3) (Clementz et al., 2011) in TNBC or by p53 in keratinocytes and epithelial cells (Lefort et al., 2007). Furthermore, E2A (E-proteins E12 and E47) in conjunction with Notch-1 induce *NOTCH1* gene expression in the early stages of T cell development (Yashiro-Ohtani et al., 2009), indicating that Notch-1 can regulate its own expression during T cell maturation. Additionally, during T cell selection, the basic helix-loop-helix (bHLH) transcription factor, Id3, is upregulated by the MAPK pathway to inhibit Notch-1 transcription.

The mature Notch receptor (272,505 Da.) is constructed as a heterodimer comprised of two domains bound by a non-covalent bond that is typically mediated by a calcium (Ca^{2+}) ion (Figure 6). During maturation, the nascent, full length Notch polypeptide enters the Golgi apparatus where it is cleaved at the S1 (scissile bond

1) site by a furin-like convertase, furin / proprotein convertase 5 / 6 (PC5/6), into two domains: the Notch extracellular domain (NECD) and the Notch transmembrane / intracellular domain (NTMICD). The extracellular domain of the Notch receptor contains a series of EGF-like repeats that arbitrate ligand interactions which are facilitated by Notch ligand expression on the surface of a neighboring cell. The EGF repeats are preceded by the Negative Regulatory Region (NRR) that inhibits Notch receptor activation in the absence of a Notch ligand. In humans, there are five known Notch ligands: Jagged-1, -2 (Jag-1, -2), Delta-Like-1, -3, and -4 (DLL-1, -3, -4) that are able to bind to and activate the Notch receptor when expressed on the surface of a cell in close proximity to the Notch receptor of a neighboring cell (Figure 7). The affinity of a Notch ligand to a Notch receptor can be attenuated by the addition of sugar moieties to the Notch receptor which is facilitated by Fringe glycosyltransferases (Lunatic, Manic, or Radical). Fringe glycosyltransferases add N-acetylglucosamine moieties to the NECD region.

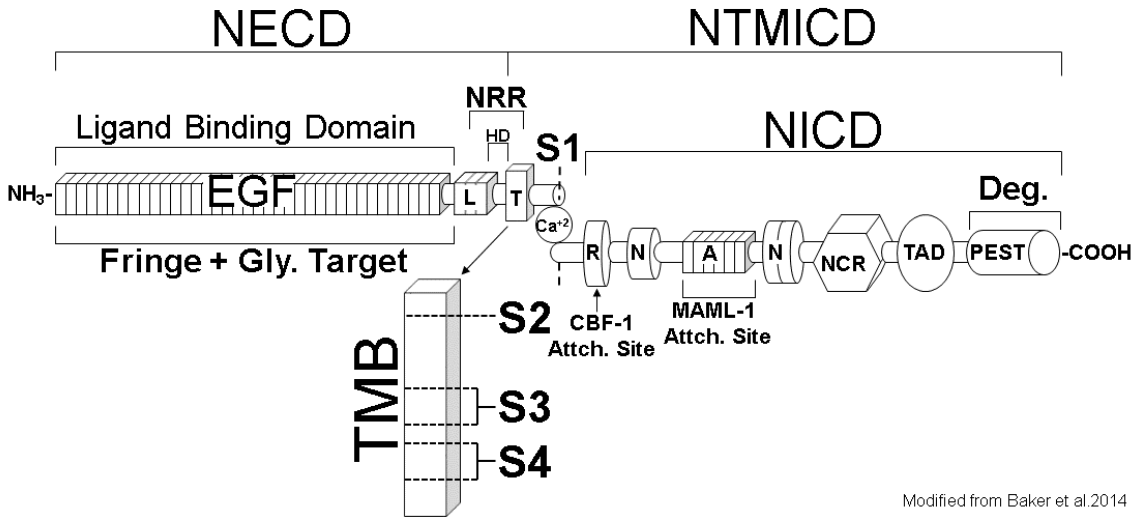

Figure 6: The Notch Receptor.

The Notch receptor is composed of several domains that can be divided by the extracellular domain (NECD) and the transmembrane and intracellular domain (NTMICD). The NECD starts at the N-terminal (NH_3) end and contains ligand binding epidermal growth factor-like (EGF) repeats as well as the negative regulatory region (NRR). Fringe proteins are able to target the EGF repeats for glycosylation, which can change their ligand binding ability to the Notch receptor. The NRR is composed of Lin12-Notch repeats (L) and the heterodimerization domain (HD). The NTMICD contains: the transmembrane domain (T, TMB), the RBP-Jκ associating module (R) or RAM domain, one of two nuclear localization signals (N), the ankyrin domain, a second nuclear localization signal, the cysteine response region (NCR), the transactivating domain (TAD), and a proline/glutamic acid/serine/threonine-rich motif (PEST) at the C-terminal (COOH) end of the Notch receptor. Ligand bound Notch is cleaved at a series of scissile sites (S1, 2, 3, 4) represented just outside of S1 and inside the transmembrane domain [S2, 3, 4 (TMB enlargement)]. Furin-like convertase cleaves immature Notch at S1 to create the mature, heterodimeric Notch receptor, which is stabilized by a non-covalently bound calcium ion (Ca^{+2}). The RAM domain facilitates core binding factor-1 (CBF-1) binding. NICD binding to CBF-1 allows binding of mammal-like mastermind-1 (MAML-1) to the Ankyrin domain to form a ternary complex. The histone acetyltransferase, p300, is recruited to NICD bound CBF-1 to form the Notch transcriptional complex. The PEST domain is responsible for protein stability of the Notch intracellular domain (NICD).

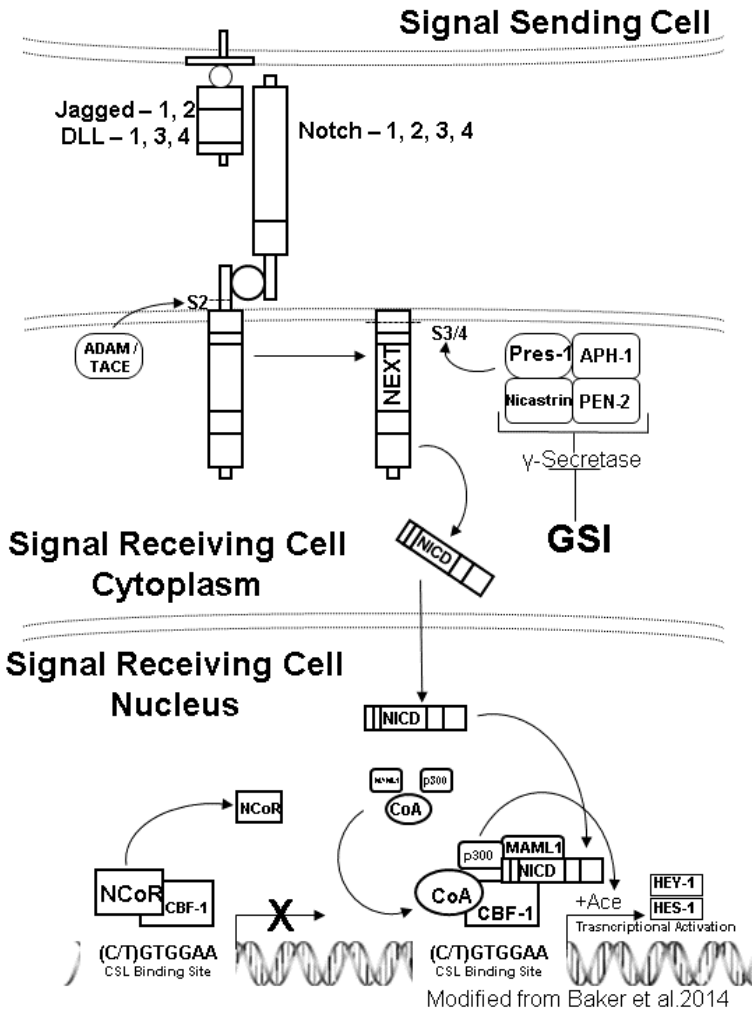

Figure 7: The Notch Pathway.
The Notch pathway is activated by a Notch ligand (Jagged-1, -2, DLL-1, -3, -4) on the surface of a signal sending cell (top) binding to a Notch receptor (Notch-1, -2, -3, -4) on the surface of a signal receiving cell (bottom). The ligand bound Notch receptor undergoes a series of cleavages. ADAM/TACE-mediated cleavage at S2 generates the NEXT fragment then γ-secretase-mediated cleavage at S3/4 generates NICD. NICD is translocated to the nucleus to bind to CBF-1 allowing the release of negative co-regulators (NCoR) and the recruitment of co-activators (CoA) such as MAML-1 and p300 to form the Notch transcriptional complex (NTC). Proper NTC formation allows NICD to act as a transcriptional activator of Notch target genes (HEY-1, HES-1).

The intracellular domain of the Notch receptor is comprised of the active portion of the Notch receptor, the Notch Intracellular domain [NICD (110 kDa.)]. NICD is comprised of the: Recombining Binding Protein-Jκ (RBP-Jκ) associating molecule (RAM) domain, Ankyrin domain, cysteine response region (NCR), transactivation domain (TAD), proline/glutamic acid/serine/threonine-rich motifs (PEST domain), and a pair of nuclear localization sequences (NLS) flanking the Ankyrin domain. The NLSs direct the cleaved NICD to enter the nucleus where it facilitates transcriptional activation of Notch target genes.

Activation of the Notch pathway requires a series of events beginning with the expression of the Notch receptor on the surface of the signal receiving cell binding to a Notch ligand on the neighboring signal sending cell. Subsequently, ligation of the Notch ligand to the Notch receptor is necessary for removal of inhibitory NECD from the NTMICD portion of the Notch receptor which results in two subsequent cleavage events: S2 and S3 cleavage. S2 cleavage is mediated by a disintigrin and matrix metalloproteinase 10 or 17 (ADAM10/17) which results in the Notch Extracellular Truncated (NEXT) Fragment. The S3 cleavage is mediated by the γ-secretase complex. Cleavage by γ-secretase results in the release of the NICD portion of the Notch receptor (Figure 7). NICD is translocated to the nucleus where it binds the constitutive repressor, RBP-Jκ/Core Binding Factor-1 (RBP-Jκ/CBF-1) poised on promoter regions of Notch target genes. In the absence of NICD, CBF-1 is bound to co-repressor proteins such as SMRT/HDAC-1 associated repressor protein (SHARP) and C-terminal-binding protein (CtBP)

(Oswald et al., 2005). This CBF-1-co-repressor complex represses gene transcription until NICD is available. Upon the binding of NICD to CBF-1, co-repressors are replaced by co-activator proteins. NICD binding to CBF-1 results in the recruitment of transcriptional co-activators beginning with Mammal Mastermind-like-1 (MAML-1) followed by the Histone Acetyltransferase (HAT), p300. Recruitment of the transcriptional co-activators to NICD1 results in unwinding of chromatin and exposing the promoter region of the target gene thereby initiating transcription. The binding of NICD to CBF-1 and the recruitment of transcriptional co-activators forms the Notch transcriptional complex. Canonical Notch target genes are predominantly helix-loop-helix transcriptional repressors including: Hairy/Enhancer of Split (HES-1-5) and Hairy/Enhancer of split with a unique YRPW motif (HEY-1, HEY-L). In mammals, there are four Notch paralogs, Notch-1, -2, -3, and -4. Each Notch paralog has unique structures that may facilitate synergistic and/or counteracting functions depending on the context of their expression (Figure 8).

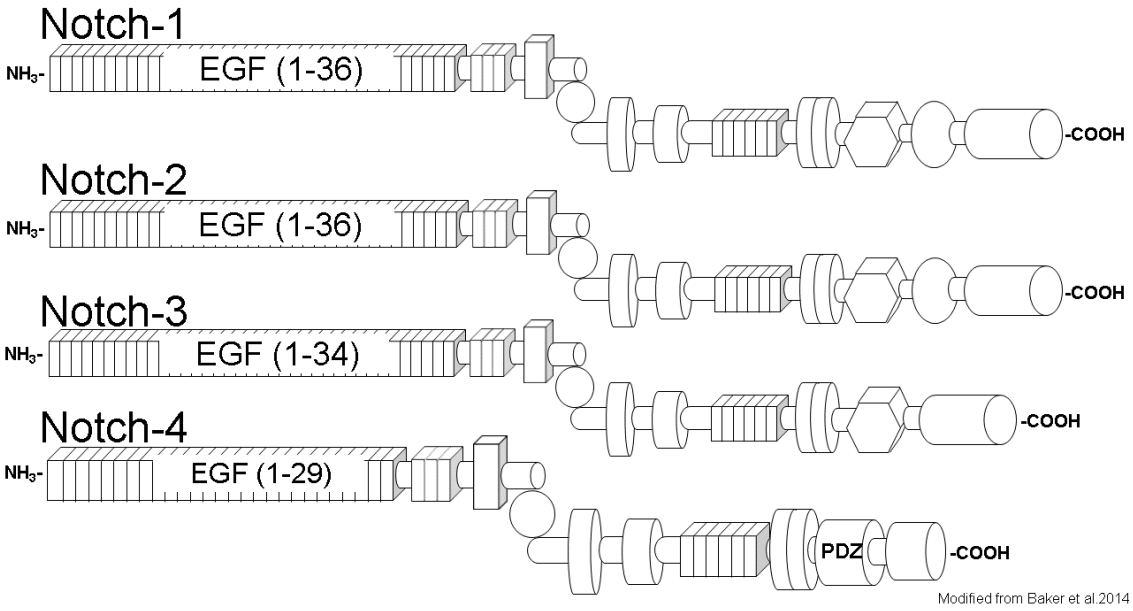

Figure 8: Notch Receptor Paralogs.
The Notch receptor paralogs (Notch-1-4) have a similar overall composition but slight differences in their structure to facilitate diversification of their attributes. Differences amongst the Notch paralogs include changes in the number of EGF-like repeats or the subtraction or addition of domains such as the PDZ domain or cysteine response region.

The Notch pathway is most frequently associated with lateral inhibition in which the Notch ligand, Delta, if expressed in one cell is capable of inhibiting Delta expression on a neighboring cell. In a cluster of undifferentiated cells, when one cell begins to differentiate into an epithelial cell, it signals the surrounding cells to also differentiate into epithelial cells. Originally, an undifferentiated cell can express both the Notch receptor and the Delta ligand. Stimulation of the Notch receptor will inhibit Delta production in the same cell. Through juxtacrine signaling, adjacent cells compete to produce Delta, causing a feedback loop that drives the two neighboring cells to assume dissimilar cell fates established by the number of Delta ligands or Notch receptors expressed by each cell. Notch signaling is used

to institute a border between two different populations of cells such as progenitor and stromal cells. Boundary development by oscillating Notch activity is most prominent in somite formation in which the continuous inactivation and activation of Notch-mediated transcriptional activity results in the segmented formation of somites in vertebrates (Pasinia et al., 2001; Palmeirim et al., 1997). Notch is able to direct cell lineage via asymmetrical inheritance of Notch regulators between two dividing, daughter cells, and is most apparent during neurogenesis (Lowell et al., 2006).

The Numb drosophila homolog (Numb) is encoded by the *NUMB* gene and acts as an inhibitor/negative regulator of Notch activity. Asymmetric distribution of Numb and Notch between two dividing cells determines if one daughter cell is a signal receiving cell and if the other is a signal sending cell. This uneven distribution of Notch and Numb is inherited through multiple cell divisions to sustain stem cell populations as well as influence cell lineage decisions (Reviewed in Chiba, 2006). The culmination of the Notch pathway outlines its capacity to act as a method of short range communication between cells. Notch juxtacrine signaling has been found to be involved in a variety of processes, most notably in stem cell differentiation, self-renewal, and cell fate determination as well as cell growth, proliferation, and survival (Reviewed in Artavanis-Tsakonas, 1999).

Notch targeted treatments include the use of γ-secretase inhibitors (GSIs). GSIs inhibit the critical S3 cleavage mediated by the γ-secretase complex preventing release of NICD and consequently, NICD-mediated transcriptional

activation. First generation GSIs were chemically designed to mimic the transition state of the γ-secretase substrate bound to the catalytic subunit, presenilin-1, such as Notch or the amyloid precursor protein (APP) (Figure 7). Second generation GSIs were designed as non-transition state analogs. These small molecule inhibitors bind allosterically to γ-secretase and attenuate γ-secretase enzymatic activity that is independent of direct competitive binding. Both types of GSIs ultimately interfere with the ability of γ-secretase to cleave and activate the Notch receptor (Clarke et al., 2006). There are several distinct GSIs including MRK-003, DAPT, and Compound E as well as γ-secretase modulators (PF-03084014), which are designed to specifically inhibit specific Notch paralogs (Zhang et al., 2012).

Role of Notch in breast cancer. There is a growing body of evidence indicating that an increase in Notch expression or mutation results in a series of events that enable breast cancer cells to: undergo EMT, become resistant to targeted treatments, metastasize, and promote BCSC survival and self-renewal (Reviewed in Nickoloff et al., 2003). Initial discovery of a role for Notch in breast cancer was made by an observation from studies using the mouse mammary tumor virus (MMTV) model of breast cancer. It was found that MMTV was inserted in the in the Int3/Notch-4 locus of murine mammary tumors (Gallahan and Callahan, 1987). The insertion of MMTV into the Notch-4 locus resulted in a Notch-4 mutation that made it constitutively active. Further investigations revealed that MMTV-driven NICD1 or NICD4 expression in the mammary gland induced spontaneous mammary tumors in mice (Dievart et al., 1999). Aberrant activation

of the Notch pathway has been shown to transform normal breast epithelial cells into cancerous cells which can be inhibited by targeting Notch-1 (Stylianou et al., 2006). Additionally, the expression of Numb is frequently lost in breast tumors (Pece et al., 2004). Increased co-expression of Jagged-1 and Notch-1 in breast tumors has been associated with worse overall survival in women with breast cancer (Reedijk et al., 2005). Increased Notch-1 expression has been shown to occur early in the development of HER2+ breast cancer (Zardawi et al., 2010) and both Notch-1 and Notch-4 have been identified as prognostic markers for breast cancer (Yao et al., 2012). Notch signaling has been shown to inhibit apoptosis by activation of the Akt pathway via an autocrine loop in breast cancer cells (Meurette et al., 2009). Notch-1, -3, and -4 are bonafide oncogenes in T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991), cervical (Zagouras et al., 1995), B-cell lymphoma (Fabbri et al., 2011), lung (Konishi et al., 2007), colon (Akiyoshi et al., 2008), and ovarian (Rose et al., 2010) cancers. In contrast, Notch-2 has tumor suppressive activity in some breast cancer subtypes (O'Neill et al., 2007; Parr et al., 2004; Shimizu et al., 2002). Notch-3 has been shown to facilitate proliferation and survival of HER2 negative tumors (Yamaguchi et al., 2008) as well as promotion of BCSC survival and self-renewal in a hypoxic environment (Sansone et al., 2007) (Figure 8). There are a number of studies suggesting that Notch functions as a tumor suppressor in myeloid malignancies (Klinakis et al., 2011) and skin cancer (Lefort et al., 2007). Research in melanoma indicates that Notch-1 acts as a master regulator of several signaling pathways. Notch-1 has

been shown to activate both Akt and MAPK pathways as well to induce expression of the EMT marker, N-Cadherin, both *in vitro* and in primary melanoma tumors (Liu et al., 2006). These data indicate that Notch acts as an oncogene or a tumor suppressor in a cell and/or tissue-dependent context.

The Notch pathway has been referred to as the Notch-survival pathway due to its ability to stimulate cell proliferation and growth. One of the Notch target genes responsible for regulation of cell longevity is called survivin. Survivin is an inhibitor of apoptotic protein (IAP) and functions to downregulate caspase 3, 7, and 9-mediated apoptotic activities. Survivin has also been shown to regulate the cell cycle by interacting with spindle microtubules during mitosis (Li et al., 1998). Cyclin dependent kinase 1 (CDK1) phosphorylates the threonine 34 residue of survivin to increase its protein stability (O'Connor et al., 2000) enabling survivin to evade degradation arbitrated by the X-linked IAP-X-linked association factor-1 complex (XIAP-XAF1). The XIAP-XAF1 complex induces E_3 ligase activity that targets survivin for ubiquitinylation and subsequent proteasome-mediated degradation. Prolonged survivin expression has been associated with breast cancer survival through PI3K/Akt and MAPK/ERK pathway activities (Siddiqa et al., 2008). There is a clear association between Notch-1 and survivin as women with ER+ breast tumors that overexpress both Notch-1 and survivin have poorer survival outcomes than women whose tumors do not co-express both transcripts (Lee et al., 2008).

Role of Notch in BCSCs. Notch plays a critical role in determining cell fate of both normal SCs and cancer SCs. It was found that Notch-4 plays a critical role in proper mammopoiesis suggesting that it directs normal breast SC and BCSC fate. It has been demonstrated that Notch, particularly Notch-4, plays a prominent role in BCSC self-renewal, survival, and cell fate, or differentiation (Dontu et al., 2004). In particular, Notch-4 was found to promote BCSC self-renewal and promote side branching of breast cells *in vitro*. Additionally, breast stem cells are hypothesized to be at the tips of extending branches in the developing mammary gland. Conversely, specific inhibition of Notch-4, or Notch-1, reduced BCSC enrichment as well as tumor initiation *in vitro* and *in vivo* yet inhibition of Notch-4 had a greater effect than inhibition of Notch-1 (Harrison et al., 2010) (Figure 5).

It has been shown that Notch-1 promotes HER2+ tumor recurrence post trastuzumab treatment in dormant residual tumor cells (Abravanel et al., 2015) suggesting that these dormant cells were BCSCs. It has been shown that Notch-1 expression in BCSCs is low compared to differentiated progenitor cells yet particular expression of the Notch paralogues appears to vary by breast cancer subtype (Grudzien et al., 2010). Inhibition of Notch signaling by a GSI, or a Notch decoy, reduces BCSC mammosphere formation *in vitro* suggesting that blockade of Notch signaling is a viable treatment option to eliminate BCSCs. One way Notch may promote BCSC survival is by regulating the differentiation of mammary progenitor cells to either myoepithelial cells or epithelial cells (Reviewed in Dontu et al., 2003). Constitutive expression of Notch-1 was shown to promote the luminal

cell fate and inhibited side branching of the developing breast tissue in a doxycycline regulated Notch-1 mammary tumor model in mice (Simmons et al., 2012). In this model, inhibition of Notch-1 promoted tumor regression and prevented tumor metastasis. Notch-1 was found to regulate the embryonic stem cell marker Nanog which is suggested to be the mechanism by which Notch-1 regulates the BCSC population *in vivo*. An increase in Notch-1 was shown to increase BCSC markers, ALDH1 and ABC transporters, which have been show to facilitate excretion of chemotherapeutic drugs from the cancer cell. Notch-1 was shown to facilitate the transcription of the embroyonic stem cell marker Sox2, which establishes a Notch-1/Sox2 signaling axis that is critical for ovarian CSC activity. Similar work has been done in TNBC BCSCs, demonstrating the ability of Notch-1 to activate transcription of Sox2 resulting in post trastuzumab treatment enrichment of the BCSC population (Azzam et al., 2013).

In an interesting study, Notch was shown to regulate HER2 expression thereby possibly regulating the survival and differentiation of BCSCs (Chen et al., 1997). Work in a HER2+ DCIS model showed that Notch inhibition reduced mammosphere formation and acini size yet had no effect on the HER2- DCIS model (Burchell et al., 2013). Conversely, gefitinib or lapatinib reduced mammosphere formation and acini size in HER2- DCIS with no effect on the HER2+ DCIS cell model. Notch-EGFR crosstalk has been implicated as a paracrine mediator of estrogen action to increase ER-/ER low BCSC survival as well as to promote proliferation of ER+ breast cancer cell lines (Harrison et al.,

2013). Estrogen has been shown to increase expression of FGFR, EGFR, and Notch ligands in ER+ bulk breast cancer cells to act in a paracrine manner to regulate expression via FGF/FGFR/Tbx3, EGFR/MAPK/ERK, and DLL-Jag/Notch/Pea3 pathways in the ER-/ER low BCSC population. Estrogen-mediated BCSC regulation can be attenuated by blocking estrogen with tamoxifen, EGFR with gefitinib, or Notch with a GSI to inhibit BCSC survival and early progenitor cell differentiation, and possibly dedifferentiation, respectively. Crosstalk between FGFR, EGFR, and Notch pathways are responsible for estrogen induced changes to ER-/ER low BCSC population and may play a part in endocrine resistance as well as offer possible therapeutic targets for the treatment of ER+ breast tumors.

Notch-1-HER2 crosstalk in trastuzumab resistance. Notch-HER2 crosstalk was initially observed in HER2+ breast cancer cells treated with trastuzumab which resulted in an increase in Notch-1 expression and activation (Osipo et al., 2008). Similarly, chronic trastuzumab treatment of HER2+ breast cancer cells to induce trastuzumab resistance resulted in an increase in Notch-1 as well as upregulation of genes transcribed by Notch-1. Targeted inhibition of Notch-1, or pan-inhibition of Notch by a GSI, enhanced trastuzumab sensitivity and reversed trastuzumab resistance *in vitro*. Combinatorial targeting of HER2 by trastuzumab and Notch by a GSI inhibited trastuzumab resistant HER2+ tumor growth as well as tumor recurrence (Pandya et al., 2011). Together, these data suggest an inverse

relationship between Notch-1 and HER2 in breast cancer in which inhibition of HER2 upregulates Notch-1 expression.

Interestingly, there is evidence that Notch-HER2 crosstalk can be stimulatory rather than inhibitory. The HER2 promoter contains a CBF-1 binding site and overexpression of NICD1 was shown to induce transcription of the *HER2* gene indicating that Notch-1 positively regulates HER2 expression (Chen et al., 1997). HER2 overexpression in DCIS increased Notch-3-mediated transcription of Notch target genes (Pradeep et al., 2011). In the HER2+ DCIS model, effectors of the Notch pathway: ADAM17, Jag-1, DLL-1, presenilin-1, and Notch-1 were increased upon Notch-3 nuclear localization. Notch-3 inhibition resulted in reduced spheroid formation, cell proliferation, and luminal spheroid filling indicating that HER2-mediated upregulation of Notch-3 promotes HER2+ DCIS survival and cell proliferation. In addition to these studies, others have demonstrated that HER2 may enable increased expression of effectors of the Notch pathway such as Notch activating matrix metalloproteinases (presinilin-1, ADAM) or Notch ligands (DLL-1, Jag-1, -2).

Notch-1-HER2 crosstalk through the MAPK pathway. HER2 is a potent activator of the Ras-MAPK pathway and communication between the Notch and MAPK pathway has been investigated. TNBC cell lines use the MAPK/ERK pathway to promote upregulation of Jagged-1 to activate Notch. TGFα-mediated EGFR activation of the MAPK pathway has been shown to enhance Jagged-1 expression in breast cancer cells to promote metastasis in the bone (Sethi et al.,

2011). The pseudokinase Tribble-3 (TRB3) was identified to activate both TGF-β and MAPK/ERK pathways (Izrailit et al., 2013). Inhibition of TRB3 reduced TNBC (MDA-MB-231) cell proliferation while Jagged-1 overexpression in TRB3-silenced cells rescued cell proliferation. TRB3 knockdown was found to reduce tumor growth in TNBC tumor xenografts in mice *in vivo*. These results indicate that TRB3 elevates Jagged-1 expression through the MAPK/ERK pathway and is critical for TNBC cell proliferation and tumorigenesis.

Notch-1-HER2 crosstalk through the Akt pathway. It is well established that HER2 activates a multitude of signal transduction pathways including PI3K/Akt/mTOR signaling. This is critically important as most of the work to date has focused on mechanisms of crosstalk between the PI3K/Akt pathway and Notch. Hyperactivation of mTOR signaling has been associated with elevated Notch signaling in poorly differentiated breast cancers as well as a poor clinical outcome for women with mTOR/Notch overexpressing tumors (Ma et al., 2010). The correlation between hyperactivation of mTOR and Notch pathways revealed that RTK activation of PI3K/mTOR pathway promoted upregulation of several mTOR targets: p63 (a member of the p53 family), signal transducer and activator or transcription 3 (STAT3), Notch, Jagged-1, and HES-1. Aberrant RTK/PI3K/Akt/mTORC1 signaling was found to increase Stat3 expression and activation resulting in an increase in p63 transcription. This increase in p63 expression was found to induce Jagged-1-mediated Notch activation and consequently HES-1 expression. This study demonstrates the mechanism by

which the upregulation of the RTK/PI3K/Akt/mTOR signaling axis activates the STAT3/Jag-1/Notch signaling cascade to promote breast tumorigenesis. Conversely, Notch can activate the PI3K/Akt/mTOR pathway in T-ALL (Calzavara et al., 2008; Palomero et al., 2007), indicating that Notch and mTOR may cooperate in a positive regulatory loop to promote cancer cell proliferation or differentiation.

Investigators have elucidated crosstalk between the PI3K/Akt/mTOR and Notch pathways in several different cancer cell lines including a chemoresistant ER+/HER2-, MCF-7 cell line, which displayed elevated Notch-1 expression and activity (Mungamuri, 2006). Notch-1 activation inhibited p53 nuclear localization and prevented phosphorylation of p53 at Ser15, Ser20, and Ser392 residues. This Notch-1-mediated blockade of p53 activity was found to be dependent on the mTOR/eIF4E signaling axis as the mTOR inhibitor, rapamyacin, reversed chemoresistance in these cells and restored p53 activity. These studies reveal an important role for the PI3K/mTOR pathway on Notch-1-mediated regulation of drug resistance and p53-dependent apoptosis.

PTEN.

Role of PTEN in breast development and tumorigenesis. The *PTEN* gene is located on chromosome 10 in the 10q23 region. PTEN expression is frequently lost in a variety of cancers including breast cancer. To better understand the role of PTEN in mammary gland development, mice with a conditional PTEN knockout were bred (MMTVCrePTEN$^{flox/flox}$) and assessed for development of mammary

tumors (Li et al., 2001). PTEN null mice displayed hyperplastic mammary gland ducts, an increase in side branching, and amplified lobulo-aveolar budding compared to the mammary tissue of control mice. Such developmental changes were also observed at puberty and during pregnancy as the PTEN deficient mice also had defective mammary gland involution after pregnancy. In addition to defects in mammary gland development, the PTEN knockout mice developed cancer earlier as well as benign lesions compared to control mice. Interestingly, the PTEN null mice displayed increased staining for cytokeratins 5 and 6 (Li et al., 2002), markers for the basal subtype of breast cancer (Sørlie et al., 2001). Furthermore, mammary tumors from the PTEN null mice showed similar characteristics to breast cancers containing BRCA-1 mutations (Saal et al., 2008). Loss of PTEN is associated with Cowden syndrome which is an uncommon autosomal dominant, inherited disorder and is categorized by the growth of non-cancerous hamartomas. Cowden syndrome is associated with PTEN mutations which results in an increase in mTOR activity and a predisposition to cancers, including breast cancer. These data present the role for PTEN in proper mammary gland development as well as the development of breast cancer when PTEN expression is lost.

Role of PTEN in HER2+ breast cancer and trastuzumab resistance. Loss of PTEN has been associated with worse overall outcome for women with HER2+ breast cancer (Stern et al., 2015). Loss of PTEN occurs in approximately 12% to 22% of HER2+ breast cancer patients (Esteva et al., 2010) while mutation of PTEN

occurs in only 3.5% of breast cancers (Rexer et al., 2013; TCGA, 2012). Detection of PTEN mutations in breast cancers had little overlap with HER2 amplification indicating that a majority of the PTEN mutations do not occur in the HER2+ subtype. Transcription of the *PTEN* gene is regulated by a number of different mechanisms and pathways, particularly during development and tumorigenesis. *PTEN* transcription is regulated by p53, SMADs, and NFκB for proper organogenesis and immune function (Knobbe et al., 2008; Luyendyk et al., 2008). Loss of PTEN expression can occur a number of ways besides loss-of-function mutations such as: loss of heterozygosity, RNA degradation, miRNA-mediated transcriptional inhibition, epigenetic modification, or post translational modification of PTEN. Additionally, transcriptional repression of *PTEN* could be due to the loss of other tumor suppressors critical to breast cancer located in the vicinity of the *PTEN* locus (Feilotter et al., 1999).

Trastuzumab treatment has been shown to increase expression of the tumor suppressor, PTEN. PTEN loss and the resulting increase in PI3K/Akt activity is a fairly good predictor of trastuzumab resistance and is improved when combined with *PIK3CA* activating mutation status in women with HER2+ breast cancer (Berns et al., 2007; Loibl et al., 2016). Women with tumors expressing low PTEN were less likely to achieve pCR when treated with trastuzumab compared to women with tumors expressing elevated PTEN (Dave et al., 2010). There are many reports showing that loss of PTEN's tumor suppressive activity results in increased Akt signaling, trastuzumab resistance, poor prognosis, and decreased

overall survival in women with HER2+ breast cancer (Fujita et al., 2006; Nagata et al., 2004).

Trastuzumab-mediated activation of PTEN. Trastuzumab can increase PTEN activity by disrupting the association between HER2 and Src (Nagata et al., 2004). Trastuzumab treatment of HER2+ breast cancer cells, such as BT474 and SkBr3, resulted in activation of PTEN. Trastuzumab was shown to inhibit phosphorylation of tyrosine residues (Y240 and Y315) on the C2 domain of PTEN (Figure 9). This blockade of tyrosine phosphorylation resulted in PTEN translocating to the cell membrane where it is positioned to dephosphorylate PIP_3 to PIP_2. The non-receptor tyrosine kinase Src has been shown to bind to and activate HER2 (Belsches-Jablonski et al., 2001) as well as increase tyrosine phosphorylation of PTEN (Lu et al., 2003) in HER2+ breast cancer cells. Trastuzumab-mediated inhibition of Src kinase activity resulted in reduced phosphorylation of PTEN as well as decreased phosphorylation of Akt. Inhibition of Src kinase activity was found to be a major contributor to the anti-proliferative effect of trastuzumab in HER2+ breast cancer *in vitro* as well as reducing tumor growth *in vivo*. Importantly, reduced PTEN expression in HER2+ tumors, by PTEN siRNA, promoted HER2+ cell proliferation *in vitro* as well as tumor growth *in vivo,* regardless of trastuzumab or paclitaxel treatment. HER2+ cell proliferation and tumor growth can be inhibited by a PI3K inhibitor, LY294002, thereby re-sensitizing PTEN deficient tumors to trastuzumab treatment. PTEN deficient tumors from women with HER2+ metastatic breast cancer showed the least clinical benefit from

trastuzumab treatment compared to PTEN expressing tumors, making PTEN deficiency a potential predictor of trastuzumab resistance.

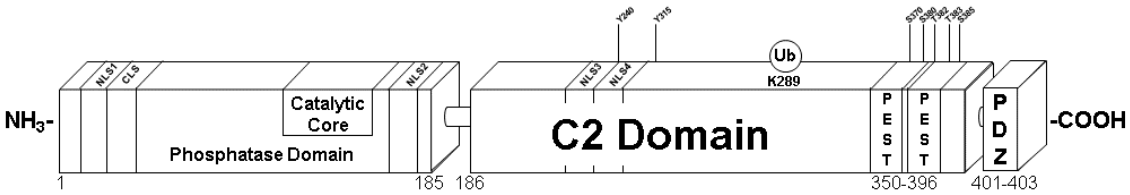

Figure 9: PTEN.
The PTEN protein is composed of two domains, the phosphatase and C2 (C-terminal) domains. The N-terminus contains a PIP_3 binding domain followed by a nuclear localization signal (NLS1), a cytoplasmic localization sequence (CLS), and a second nuclear localization signal (NLS2). The phosphatase domain is responsible for lipid and protein phosphatase activity. The catalytic core binds PIP_3 to facilitate its dephosphorylation. The C2 domain binds to lipids and contains NLS3 and 4, two proline/glutamic acid/serine/threonine-rich motifs (PEST), and the PDZ domain. Monoubiquitylation can occur at residue K289 as well as residues S370-S385. Phosphorylation can occur at residues Y240 and Y315.

Mechanisms of Notch-1-mediated inhibition of PTEN. Notch-1 has demonstrated the ability to regulate the PI3K/Akt pathway through repression of PTEN in several different cancers. Aberrant activation of Notch-1 is common in T-ALL (Reviewed in Grabher et al., 2006). Thus, GSI treatment was proposed as a possible therapeutic for T-ALL, but half of T-ALL cells failed to respond to GSI treatment (Palomero et al., 2007). Interestingly, GSI effectively reduced NICD1 levels and Notch target gene expression suggesting that compensatory signaling pathways may be responsible for continued T-ALL cell growth. Palomero et al. analyzed GSI sensitive and resistant T-ALL cell lines to find decreased expression of PTEN in both cell types. GSI resistant cells were found to have a loss-of-function PTEN mutation that resulted in hyperactivation of Akt and T-ALL cell growth.

Notch-1 activity in GSI sensitive cells was found to indirectly represses PTEN. In the GSI sensitive cells, constitutive activation of Notch-1 increased transcription of the transcriptional repressor, and Notch-1 target gene, HES-1. HES-1 is directly recruited to the PTEN promoter where it is able to inhibit transcription of PTEN. These data demonstrate a parallel relationship between the Notch-1 and PI3K/Akt pathway in which they both promote T-ALL cell growth. Accordingly, the inverse relationship of Notch-1 and PTEN in T-ALL predicts a worse overall outcome for T-ALL patients.

Repression of PTEN by Notch-1 has been shown to occur in melanoma cell lines. Pan Notch inhibition by GSI (RO4929097) resulted in elevated PTEN expression, reduced Akt pathway activity, and an increase in senescence and apoptosis in PTEN wild type expressing melanoma cancer cells (Nair et al., 2013b). PTEN null or mutant melanoma cell lines did not enter apoptosis or senesce when treated with GSI suggesting that PTEN status dictates sensitivity to GSI. As such, wild type PTEN is inhibited by Notch-1 and is sensitive to GSI treatment but neither mutant nor PTEN null cells are dependent on Notch-1-mediated repression of PTEN and are therefore, resistant to GSI. Additionally, in wild-type PTEN expressing melanoma cells, GSI treatment improved the effect of chemotherapeutic temozolomide treatment both *in vitro* and *in vivo*. These studies highlight the importance of Akt pathway activity in dictating melanoma tumor susceptibility to GSI alone or in combination with chemotherapy.

Notch-1-mediated repression of PTEN can occur through post translational modification of PTEN. In gastric cancer, Notch-1 inhibition results in increased PTEN activity, cell cycle arrest at the G2/M checkpoint, and reduced tumor growth *in vivo* (Kim et al., 2015). Notch-1 was shown to induce phosphorylation of the PTEN C-terminus to promote accumulation of PTEN in the nucleus. Nuclear PTEN accumulation resulted in cell apoptosis through interaction with the cyclin-1/CDK1 complex. Increased Akt activation through PTEN inhibition was also found to be important in other cancer models such as mesothelioma, in which Notch-1 and Notch-2 were found to have contrasting effects (Graziani et al., 2008). Interestingly, Notch-1 was required for PTEN downregulation, Akt activation, and DNA synthesis in mesothelioma cell models. However, Notch-2 had the opposite effect by increasing PTEN expression, Akt inhibition, reduced DNA synthesis, and cell death. Interestingly, Notch-1 was found to directly bind to CBF-1 at the PTEN promoter thereby directly inhibiting PTEN transcription in mesothelioma cells. Together, these data suggest several mechanisms in which Notch can inhibit PTEN activity to regulate cancer cell growth and survival as well as tumorigenesis *in vivo*.

Role of PTEN in BCSCs. Given the frequency of PTEN loss or downregulation in breast cancer, it is probable that changes in PTEN activity can affect the BCSC population. PTEN has been shown to regulate self-renewal of neuronal and hematopoietic stem cells (Figure 5). Case in point, PTEN is an acute regulator of FOXO transcription factors which control hematopoietic stem cell proliferation and

survival (Sengupta et al., 2011). Inhibition of PTEN in malignant and normal BCSCs from HER2 negative / ER+ cell lines activates the Akt pathway and phosphorylation of GSK-β resulting in activation of the Wnt/β-catenin pathway (Jordan et al., 2009). The PTEN/Akt/GSK-β/Wnt/β-catenin signaling pathway is involved in enrichment of the mammary stem/progenitor cell population. Targeted inhibition of Akt activity, by perifosine and not the mTOR inhibitor rapamycin, reduces the BCSC population in vitro as well as tumor growth and recurrence in vivo.

MicroRNAs have been shown to play a role in regulating PTEN expression and consequently effecting enrichment of the BCSC population. Elevated miR-205 expression increased the mouse mammary epithelial progenitor cell population and the CSC population through regulation of Zeb-1, -2, and PTEN expression (Greene et al., 2010). Interestingly, it has been shown that overexpression of HER2 attenuates miR-205 and may be essential for HER2-driven tumorigenesis (Adachi et al., 2011). miR-10b is capable of increasing BCSC stemness, self-renewal, and EMT markers by sustaining Akt pathway activation through PTEN inhibition in MCF-7 cells (Bahena-Ocampo et al., 2016). The BCSC population and EMT have been shown to be dependent on miR-21 activity as inhibition of miR-21 reverses EMT. This reversal of EMT was shown to require both the PI3K/Akt and MAPK/ERK pathway inactivation through upregulation of PTEN (Han et al., 2012). Together these data indicate that PTEN attenuates both the Akt and MAPK pathways to limit the BCSC population.

Subcellular location of PTEN determines its phosphatase activity. Lipid or protein phosphatase activity of PTEN depends on its location within the cell. Canonically, PTEN acts as a lipid phosphatase by dephosphorylating the D3 position of PIP3 reversing PIP3 to PIP2. PTEN attenuation of PI3K signaling occurs in the cytoplasm. Noncanonically, PTEN has been shown to act as a protein phosphatase in the nucleus by regulating MAPK activity through dephosphorylation of ERK1/2. In MCF-7 cells, expression of a mutant PTEN that is localized in the nucleus resulted in cell cycle arrest in the G0/G1 phase through downregulation of cyclin D1 (Chung et al., 2005b). Whereas, expression of a wild type PTEN, that localized in the cytoplasm, resulted in a higher G1/S ratio of cells compared to nuclear PTEN expressing cells (Figure 10). Cytoplasmic PTEN was found to be pro-apoptotic while nuclear PTEN promoted cell cycle arrest. The diversity of PTEN phosphatase activity makes it an attractive target for suppression in cancer cells.

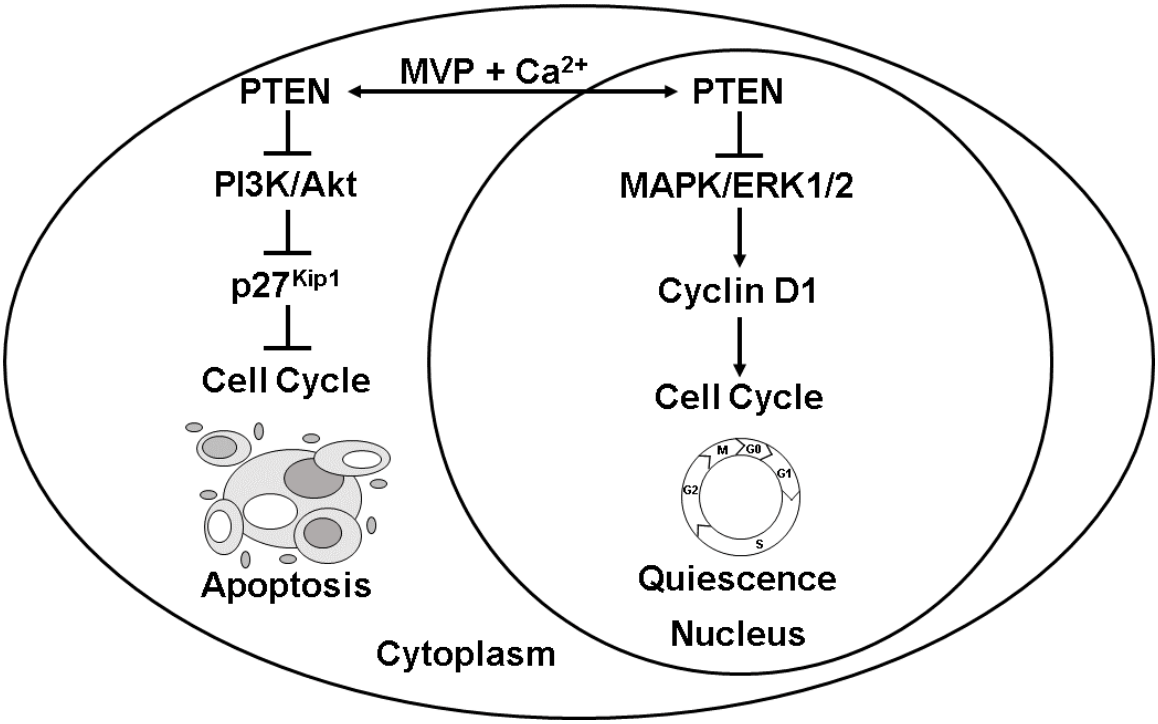

Figure 10: Subcellular Phosphatase activity of PTEN.
The phosphatase functionality of PTEN can change by its location within the cell. Cytoplasmic PTEN dephosphorylates Akt resulting in upregulation of $p27^{Kip1}$ and apoptosis of the cell. Nuclear PTEN dephosphorylates ERK1/2 resulting in downregulation of cyclin D1. Reduced cyclin D1 results in arrest of the cell cycle at the G0-G1 phase and the cell to enter quiescence.

Nuclear PTEN acts as a protein phosphatase in which it regulates ERK1/2 phosphorylation and cyclin D1 expression (Weng et al., 2001). Ras mutations are infrequent in breast cancer but increased Ras activation can occur through overexpression of EGFR or HER2 amplification in HER2+ breast cancer (von Lintig et al., 2000). Hyper-activation of the MAPK pathway through Ras correlates with increased cancer stem cell enrichment and poor patient survival in TNBC (Mittal et al., 2014). Recently it has been reported that a loss of PTEN in HER2+ breast

cancer promotes dependency on the MAPK pathway through elevated MEK/ERK activity (Ebbesen et al., 2016).

The mechanism of PTEN entry into the nucleus remains controversial. There are examples of PTEN entering the nucleus by simple transport (Li et al., 2005). However, PTEN binding to a major vault protein (MVP) has been shown to transport PTEN from the cytoplasm into the nucleus in MCF-7 cells (Chung et al., 2005a). Further assessment of PTEN nuclear translocation revealed 4 putative nuclear localization signal (NLS)-like sequences (NLS-1, -2, -3, -4) (Figure 9). PTEN localization was not affected by the mutation of one of these sequences but mutation of a pair of the NLS sequences (NLS-2 and -3; NLS-3 and -4) inhibited binding to MVP as well as entry into the nucleus. Calcium ions were found to positively regulate MVP binding to PTEN (Minaguchi et al., 2006). PTEN entry into the nucleus may also be regulated by the E_3 ligase, Neural precursor cell Expressed Developmentally Downregulated 4 -1 protein (NEDD4-1). Monoubiquitinylation prevents PTEN nuclear entry whereas polyubiquitinylation by NEDD4-1 results in PTEN degradation and loss of its tumor suppressive activity (Wang et al., 2007). Together these data present the hypothesis that PTEN functionality changes based on its location within the cell and that nuclear PTEN may play a role in breast cancer.

The MAPK Pathway.

Role of the MAPK pathway in breast development. The MAPK pathway plays a role in mammary gland development as well as tumorigenesis. Particular

effectors of the MAPK pathway have been implicated in mammary gland development, specifically ERK1/2, c-Jun N-terminal kinase (JNK), and p38 (Reviewed in Whyte et al., 2009). As described above, the phosphorylation cascade of Raf/MEK/ERK results in translocation of ERK1/2 into the nucleus to phosphorylate a number of targets including hormone receptors as well as regulators of apoptosis and transcription. Biologically, ERK1/2 phosphorylation of target substrates encourages cell proliferation, survival, differentiation (Reviewed in Pearson et al., 2001), angiogenesis (Zeng et al., 2005), invasion, and motility (Joslin et al., 2007). MAPK/ERK1/2 is involved in a number of cellular functions important to organogenesis making activation of the MAPK pathway necessary for proper mammary gland development as well as induction of breast cancer if dysregulated. For example, normal mammary epithelial cells form spherical acini that organize into branching alveolus structures when supported by a healthy ECM. Alveolar structuring by breast epithelial cells can be recapitulated in vitro by three dimensional culturing techniques (Blatchford et al., 1999). ERK1/2 signaling is necessary for bidirectional crosstalk between β1-integrins and EGFR for the proper polarization and placement of mammary epithelial cells in acinar formation (Wang et al., 1998). Conversely, previous work has shown that HER2, and not EGFR, plays an important role in acinar development in the early stages of breast cancer disease progression (Muthuswamy et al., 2001). Inhibition of ERK1/2, EGFR, or β1-integrins resulted in malformation of spherical acini as well as inhibition of PI3K indicating that both MAPK/ERK1/2 and PI3K/Akt pathways play

important roles in the proper mammary gland formation. Together these data present the importance of the MAPK/ERK1/2 pathway in proper mammary gland development and tumorigenesis.

Role of the MAPK pathway in HER2+ breast cancer. Increased HER2 activity in HER2+ breast cancer cells upregulate both downstream PI3K/Akt and MAPK pathways. Downstream effects of trastuzumab treatment on MAPK pathway activity have been controversial. One study shows that trastuzumab treatment does not affect downstream MAPK activity in HER2-positive SKBr3 and MDA-MB-453 cell lines (Cuello et al., 2001). The trastuzumab treatment concentration for this study, 1 μM, and length of treatment, 4 days, was very low. In vitro trastuzumab treatment is typically between 10 and 20 μM in order to mimic physiological treatment conditions. Conversely, a three-day course of 10 μM trastuzumab treatment resulted in inhibition of MAPK phosphorylation and activation in BT474 and SkBr3 cells (Yakes et al., 2002) as well as in metastatic breast cancer patient samples (Gori et al., 2009). These data suggest that trastuzumab affects downstream MAPK pathway activity when given at the proper dose.

MAPK pathway activation has been shown to be the central node in a variety of breast cancer subsets under varying treatment conditions. For instance, alcoholism has been shown to increase the risk of breast cancer. Alcohol exposure was shown to increase HER2+ cell migration and invasion (Xu et al., 2016). Increased aggressiveness of HER2+ cells exposed to alcohol occurred through the phosphorylation of p38γ (P-p38γ), a MAPK effector that typically responds to

DNA damage and cell stress. Activation of p38 interacted with downstream synapse associated protein 97 (SAP97) to promote cell migration and invasion as well as enrichment of the BCSC population in a HER2+ MMTV mouse model. Similarly, in a TNBC cell and mouse xenograft model, p38γ-MAPK inhibition was found to reduce the BCSC population as well as downregulate Nanog, Sox2, and Oct 3/4 cancer stem cell drivers (Qi et al., 2015). MAPK phosphatases (DUSP/MKP1) are overexpressed in HER2+ breast cancers (Candas et al., 2014). MKP1 attenuates MAPK activity by removing phosphate groups necessary to progress the MAPK phosphorylation cascade. MKP1 has been shown to induce chemoresistance and radioresistance by preventing apoptosis in HER2+ bulk and cancer stem cells. These data represent how the MAPK pathway can contribute to expansion of the BCSC population, resistance, and metastasis of HER2+ breast cancer.

Notch-1- MAPK crosstalk in breast cancer. Here we assess bi-directional crosstalk between Notch-1 and MAPK pathways in an effort to better understand their involvement in mechanisms of cancer bulk and stem cell proliferation as well as drug resistance in breast cancer. Ras (specifically H-Ras) can activate Notch-1 signaling in both in vitro and in vivo cancer models (Weijzen et al., 2002). Notch-1 activation by Ras was necessary to maintain the abnormal tumor phenotype in Ras-transformed cells. Aberrant activation of Ras signaling through p38-MAPK was found to upregulate proteins involved in Notch signaling, specifically presanilin-1 and DLL-1. Inhibition of Notch-1 reduced proliferation and tumor

growth of Ras transformed cells and tumor xenografts, respectively. Interestingly, in cells with null or low Ras expression (human foreskin fibroblasts and HER2-negative MCF-7 cells) overexpression of Notch-1 was unstable and toxic to these cells. Additionally, NICD1 overexpression in hTERT/SV40 immortalized fibroblasts was found to increase cell proliferation and anchorage independent growth compared to the vector control but not to level of Ras transformed cells. This result suggesting that oncogenic Ras allows cells to tolerate elevated Notch-1 expression. Together these data present a potential mechanism for Ras-mediated upregulation of Notch-1 to drive and maintain the neoplastic phenotype in breast cancer.

Immunohistological assessment of Notch in progressive stages of breast carcinogenesis revealed that Notch-1, and downstream Notch targets, were increased in approximately 75% of breast cancer tissue samples compared to normal breast tissue samples (Mittal et al., 2009). Furthermore, aberrant activity of Notch was apparent in DCIS and hyperplasia suggesting that abnormal Notch activity occurs early in breast cancer progression. HMLE (Human Mammary Epithelial cells overexpressing hTERT and SV40 large and small T antigens) cells have low Notch-1 and Ras expression. Transformation of HMLE cells with Notch-1 failed to form mammospheres *in vitro* or tumors *in vivo*. Conversely, NICD1 overexpression in Ras transformed cells (HMLER) increased mammosphere formation *in vitro* and tumor growth in a mouse xenograft model compared HMLE or HMLE with NICD1 overexpression alone. Inhibition of either Notch by GSI or

Ras/MAPK pathway by the MEK inhibitor PD 98059 reduced BCSC survival and self-renewal. Positive staining for both Notch and/or HES-5 as well as nuclear P-ERK1/2 in breast tumor tissues correlated to aggressive grade III carcinomas with a high risk of metastasis. These results suggest an important role for the co-expression of Notch-1 and Ras/MAPK to promote BCSC survival and self-renewal as well as tumor progression.

Conclusion.

These data presented here begin to reveal the complexity of the Notch-1/PTEN/MAPK signal transduction pathway. As described above there are numerous interactions between Notch-1 and PTEN, as well as downstream PI3K/Akt and MAPK/ERK1/2 pathways. Being that both Notch-1 and PTEN are context dependent we have limited our scope to trastuzumab resistant HER2+ breast cancer which may draw from more aggressive cancer models such as TNBC or endocrine resistant ER+ breast cancer. However, little work has been done on the role of Notch-1, PTEN, and the MAPK/ERK pathway in trastuzumab resistant breast cancer. The results presented here may shed some light on the mechanics of more aggressive cancers by presenting a novel role for Notch-1 in PTEN regulation and MAPK activation in trastuzumab resistant HER2+ breast cancer.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture.

BT474TS and HCC1954 breast cancer cells were purchased from American Type Culture Collection (ATCC). BT474TR, trastuzumab resistant, cells were generated *in vitro* by treating parental BT474TS, trastuzumab sensitive, cells with increasing concentrations of trastuzumab for 6 months till the cells could survive under constant trastuzumab treatment of 10μg/mL in PBS (Osipo et al., 2008). Cells were cultured in Dulbecco's minimal essential media with high glucose [1x DMEM (Fisher Scientific, Hampton, NH)] 10% fetal bovine serum (FBS), 100μM L-Glutamine, and 100μM nonessential amino acid. Cells were maintained at 37ºC with 5% CO_2 and 95% O_2. Cells were authenticated by STR allelic profiling (DCC Medical).

Drugs.

Trastuzumab is a humanized monoclonal antibody against extracellular domain IV of the HER2 receptor (Carter et al., 1992). Lyophilized trastuzumab was resuspended in sterile PBS for a concentration of 22mg/mL. Cells in culture were maintained at 7 to 10μg/mL in media. Experimental trastuzumab treatments were 20μg/mL. U0126 inhibits MAPK pathway activity by noncompetitive inhibition of the dual specific kinases (threonine and tyrosine) MEK1 and MEK2. U0126-mediated

inhibition of MEK1/2 results in reduced phosphorylation of the MEK1/2 kinase substrate, ERK1/2. U0126 was resuspended in DMSO for a concentration of 20mg/mL and used in treatments at concentration of 10µg/mL *in vitro*.

Expression Vectors.

The pcDNA and pcDNA-NICD1 expression vectors were kindly provided by Dr. Lucio Miele (Department of Genetics, Louisiana State University, New Orleans, LA). The MEK1 S218D/S222D phosphomutant pEXP304-MEK1DD/V5, and control vector pEXP304-LacZ/V5, were kindly provided by Dr. Takeshi Shimamura (Department of Molecular Pharmacology and Therapeutics, Maywood, IL). The pUSEamp and N-terminal Myrstoylated-Akt1 expression vectors were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY).

RNA Interference and Transfection Reagents.

Notch-1, PTEN, and HES-1 siRNAs were purchased from Santa Cruz Biotechnology (Dallas, TX). Secondary Notch-1 siRNA (Notch-1i #2) was purchased from Thermo Fisher Scientific [Dharmacon (Waltham, MA)] and scrambled siRNA (SCBi) was purchased from Qiagen (Hilden, Germany). Transfection reagents, Lipofectamine 2000, Lipofectamine 3000, and RNAiMax were purchased from Invitrogen (Carlsbad, CA) as well as Polyethylenimine (PEI) form PolySciences (Niles, IL).

BT474 (3×10^5) or HCC1954 (1×10^5) cells per well were seeded into a 6 well tissue culture treated plate. The following day, 1µg of vector DNA was mixed with 250µL of Opti-MEM (Life Technologies, Carlsbad, CA) in a 15mL tube. In a

separate microcentrifuge tube, 4μL of Lipofectamine 2000 or PEI were mixed with 250μL of Opti-MEM. The two tubes were incubated for 5 minutes at room temperature then mixed together and incubated for an additional 20 minutes at room temperature. The total volume of the transfection reaction was 500μL. The cells were washed with PBS. 1.5mL of media was added to the transfection reaction and then added to the washed cells. Similarly, transfections using Lipofectamine 3000 in a 6 well plate, mixed 5μg of vector DNA, 5μL of P3000, and 250μL of Opti-MEM in one tube. 3.75μL of Lipofectamine 3000 and 250μL of Opti-MEM were mixed in a separate tube followed by incubation and mixing in the same manner as Lipofectamine 2000. siRNA transfections using RNAiMax were performed in a similar manner. In a 6 well plate, 10μM of siRNA was added to 250μL of Opti-MEM in a 15mL tube. 10μM of RNAiMax was added to 250μL of Opti-MEM in a separate tube. Both tubes were mixed, incubated, and added to the cells in the same manner as previous transfections. Co-transfections used halved ratios of siRNA and RNAiMax, 5μM and 5μL, respectively. The siRNA sequences released by their respective companies are shown in Table 1.

siRNA	Company	Catalog #	Sense Sequence (5’ – 3’)	Antisense Sequence (5’ – 3’)
Notch-1	Santa Cruz	Sc-36095A	CACCAGUUUGAA UGGUCAAtt	UUGACCAUUCAA ACUGGUGtt
		Sc-36095B	CCCAUGGUACCA AUCAUGAtt	UCAUGAUUGGUA CCAUGGGtt
		Sc-36095C	CCAUGGUACCAA UCAUGAAtt	UUCAUGAUUGGU ACCAUGGtt
Notch-1	Thermo Fisher (Dharamcon)	HSS107248	ACGAAGAACAGA AGCACAAAGGCG G	CCGCCTTTGTGC TTCTGTTCTTCGT
PTEN	Santa Cruz	Sc-29459A	CAGUGGCACUGU UGUUUCAtt	UGAAACAACAGU GCCACUGtt
		Sc-29459B	GUGUGGUGAUAU CAAAGUAtt	UACUUUGAUAUC ACCACACtt
		Sc-29459C	GAAGAUCAGCAU ACACAAAtt	UUUGUGUAUGCU GAUCUUCtt
Scrambled Control (SCBi)	Qiagen	1027281	Sequence held by Qiagen	

Table 1: siRNA sequences

Antibodies.

PTEN (9559S), Phosphorylated Akt1 (Ser473, 9271S and Thr308, 9275S), Pan Akt (4691S), Total p44/42 ERK1/2 (4695), Histone H3 (4620) and Phosphorylated p44/42 ERK1/2 (Tyr202/Tyr204, 4370) were purchased from Cell Signaling Technologies (Danavers, MA). Notch-1 (C-20, sc-6014), MEK-1 (C-18, sc-219), and normal rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Total HER2 [(ErbB2) MS0730-P] was purchased from Thermo Fisher Scientific (Waltham, MA). Phosphorylated HER2 at tyrosine 1248 [PY1248-ErbB2 (AF1768)] was purchased from R and D Systems (Minneapolis, MN). β-Actin (A5441) was purchased from Sigma Aldrich (St. Louis, MO) and used as an endogenous control for all Western blots. Secondary

antibodies used include donkey anti-rabbit (1:1000, sc-2313) and donkey anti-mouse (1:5000, sc-2314) purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Reverse Transcription Real-Time Polymerase Chain Reaction (RT-PCR).

BT474 (3×10^5) or HCC1954 (1×10^5) cells were plated in a 6-well tissue culture treated dish. The next day, the cells were transfected using the appropriate transfection reagents and incubated at 37 ºC with 5% CO_2 and 95% O_2 for 2 days. Similarly, co-transfections combining siRNA and expression vectors were staggered meaning that one transfection was done in the morning and one in the evening. 2 days later, the cells were harvested in TRIzol® (Thermo Fisher Scientific, Waltham, MA). The cells were harvest on ice, by scraping the cells into the TRIzol® (500µL) solution. Total RNA was extracted from the harvested cells using the RiboPure™ Kit (Thermo Fisher Scientific). The cells in TRIzol® were incubated at room temperature for 5 minutes then 50µL of 1-bromo-3-chloropropane [(BCP) Sigma Aldrich, St. Louis, MO] was added to each sample. The sample was vortexed and incubated for 5 minutes at room temperature. Next, the samples were centrifuged at 15,000 RPM for 10 minutes at 4ºC. On ice, the clear aqueous phase was transferred to a new tube to which 200µL of 100% ethanol was added and mixed immediately. The sample was passed through a filter cartridge by centrifugation at 12,000 RPM for 30 seconds at room temperature. Each sample was washed twice with 250µL of wash buffer and spun at 12,000 RPM for 30 seconds at room temperature. After the second wash, the

tubes were spun one more time at 12,000 RPM to discard any access ethanol/wash buffer. Each filter cartridge was added to a new tube and 100µL of elution buffer was added. The tubes were incubated in a 37 ºC water bath for 5 minutes then the RNA was extracted from the filter cartridge into a new microcentrifuge tube via centrifugation at 12,000 RPM for 30 seconds at room temperature. Total RNA quality and quantity was determined by measuring the UV absorbance at 260 nm using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

The harvested total RNA was reverse transcribed (RT) into total cDNA using the TaqMan Reverse Transcriptase Kit (Applied Biosystems, Ford City, CA). 1µg of total RNA was reverse transcribed into cDNA in a total volume of 100µL containing: 1x RT buffer, 5.5mM $MgCl_2$, 500µM dNTPs, 2.5µM random hexamers, 0.4 U/µL RNase inhibitors, and 1.25 U/µL RT enzyme (Multiscribe™ Reverse Transcriptase Kit, Applied Biosystems). The RT reaction was incubated in a thermal cycler under the parameters:10 minutes at 25 ºC, 30 minutes at 48 ºC, 5 minutes at 95 ºC, 60 minutes at 25 ºC, and held at 4 ºC until use.

Real time PCR was performed using iTaq™ SYBR® Green Supermix with ROX (BioRad, Hercules, CA) in a 96-optical PCR plate. A mastermix solution of 22.5µL per sample was made of: 50µM forward and reverse primers, and 2x SYBER® Green Universal Master Mix. 2.5µL of cDNA was added to 22.5µL of mastermix in triplicate wells. Quantitative PCR was run using the StepOnePlus thermocycler (Applied Biosystems, Foster City, CA) under the following

parameters: initial denature at 95 ºC for 10 minutes, PCR cycling for 10 seconds at 95 ºC for 40 cycles, and annealing for 45 seconds at 60 ºC. Upon completion of the PCR cycling, melt curves were recorded and analyzed to determine the average cycle threshold (Ct) value. The Ct value is the number of cycles necessary for the fluorescent signal to overcome the background level, or threshold, of fluorescent signal. Ct values were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT), an endogenous control, to discern ΔCt. ΔΔCt was calculated by normalizing the ΔCt values to a control sample, such as the scrambled control siRNA treated cells. Relative quantification (Rq) was calculated using the $2^{-\Delta\Delta Ct}$ method to determine relative fold increases or decreases in transcript compared to the control sample (Rao et al., 2013). The PCR primers used for the detection of specific transcripts are shown in table 2.

Real Time PCR Primers		
cDNA Primers	Forward Primer 5'-3'	Reverse Primer 5'-3'
Notch-1	ATCAACGCCGTAGATGACC	TTGTTAGCCCCGTTCTTCAG
PTEN	ACCAGGACCAGAGGAAACCT	GCTAGCCTCTGGATTTGACG
HEY-1	TCATTTGGAGTGTTGGTGGA	CTCGCACACCATGATCACTT
HPRT	ATGAACCAGGTTATGACCTTGAT	CCTGTTGACTGGTCATTACAATA
ChIP Primers		
PTEN	TTCTCCTGAAAGGGAAGGTG	GGAGGCAGTAGAAGGGGAGAG
HEY-1	AGCGTGGGAAAGGATGGTTG	CTCGCTTCATGCTGGCTCCC

Table 2: PCR Primer sequences

Western Blot Analysis.

BT474 or HCC1954 were plated in 10 cm^2 (3 x 10^6 or 1 x 10^6) tissue culture treated dish. The following day, the cells were transfected with plasmid DNA or siRNA, treated with 20µg/mL trastuzumab or 10µg/mL U0126 and incubated at 37 ºC with 5% O_2 and 95% CO_2 for 48 hours. After transfection, the plates were put on ice, media was aspirated, and the cells were washed with cold PBS. Cells were lysed by adding 300µL per 10 cm^2 dish of Triton X-100 lysis buffer or Radioimmunoprecipitation Assay (RIPA) buffer, scraped, and collected in appropriately labeled microcentrifuge tubes. Triton X-100 buffer consists of: 50mM HEPES (pH to 7.4), 1% Triton X-100, 150mM NaCl, 5mM EDTA, 1mM Na_3VO_4, 10mM NaF, 1mM PMSF, 1 protease inhibitor cocktail tablet. RIPA buffer consists of: [0.1 % SDS, 50mM Tris-HCl, 1% NP-40, (pH to 7.0)] 0.5% Na deoxycholate, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, 1 protease cocktail tablet. Little to no

difference was observed between using Triton X-100 and RIPA buffer but both were used. The harvested samples were vortexed for 5 seconds at a setting of 10 followed by incubation on ice for 20 minutes. After incubation, the samples were sonicated two times for 10 seconds at 20% amplitude using the Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA). 10µL of each sample were plated into a 96-well plate, along with 0, 0.5, 1, 2, 4, 6, 8, and 10mM BSA protein standards, to determine protein concentration by BCA protein assay [50:1 Reagent A:Reagent B (Thermo Fisher Scientific)]. The plate was incubated at 37 ºC for 30 minutes then read on a 96-well plate fluorescent plate reader. Protein concentrations were calculated [regression line from protein standard curve (y=mx+b) used to calculate Average protein concentration – b / m)] and made into 10 – 20 µg aliquots in beta-mercaptoethanol (Fisher, Waltham, MA) and 2x lamelli buffer (BioRad, Hercules, CA). Samples were heated for 5 minutes at 95 ºC.

Proteins were detected by Western blot using a 7% Tris-Acetate or 4-12% Bis-Tris pre-cast gel (Invitrogen, Carlsbad, CA) as the proteins are detectable in either gel. 10 to 40µg of prepared protein lysate as well as a pre-stained protein ladder (SeeBlue® Plus 2, Thermo Fisher Scientific) were loaded into separate lanes of an SDS-PAGE gel in 1x Tris-Acetate SDS running buffer (Invitrogen). The 7% Tris acetate gel was run at 150V for 1 hour or the 4-12% Bis-Tris gel was run at 200V for 50 minutes using the XCell SureLock © MiniCell apparatus (Thermo Fisher Scientific). The gel was transferred to a polyvinylidene fluoride (PVDF) membrane using 1x transfer buffer [50 mL. of 10x NuPAGE © transfer buffer

(Invitrogen), 200 mL methanol, 750 mL water] at 40 volts for 2 hours using the XCell II™ Blot Module (Thermo Fisher Scientific). After completion of the gel transfer, the PVDF membrane was hydrated in methanol for 30 seconds then rinsed in deionized water. The membrane was blocked in 5% non-fat milk (Bio-Rad) or Roche blocking solution [Roche buffer (Roche, Basel, Switzerland)] in 1x Tris Buffered Saline with Tween 20 (TBST) [(10x TBST (12.1 g Tris Base, 87 g sodium chloride, ph to 8.0 in 1 L) 0.2% Nonidet P-40, 0.05% Tween-20] or 1x Tris Buffered Saline [(TBS) no Tween-20 added], respectively, for 1 hour at room temperature under constant agitation. The membrane was incubated with a primary antibody at 4ºC overnight under constant agitation. The following day, the membrane was washed 3 times in 1x TBST solution for 10 minutes while under constant agitation at room temperature then the appropriate secondary antibody conjugated to horseradish peroxidase was added. The secondary antibody was diluted in the appropriate blocking solution for 1 hour at room temperature under agitation. Membranes were washed 3 times in 1x TBST for 10 minutes. Proteins were detected by adding Enhanced Chemiluminesence (Thermo Fisher Scientific) or SuperSignal West Extended Duration substrate (Thermo Fisher Scientific) in a 1:1 volume, incubated for 1 or 5 minutes, respectively, at room temperature. Stained bands were visualized by exposing the membrane to radiography film (MedSci) in the dark room.

Membranes were regularly stripped and re-probed to detect additional proteins. The membrane was washed once in 1x TBST. Stripping buffer (Thermo

Fisher Scientific) was added to cover the membrane and incubated for 30 minutes at room temperature under constant agitation. After 30 minutes, the membrane was hydrated in methanol for 30 seconds then rinsed in deionized water. The membrane was washed 3 times for 10 minutes in 1x TBST then blocked in 5% non-fat milk for 1 hour at room temperature. The appropriate primary antibody was added and the procedure was continued as described previously.

Densitometry of Wester blots was done using ImageJ software to quantitate protein amounts in three separate experiments (Rasband, 1997). Notch-1 and PTEN proteins were normalized to the loading control, β-actin. Phosphorylation of Akt at Serine473 was normalized to total Akt.

Chromatin Immunoprecipitation (ChIP).

Chromatin Immunoprecipitation (ChIP) assays were performed using the SimpleChIP© Plus kit (Cell Signaling Technology, Danvers, MA). Specifications for some of the components used in this kit would not be disclosed by Cell Signaling Technology. BT474 (5×10^6) were seeded in 3x 15 cm^2 tissue culture treated dishes. The following day, BT474TS cells were transfected with NICD1 (7.5µg) or pcDNA3 using PEI (30µL) for 48 hours. After 2 days, the cells were washed in cold PBS, trypsinized (2x trypsin), and the 3 plates were harvested into one appropriately labeled 50mL canonical tube on ice. Cells were counted using the Countess Automated Cell Counter (Life Technologies, Carlsbad, CA) by Trypan Blue staining and 25×10^6 live cells were used for one ChIP sample. The cells were cross-linked by adding 1% formaldehyde (Sigma Aldrich, St. Louis, MO)

for 30 minutes at room temperature under constant agitation. After 30 minutes, the cross-linking reaction was quenched with 1.25M glycine, swirled, and incubated for 5 minutes at room temperature. The cross-linked cells were centrifuged at 1500 RPM for 5 minutes at 4ºC then washed twice with ice cold PBS. The cells were pelleted by centrifuged at 1500 RPM for 5 minutes at 4ºC. Untransfected BT474TS and BT474TR cells were seeded and harvested in the same manner.

The cells were resuspended in 1mL of ice cold Buffer A (750µL deionized water, 250µL 4x Buffer A, 0.5µL (1M) DTT, 5µL (200x) PIC) for each immunoprecipitation (IP) and incubated on ice for 10 minutes. The cells were mixed by inversion and lightly vortexed every 3 minutes during the 10-minute incubation period. The nuclei were pelleted by centrifugation at 3000 RPM for 5 minutes at 4ºC. The supernatant was aspirated from the pelleted nuclei and resuspended in 1mL of ice cold Buffer B (825µL deionized water, 275µL 4x buffer B, 0.55µL (1M) DTT). Centrifugation was repeated at 3000 RPM for 5 minutes at 4ºC, supernatant was aspirated, and 100µL of buffer B was added. 1µL of micrococcal nuclease (4000 gel units/µL) was added to the nuclei for chromatin digestion and incubated at 37 ºC for 20 minutes with intermittent mixing by inversion every 3 to 5 minutes. After 20 minutes, the digestion was stopped by the addition of 10µL of 0.5M Ethylenediaminetetraacetic acid (EDTA). The nuclei were pelleted by centrifugation at 13,000 RPM for 1 minute at 4 ºC. The supernatant was aspirated and the pellet was resuspended in 100µL of 1x ChIP Buffer (90µL deionized water, 10µL 10x ChIP buffer, 0.5µL 200x PIC) and incubated on ice for

10 minutes. After 10 minutes, the sample was sonicated 3 times for 20 seconds (pulse 20 seconds on, 30 seconds off) at 20% amplitude. The chromatin was centrifuged for 10 minutes at 10,000 RPM at 4 ºC and transferred to a fresh microcentrifuge tube. 10µL of digested chromatin was put aside at -20 ºC to be used as a 2% input control. This method of digestion should yield chromatin fragments of 150 – 900 base pairs (bp). 3 antibodies were used for chromatin immunoprecipitation: C-terminal Notch-1 (C-20), normal rabbit IgG, and Histone H3 meaning that the prepared chromatin is immunoprecipitated by 3 different antibodies in 3 separate microcentrifuge tubes. 100µL of chromatin was diluted in 400µL of 1x ChIP buffer for each ChIP. 10µL of Notch-1 (200 µg/mL), 1µL of normal rabbit IgG (200 µg/0.5 µL), or 10µL of Histone H3 antibody was added to the diluted chromatin. The antibody:chromatin mixture was rotated overnight at 4 ºC to facilitate chromatin immunoprecipitation.

The next day, condensation in the ChIP sample was lightly spun down to get all of the antibody:chomatin mixture to the bottom of the microcentrifuge tube. The ChIP sample was placed in a magnetic rack. The tube was left in the rack for 1 to 2 minutes until the beads visibly gathered on one side of the tube allowing aspiration of the supernatant from the tube. The ChIP sample was washed with low salt buffer (1x ChIP Buffer) 3 times then high salt buffer [(1x ChIP Buffer and 5M NaCl] one time. 150µL of ChIP elution buffer (2x ChIP Buffer) was added to the ChIP sample in a fresh screw top microcentrifuge tube and put in a shaker at 300 RPM at 65 ºC for 30 minutes. Condensation was lightly spun down in the ChIP

sample and placed in the magnetic rack. The supernatant contains the immunoprecipitated DNA which is placed in a new screw top microcentrifuge tube. 150µL of ChIP elution buffer was added to the 2% Input samples. 6µL of 5M $NaCl_2$ and 2µL of proteinase K (20 mg/mL) was added to both the ChIP and 2% input samples followed by incubation at 65 ºC for 2 hours.

After the 2 hour incubation, 600µL of DNA binding reagent A was added at a 4:1 ratio to each sample. The mixture was transferred to a DNA purification column in a microcentrifuge tube and centrifuged for 30 seconds at 14,000 RPM. The supernatant was discarded and the DNA in the purification column was washed twice with 700µL of wash reagent B. The spin column was put in a new, properly labeled microcentrifuge tube. 50µL of DNA elution reagent C was added to the DNA and spun down again for 30 seconds at 14,000 RPM. The DNA was diluted in deionized water at a 1:5 ratio. Quantitative PCR was performed as described in section 6, Reverse Transcription Real-Time Polymerase Chain Reaction (RT-PCR), using HEY-1 and PTEN ChIP primers that were designed to flank CBF-1 binding elements (Persson et al., 2010) (Table 2) to quantitate NICD1 enrichment of the PTEN and HEY-1 promoters.

Mammosphere Assay.

BT474 or HCC1954 were plated in a 6-well (3×10^5 or 1×10^5) tissue culture treated dish. The following day, the cells were transfected with plasmid DNA or siRNA and incubated at 37 ºC with 5% O_2 and 95% CO_2 for 48 hours. After 2 days, the cells were trypsinized and harvested in PBS. Live cells were counted using the

Countess Automated Cell Counter (Life Technologies, Carlsbad, CA) by Trypan Blue staining. 1.0 x 10^6 cells were suspended in 1 ml of PBS. 1.0 x 10^5 BT474 or 5.0 x 10^4 HCC1954 cells were seeded in mammosphere forming media [(196mL DMEM/F12 (Life Technologies), 4g methyl cellulose (Sigma Aldrich, St. Louis, MO), 10mL B27 (Life Technologies), 1µL recombinant EGF (Sigma Aldrich)] on low attachment 6 well plates (Dot Scientific, Burton, MI). Mammosphere media was pretreated with PBS, trastuzumab (20µg/mL in PBS), dimethylsulfoxide (DMSO), or U0126 (10µg/mL in DMSO) per well. Mammosphere media was added to the well followed by the cells on top of the media then vigorously swirled to assure cell individualization in the mammosphere media. The BT474 mammospheres were incubated at 37 ºC with 5% O_2 and 95% CO_2 for 10 days and the HCC1954 mammospheres for 7 days.

After incubation, pictures of the mammospheres were taken at 20x magnification using a Nikon Diaphot TMD Fluorescence Phase Contrast Inverted microscope (Nikon, Tokyo, Japan). 2mL of PBS were added to the well and incubated at 37 ºC for 30 minutes to dissolve the mammosphere media. After 30 minutes, the PBS:mammosphere solution was put in a properly labeled 50mL tube. Care was taken not to disrupt the mammospheres during collection. Addition of PBS to the mammospheres was repeated a total of three times to completely remove all mammospheres from the well. The harvested mammospheres were centrifuged at 1200 RPM for 2 minutes at 4 ºC. Care was taken to only aspirate off the mammosphere media:PBS mixture leaving the mammospheres pelleted at the

bottom of the 50mL tube. BT474TS mammospheres were resuspended in 3mL of PBS and BT474TR or HCC1954 in 5 mL of PBS. 50μL of the mammosphere:PBS mixture was plated in a flat bottom, 96 well plate. 4 to 5 pictures at 4x magnification were taken of the of the mammospheres. These pictures were used to count the number of mammospheres/50μL. Mammospheres larger than 50 μmicrons were counted as such. Percent mammosphere forming efficiency (%MFE) was calculated as follows: number of mammospheres counted/number of cells seeded x 100.

After the pictures were taken, 300μL of trypsin was added to the primary mammospheres. The mammospheres were incubated at 37 ºC for 5 minutes then disaggregated by vigorous pipetting. Once the mammospheres were disaggregated into single cells, media was added to deactivate the trypsin and the cells were washed twice in PBS. 1.0 x 10^6 live cells from primary mammospheres were resuspended in 1mL of PBS. The live cells were plated in mammosphere media and incubated to form secondary mammospheres. The secondary mammospheres were harvested, counted, and secondary %MFE was calculated in the same manner as primary mammospheres.

Trastuzumab Resistant BCSC Tumor Xenograft Growth.

3 x 10^6 BT474TR cells were seeded in a 10 cm^2 tissue culture treated dish. The following day, the cells were transfected with scrambled control, Notch-1, PTEN, or Notch-1 and PTEN siRNA for 2 days. After transfection, the cells were trypsinized (2x trypsin), counted, and 1 x 10^5 live cells were seeded in

mammosphere media as described in section 9, mammosphere assay. After a 10 day incubation period, the mammospheres were harvested and disaggregated. 1 x 10^4 live cells in 50µL of PBS were mixed in 50µL of Matrigel (Corning, Corning, NY). The Matrigel:PBS suspension of cells was injected bilaterally into the mammary fats pads of 5 to 6 week old, ovariectomized, FoxN1nu/nu athymic, nude female mice (Charles River, Chicago, IL) along with implantation of a 17β-estradiol-containing silastic release capsule of 0.3cm in length for a constant release of 83-100pg/mL as previously described (O'Regan et al., 1998). Each mouse was tagged with an ear tag to identify their treatment group (SCBi, N-1i, PTENi, N1i + PTENi) as well as record their tumor take and growth. Tumor growth was measured weekly as area (length x width) using Vernier calipers. Some tumors reached the maximum cross sectional area [(l x w)π/4)] allowed (0.5cm^2) by week 12. On week 12, mice were sacrificed, tumors were harvested, and frozen at -80 ºC. Percent tumor take as well as tumor growth was calculated and graphed. All animal study protocols were approved by Loyola University's Institutional Animal Care and Use Committee.

Statistical Analysis.

Statistical analysis of at least 3 independent experiments was performed. Means plus or minus standard deviations were calculated using Excel software (Microsoft). A two-sided student's t-test was used to compare 2 groups. A one-way ANOVA with Tukey's range test was used to compare multiple groups. Asterisk (*) represents statistical significance between control groups and sensitive cells and

(**) represents statistical significance between Notch-1 inhibition compared to other treatment groups. We performed power analysis to determine the appropriate number of mice needed per treatment group for the *in vivo* study. Based on previous experience, we determined the number of mice required for each treatment group was 6. A Kaplan-Meier curve for tumor formation was generated by Prism Version 6 (GraphPad Software). P-values <0.05 were considered statistically significant.

Meta-Analysis of microarray cohorts. The Hatzis, Bonnefoi, and Curtis breast cancer microarray cohorts were acquired from oncomine (oncomine.org). Dr. Jun Li from the University of Notre Dame analyzed the cohorts with a log rank (Mantel-Cox) test.

CHAPTER 3

HYPOTHESIS AND SPECIFIC AIMS

Resistance to anti-ErbB-2 (HER2)-targeted therapy remains a clinical problem for women with HER2+ breast cancer. Resistance has been shown to lead to enrichment of cancer stem cells, tumor recurrence, disease progression, and ultimately death. There is an immediate need to elucidate mechanisms responsible for drug resistance and breast cancer stem survival and self-renewal. Discerning these mechanisms will identify actionable targets to combat breast cancer progression. Several markers of trastuzumab resistance are known including downregulation of the tumor suppressor, PTEN. Canonically, PTEN acts as a lipid phosphatase to reduce the PI3K/Akt pathway and non-canonically as a protein phosphatase to dephosphorylate nuclear ERK1/2 resulting in diminished MAPK signaling. Women with HER2+ breast tumors expressing less PTEN and increased PI3K/Akt or MAPK activity have worse overall outcome. Our lab has previously shown that trastuzumab resistant cells have increased expression of Notch-1 which drives cell proliferation *in vitro* as well as tumor recurrence *in vivo*. Yet, we do not know the mechanism by which Notch-1 represses PTEN to increase downstream signaling and trastuzumab resistance. Initial characterization of trastuzumab resistant cells (BT474TR) display an increase in Notch-1 and a decrease in PTEN protein expression as well as upregulation of Akt and MAPK

pathway activity as measured by phosphorylation of Akt (Ser473-Akt1 and Thr308-Akt1) and ERK1/2 compared to trastuzumab sensitive cells (BT474TS) (Figure 11A). Preliminary results demonstrate an increase in PTEN protein (Figure 11B) and transcript (Figure 11C) expression upon Notch-1 knockdown. Additionally, Notch-1 knockdown results in downregulation of ERK1/2 phosphorylation (Figure 11B) indicating reduced MAPK pathway activity. Taken together, these results suggest the following hypothesis (Figure 12).

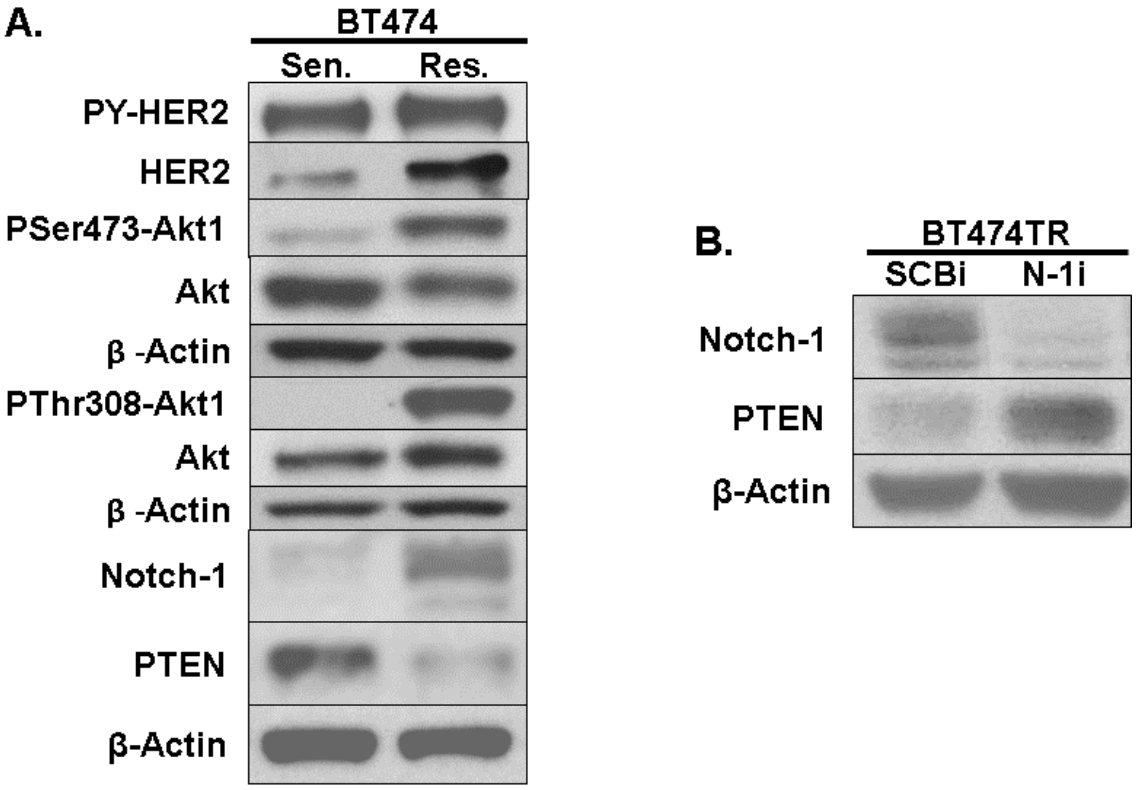

Figure 11: Characterization of the trastuzumab resistant (BT474TR) cell line.

A. Total protein lysates from BT474TS [Trastuzumab Sensitive (Sen.)] and BT474TR [Acquired Trastuzumab Resistant (Res.)] cells underwent Western blot analysis to detect HER2, HER2 phosphorylation (PY-HER2), Akt phosphorylation (PSer473-Akt1, PThr308-Akt1), Notch-1, PTEN, and β-Actin protein expression. **B.** BT474TR cells were transfected with scrambled control (SCBi) or Notch-1 siRNA (N-1i) for 48 hours. Post transfection, total cellular Notch-1, PTEN, and β-Actin protein expression was analyzed by Western blot.

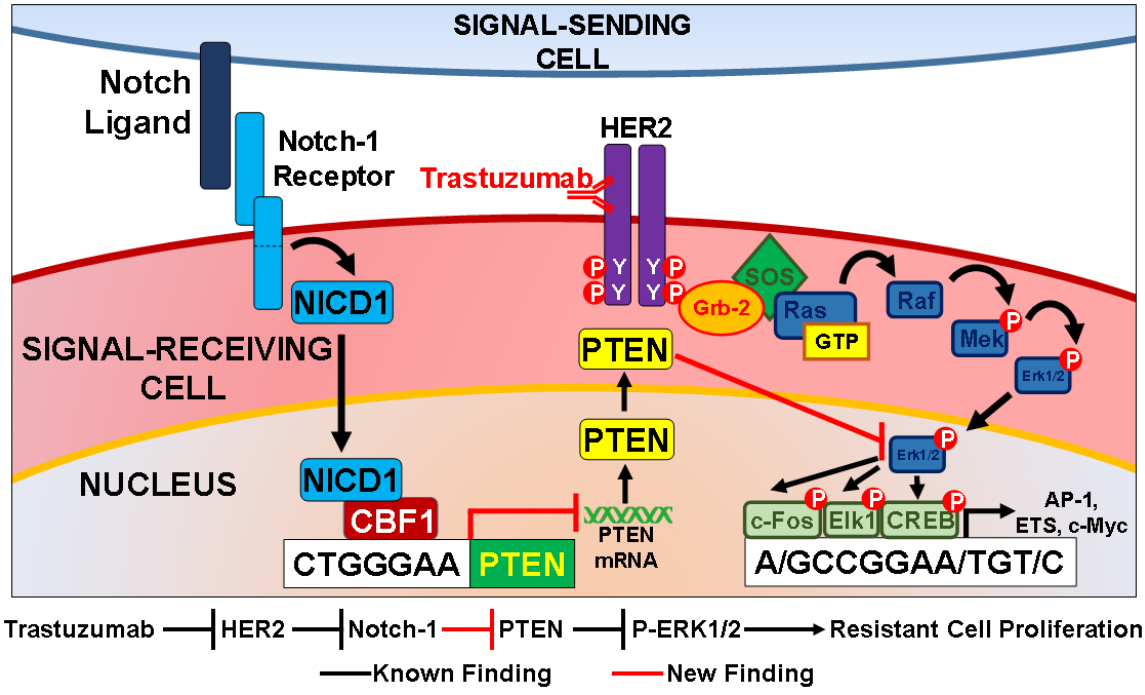

Figure 12: Notch-1 mediated inhibition of PTEN in trastuzumab resistant cells promotes activation of the MAPK/ERK1/2 pathway.

The role of Notch-1-mediated repression of PTEN in HER2+ trastuzumab resistant breast cancer

We will address this hypothesis with the following specific aims:

Specific Aim 1: Determine if Notch-1 directly binds to the PTEN promoter in trastuzumab resistant cells.

Specific Aim 2: Determine the biological significance of Notch-1-mediated downregulation of PTEN in trastuzumab resistant cells.

Specific Aim 3: Determine if Notch-1-mediated repression of PTEN is necessary for trastuzumab resistant tumor initiating potential *in vivo.*

Our studies will elucidate the mechanism by which Notch-1 inhibits PTEN in HER2+ breast cancer cells. Upregulation of Notch-1 in trastuzumab treated, as well as trastuzumab resistant, cells through inhibition of HER2 has been established. However, the role of Notch-1 in driving trastuzumab resistance remains unknown. PTEN is a well-established suppressor of the PI3K/Akt pathway which has been associated with drug resistance and a more aggressive breast cancer phenotype. A majority of work focuses on PTEN-mediated downregulation of the Akt pathway but PTEN-mediated inhibition of the MAPK pathway has not been well characterized in trastuzumab resistant HER2+ breast cancer. Here, for the first time, we demonstrate the ability of Notch-1 to mediate PTEN inhibition which results in increased MAPK activity in HER2+ trastuzumab resistant cells. Furthermore, Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant cell proliferation as well as breast cancer stem cell survival and self-renewal *in vitro*. Additionally, Notch-1 repression of PTEN promotes tumorigenesis in a trastuzumab resistant tumor xenograft model. Together, these results demonstrate the functional relevance of Notch-1 regulation of PTEN in HER2+ breast cancer. These studies present a mechanism by which Notch-1 regulation of the PTEN-ERK1/2 signal transduction pathway promotes resistance to trastuzumab treatment. Furthermore, these studies establish combined low PTEN and high Notch-1 activity as good prognostic indicators of trastuzumab resistance in HER2+ breast cancer patients. This data presents a new perspective on the role of Notch-1 in trastuzumab resistant breast cancer and further promotes Notch-1

as a therapeutic target for women who confront resistance to first line anti-HER2 therapies such as trastuzumab.

CHAPTER 4

RESULTS

Specific Aim 1. Determine if Notch-1 Directly Binds to the PTEN Promoter in Trastuzumab Resistant Cells.

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

Aim 1A: Discern if Notch-1 is necessary to inhibit PTEN expression in trastuzumab resistant cells.

Aim 1B: Determine if Notch-1 is sufficient to repress PTEN expression in trastuzumab sensitive cells.

Aim 1C: Ascertain if Notch-1 is directly recruited to the PTEN Promoter.

Aim 1A: Discern if Notch-1 is Necessary to Inhibit PTEN Expression in Trastuzumab Resistant Cells.

Rationale. We wanted to determine the mechanism by which Notch-1 maintains trastuzumab resistance in HER2+ breast cancer cells, specifically through the PI3K/Akt pathway. Previous work by our laboratory has demonstrated that resistant cells depend on the Notch pathway to promote HER2+ breast cancer cell proliferation *in vitro* and tumor growth and recurrence *in vivo* (Osipo et al., 2008;

Pandya et al., 2011). Additionally, our preliminary data shows that trastuzumab resistant cells have an increase in Notch-1 protein expression and Akt-1 phosphorylation as well as a decrease in PTEN protein expression when compared to trastuzumab sensitive cells (Figure 11A). Moreover, both sensitive and resistant cells have similar levels of tyrosine phosphorylated HER2 (PY-HER2), suggesting that continued proliferation of trastuzumab resistant cells is likely independent of HER2 activity. Importantly, upon Notch-1 knockdown, we observed an increase in PTEN protein expression as detected by Western blotting (Figure 11B). Together, these preliminary data suggest that Notch-1 is necessary for inhibition of PTEN expression in trastuzumab resistant cells. However, the exact mechanism by which Notch-1 represses PTEN in HER2+ breast cancer is unknown. The goal of aim 1A is to determine if Notch-1 is necessary to repress PTEN expression at the mRNA level.

Notch-1 inhibition increases PTEN RNA transcript and protein expression. Initial investigation into the mechanism of trastuzumab-mediated inhibition of HER2+ breast cancer demonstrated an upregulation of PTEN protein expression upon trastuzumab treatment. Trastuzumab-mediated upregulation of PTEN is hypothesized to decrease downstream PI3K/Akt pathway activation resulting in reduced HER2+ tumor growth. Our model of acquired resistance to trastuzumab include cell lines (BT474TR) that display both a decrease in PTEN protein and an increase in downstream Akt activity as measured by increased levels of P-Thr308 and P-Ser473 Akt by Western blotting compared to the parental,

trastuzumab sensitive cell line (BT474TS). These results are consistent with findings from other laboratories demonstrating similar characteristics in resistant cells as those previously described (Figure 11A) (Chan et al., 2005). Moreover, we discovered that our BT474TR cells also have an increase in Notch-1 protein expression and activity compared to trastuzumab sensitive cells (Figure 11A) (Osipo et al., 2008).

Our preliminary data demonstrated that inhibition of Notch-1 promoted PTEN protein expression (Figure 11B). In order to determine if this effect occurs at the transcript level, we asked the question: does Notch-1 inhibit PTEN RNA expression? To answer this question, we measured Notch-1 and PTEN transcript levels using real-time PCR in acquired resistant cells (BT474TR) upon Notch-1 knockdown compared to control. The results showed that inhibition of Notch-1 resulted in an increase in PTEN RNA transcripts (Figure 13A). This result suggests that Notch-1 is required for PTEN expression at the RNA transcript level. To determine if Notch-1 repression of PTEN was reproducible in other cell lines, protein and transcript levels of PTEN were measured post Notch-1 knockdown in inherently resistant HCC1954 cells by Western blot and real time PCR analysis, respectively. In agreement with results from acquired resistant BT474 cells, Notch-1 knockdown also resulted in an increase in PTEN RNA transcript (Figure 13B) and protein (Figure 13C) levels.

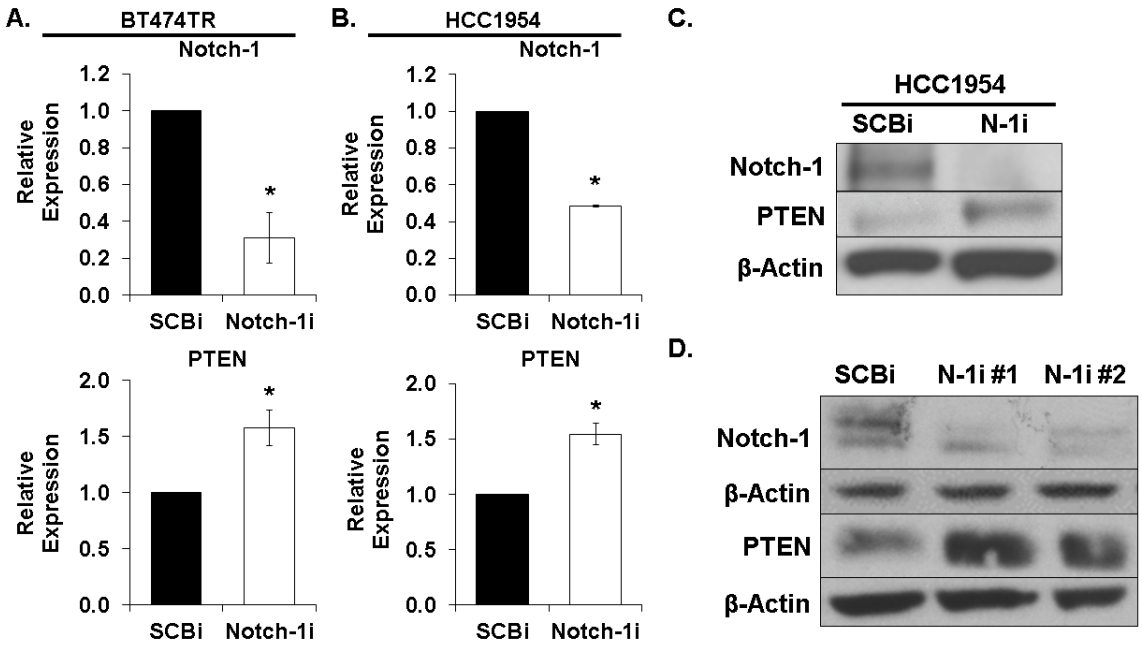

Figure 13: Notch-1 represses PTEN expression in trastuzumab resistant cells.

A. BT474TR cells were transfected with scrambled control (SCBi) or Notch-1 siRNA (N-1i) for 48 hours. Post transfection, relative expression of Notch-1 (top) or PTEN (bottom) was measured by RT-PCR. **B.** Similarly, intrinsically resistant HCC1954 were transfected with SCBi or Notch-1i and relative expression of Notch-1 (top) or PTEN (bottom) was measured by RT-PCR. **C.** Post transfection of HCC1954 cells, total cellular Notch-1, PTEN, and β-Actin protein expression was analyzed by Western blot. **D.** BT474TR cells were transfected with SCBi, N-1i, and a second Notch-1 siRNA (N-1i#2) for 48 hours. Post transfection, total cellular Notch-1, PTEN, and β-Actin protein expression was analyzed by Western blot. Asterisk (*) represents statistical significance compared to SCBi from three independent experiments using a Student's t-test.

Taken together, these results indicate that Notch-1 is necessary for PTEN inhibition at the transcript level in HER2+ trastuzumab resistant cells.

Notch-1-mediated inhibition of PTEN is not due to an off target effect by Notch-1 siRNA. It has become a common practice to validate effects of siRNAs using a second, distinct siRNA targeting the same transcript. This is now standard due to siRNAs having off target effects (Jackson et al., 2010). To ensure that knockdown of Notch-1 by the first siRNA resulting in an increase in PTEN expression was not due to an off target effect, we repeated the previous studies using a distinct Notch-1 siRNA (#2). The results using the second Notch-1 siRNA (#2) were similar to findings using the first Notch-1 siRNA (#1). Knockdown of Notch-1 using either siRNA (#1 or #2) resulted in upregulation of PTEN protein expression (Figure 13D). Notch-1 siRNA #1 is used throughout this manuscript unless otherwise noted. This result confirms that Notch-1 is necessary for inhibition of PTEN in our trastuzumab resistant cell models.

Aim 1B: Determine if Notch-1 is Sufficient to Repress PTEN Expression in Trastuzumab Sensitive Cells.

Aberrant expression of Notch-1 has been shown to regulate PTEN in T-ALL (Palomero et al., 2007) and gastric cancers (Kim et al., 2015). Similarly, trastuzumab resistant cells display an increase in Notch-1 and a decrease in PTEN expression compared to trastuzumab sensitive cells. Thus far, our results demonstrate that Notch-1 is required for PTEN inhibition in both acquired and inherently resistant HER2+ breast cancer cells. However, Notch-1 is elevated in trastuzumab resistant cells compared to trastuzumab sensitive cells (Figure 11A) suggesting that upregulation of Notch-1 may be necessary for attenuating PTEN

transcription. Under this assumption, an inverse relationship exists between Notch-1 and PTEN in which high Notch-1 expression results in PTEN inhibition and low Notch-1 expression allows increased PTEN expression. Thus, the goal for aim 1B is to determine if Notch-1 is sufficient to inhibit PTEN expression in trastuzumab sensitive cells.

Overexpression of Notch-1 results in decreased PTEN expression. Given our initial data demonstrating that Notch-1 is required to attenuate PTEN transcript and protein expression in resistant cells, we asked if Notch-1 overexpression downregulates PTEN in trastuzumab sensitive cells. To address this question, we measured PTEN protein and transcript levels expression in BT474 sensitive cells transfected with a control plasmid (pcDNA3) or a plasmid harboring cDNA for the Notch-1 Intracellular Domain 1 (pcDNA3-NICD1) by Western blot and real time PCR analysis. The results showed that NICD1 overexpression in trastuzumab sensitive, BT474 cells resulted in a significant decrease in PTEN transcript (Figure 14A) and protein (Figure 14B) expression compared to the vector control.

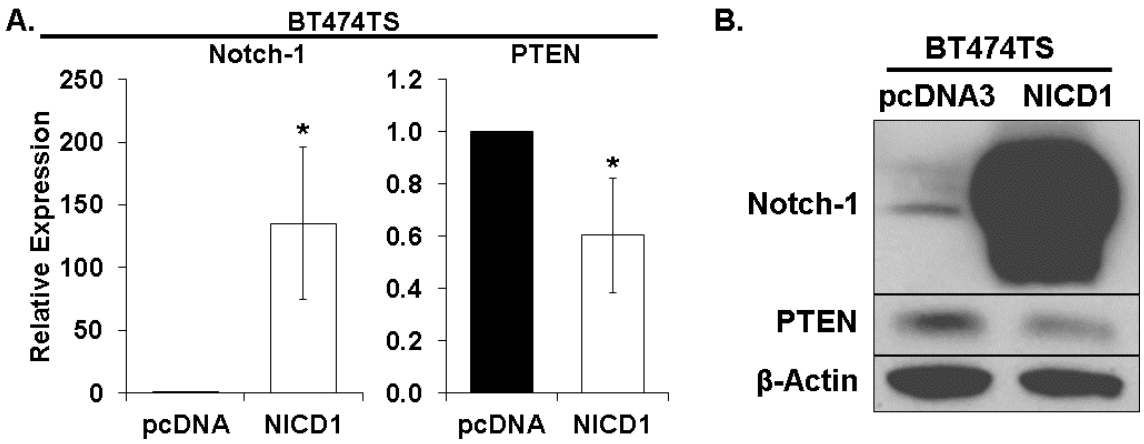

Figure 14: Notch-1 overexpression represses PTEN in trastuzumab sensitive cells.
A. BT474TS cells were transfected with a vector control (pcDNA3) or NICD1 expression vector (NICD1) for 48 hours. Post transfection, Notch-1 (left) or PTEN (right) mRNA expression was measure by RT-PCR. **B.** Total cellular Notch-1, PTEN, and β-Actin protein expression was analyzed by Western blot post transfection. Asterisk (*) represents statistical significance compared to pcDNA from three independent experiments using Student's t-test.

Taken together, these data suggest that Notch-1 is sufficient to regulate PTEN expression at the transcript and protein levels in trastuzumab sensitive, HER2+ breast cancer cells.

Aim 1C: Ascertain if Notch-1 is Directly Recruited to the PTEN Promoter.

Recruitment of Notch-1 to a CBF-1 element on a gene promoter canonically activates transcription of the target gene. Previous work reported that Notch-1 activated PTEN transcription in an embryonic kidney cell model (293T) (Whelan et al., 2007). In contrast, Notch-1 was shown to repress PTEN expression in T-ALL (Palomero et al., 2007). Canonically, Notch-1 is a transcriptional activator of CBF-1-driven gene targets. In T-ALL, Palomero et al. demonstrated that Notch-1 indirectly represses PTEN through upregulation of the

transcriptional repressor HES-1, which is recruited to the PTEN promoter to repress transcription of PTEN. Thus far, our results show that Notch-1 is both necessary and sufficient to downregulate PTEN at the transcript level. To determine if Notch-1 enrichment at the PTEN promoter correlates with reduced PTEN transcript RNA expression, we performed a series of chromatin immunoprecipitation (ChIP) assays to measure direct recruitment of NICD1 to a CBF-1 site on the PTEN promoter. The goal of aim 1C is to determine if aberrant Notch-1 expression results in an increase in NICD1 recruitment to the PTEN promoter in HER2+ breast cancer cells.

Trastuzumab resistant cells have increased recruitment of Notch-1 at the PTEN promoter. The canonical role of Notch-1 is to activate transcription, leading us to ask the question: Is there an increase in NICD1 recruitment at the PTEN promoter in resistant cells compared to sensitive cells? We measured endogenous NICD1 enrichment at CBF-1 sites on HEY-1 and PTEN promoters in both trastuzumab sensitive and resistant cells through a series of ChIP assays. Enrichment of NICD1 on the HEY-1 promoter was performed as a positive control as HEY-1 is a canonical Notch-1 target gene (Katoh and Katoh, 2007). NICD1 enrichment at the HEY-1 promoter was measured by ChIP followed by quantitative PCR (qPCR). The DNA fragment from the HEY-1 promoter region -243 bps upstream of the transcriptional start site (maps above bar graphs, Figure 16A) was amplified using a pair of primers flanking this region. Similarly, primers flanking a region -432 bps upstream of the transcriptional start site of the PTEN

gene amplified a DNA fragment enriched by NICD1. The primer pairs used in this study were shown to flank a canonical CBF-1 binding sequence (CTGGGAA) (Persson and Wilson, 2010), in the PTEN promoter as previously described by Graziani et al. (Graziani et al., 2008). Analysis of the PTEN and HEY-1 promoters revealed 5 to 6 putative CBF-1 sites and we found that putative site #4 (CTGGGAA) was the most highly enriched for NICD1 in our HER2+ breast cancer cell model (Figure 15). We detected more than a 3-fold increase in endogenous NICD1 enrichment at both the HEY-1 and PTEN promoters in resistant cells compared to sensitive cells (Figure 16B).

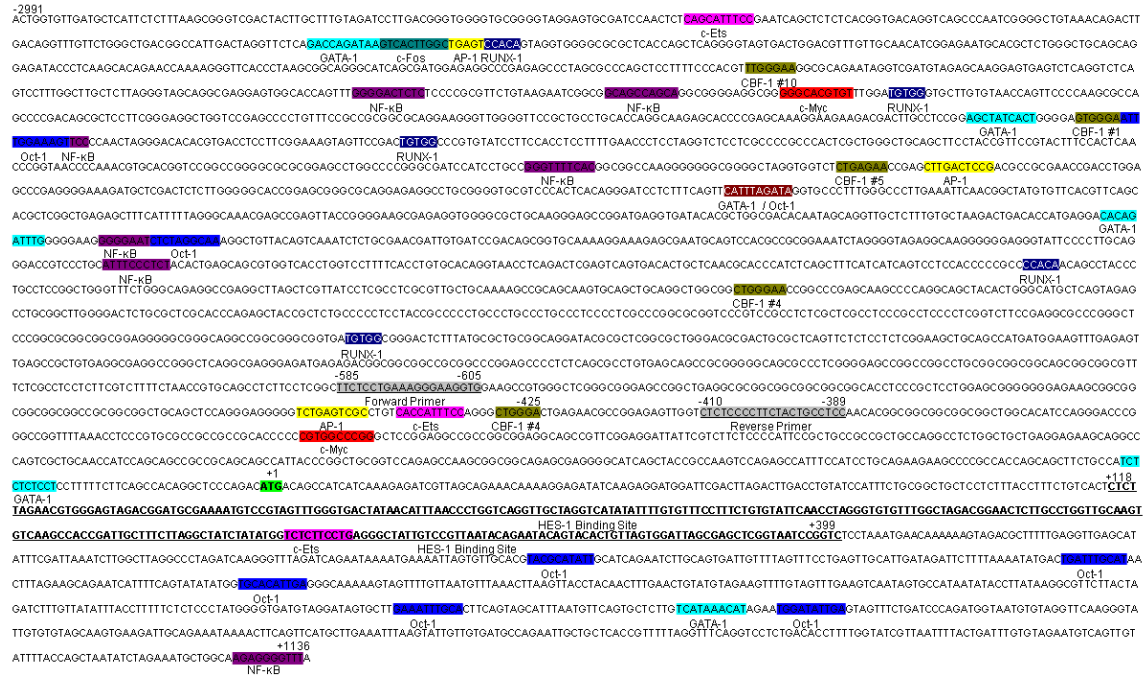

Figure 15: PTEN promoter region.
The PTEN promoter region includes the transcription initiation site [ATG(+1)], forward (-585 to -610) and reverse (-389 to -410) ChIP pimers, CBF-1 (olive green) #4 (-425 and -1160), #5 (-1930), #1 (-2210), #10 (-2566), c-Ets (pink), GATA-1 (agua blue), c-Fos (blue-green), AP-1 (Yellow), RUNX-1 (blue with white letters), NF-κB (purple), c-Myc (red), Oct-1 (blue), GATA-1/Oct-1 (Maroon with white letters), and the HES-1 binding site (+118 to +319) (Palomero et al., 2007).

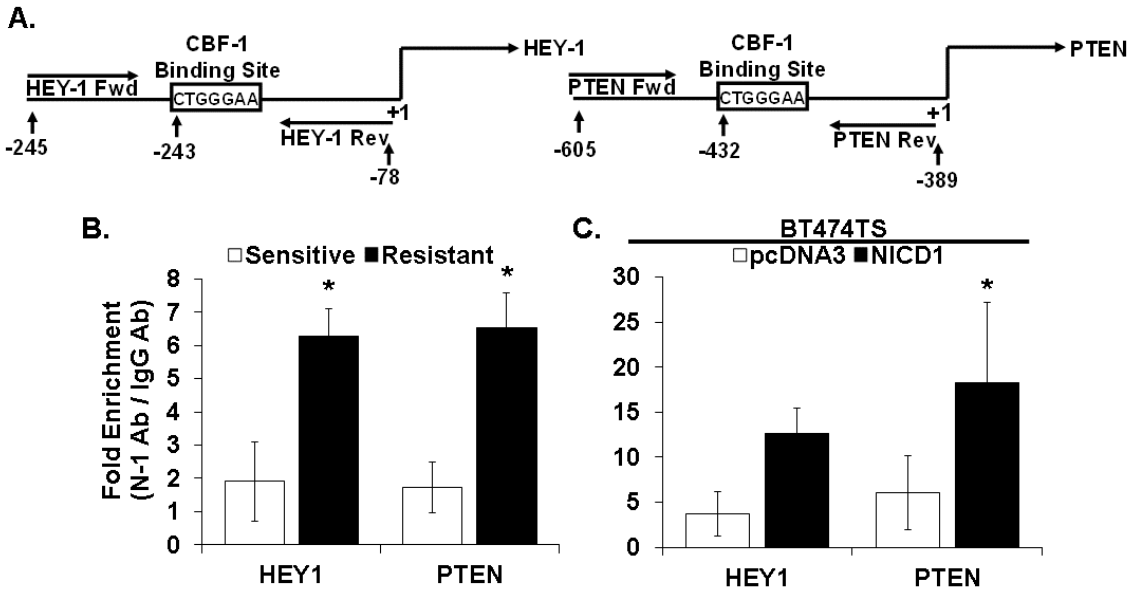

Figure 16: Notch-1 is directly recruited to the PTEN promoter.
A. HEY-1 (left) and PTEN (right) promoter maps showing CBF-1 binding sites flanked by specific primer amplification sites. These primers were used to quantify NICD1 enrichment of the HEY-1 or PTEN promoter by qPCR. **B.** BT474TR and BT474TS cells were fixed, fragmented, and chromatin was immunoprecipitated by a C-terminal Notch-1 (Santa Cruz Notch-1 Ab) or control IgG antibody. Purified DNA from Notch-1 or IgG immunoprecipitated chromatin was used to quantify NICD1 enrichment of HEY-1 or PTEN promoters by qPCR using the primers shown in A. **C.** BT474TS cells were transfected with NICD1 or pcDNA3 expression vectors for 48 hours. Post transfection, cells were harvested, fixed, fragmented, immunoprecipitated by Notch-1 or IgG antibodies, and NICD1 enrichment of HEY-1 or PTEN promoters was quantified by qPCR using the same HEY-1 or PTEN primers as in B. Asterisk (*) represents statistical significance compared to sensitive (B) or pcDNA3 (C) mean NICD1 enrichment from three independent experiments using Student's t-test.

These results are contrary to the canonical role of Notch-1 as a transcriptional activator as we observe a decrease in PTEN transcripts in resistant compared to sensitive cells. Yet, there is an increase in NICD1 enrichment at the PTEN promoter in resistant compared to sensitive cells. This result suggests that

Notch-1 is potentially acting as a direct inhibitor of PTEN transcript RNA expression in trastuzumab resistant, HER2+ breast cancer cells.

Overexpression of Notch-1 increases Notch-1 enrichment at the PTEN promoter in trastuzumab sensitive cells. Previous results indicated that Notch-1 is sufficient to repress PTEN transcripts in trastuzumab sensitive cells (Figure 14). Given this result, and the enrichment of NICD1 at the PTEN promoter in resistant cells, lead us to ask the question: is NICD1 directly recruited to the PTEN promoter in trastuzumab sensitive cells? We addressed this question by performing the same ChIP assays used to determine endogenous enrichment of NICD1 at the PTEN promoter in resistant and sensitive cells. Similar to the previous results, we observed a 3-fold increase in NICD1 enrichment at CBF-1 sites on the HEY-1 and PTEN promoters in sensitive cells-overexpressing NICD1 as compared to the vector control (pcDNA3)-transfected cells (Figure 16C).

Taken together, these data presented in aim 1C suggests that Notch-1 is directly involved in repression of PTEN expression in HER2+ breast cancer cells.

Specific aim 2. Determine the Biological Significance of Notch-1-Mediated Downregulation of PTEN in Trastuzumab Resistant Cells.

The goal of specific aim 2 is to test the following hypotheses:

Aim 2A: Determine if Notch-1-mediated repression of PTEN promotes cell proliferation of trastuzumab resistant cells.

Aim 2B: Determine the role of the MAPK pathway in Notch-1-mediated repression of PTEN in trastuzumab resistant cells.

Aim 2C: Deduce if Notch-1-mediated repression of PTEN promotes trastuzumab resistant breast cancer stem cell survival and self-renewal.

Aim 2A: Determine if Notch-1-Mediated Repression of PTEN Promotes Proliferation of Trastuzumab Resistant Cells.

Rationale. Notch-1 has been shown to promote tumorigenesis in a number of different cancers. Additionally, PTEN is a well-known inhibitor of the PI3K/Akt pathway which has presented itself as a central node in drug resistant, HER2+ breast cancer (Berns et al., 2007). Thus far, we have shown that Notch-1 is required for the inhibition of PTEN expression potentially through direct recruitment of NICD1 to the PTEN promoter. Moreover, we have shown that there is an increase in Akt activation in resistant compared to sensitive cells. Thus, we wanted to determine the biological significance of Notch-1-mediated repression of PTEN on cell growth and the PI3K/Akt pathway in trastuzumab resistant cells. The goal of aim 2A is to determine if Notch-1-mediated repression of PTEN is responsible for proliferation of trastuzumab resistant cells *in vitro*.

Trastuzumab reduces trastuzumab sensitive cell proliferation yet has no effect on resistant cell proliferation. First, we wanted to ensure that our trastuzumab resistant cells were resistant to trastuzumab treatment and that our trastuzumab sensitive cells remained sensitive to trastuzumab treatment. To assess trastuzumab sensitivity, we measure cell proliferation of resistant and sensitive cells treated with 0, 2.5, 5, 10, 20, or 40 µg/ml of trastuzumab for 10 days. The results demonstrate that our resistant cells are able to proliferate under high doses of trastuzumab treatment while our sensitive cells are unable proliferate when exposed to low doses of trastuzumab (Figure 17). These data show that our sensitive cells are indeed sensitive to trastuzumab treatment while our resistant cells are able to proliferate in response to high doses of trastuzumab treatment.

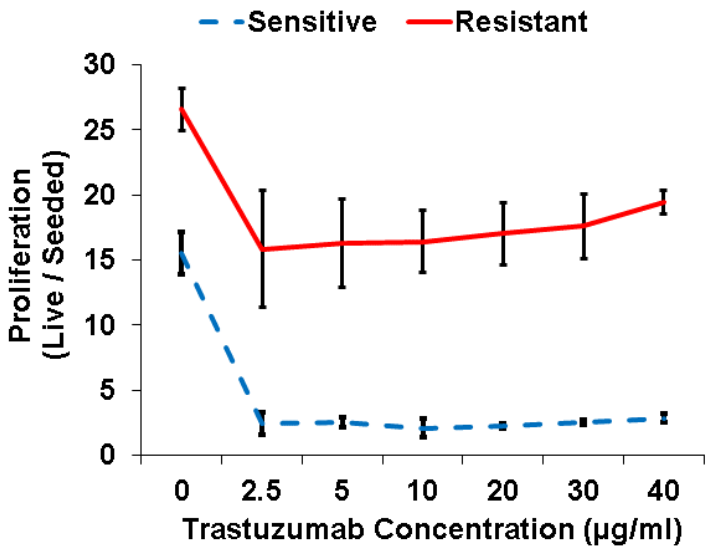

Figure 17: Trastuzumab reduces trastuzumab sensitive cell proliferation but has no effect on resistant cell proliferation.
100,000 BT474S and BT474TR cells / well were seeded in 6 well plates and treated daily with 0, 2.5, 5, 10, 20, 30, or 40 µg/mL of trastuzumab for 10 days. Mean cell proliferation was calculated as the number of live cells / number of cells seeded from triplicate wells.

Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant cell proliferation regardless of trastuzumab treatment. Previous work showed that Notch-1 was required for cell proliferation of trastuzumab resistant breast cancer cells (Osipo et al., 2008). Thus far, we have shown that Notch-1 is directly recruited to the PTEN promoter which associates with reduced PTEN transcript RNA expression in the same trastuzumab resistance cells. Additionally, we have shown that low PTEN expression in trastuzumab resistance cells correlated with hyperactivation of Akt. What is not known is if Notch-1-mediated repression of PTEN is responsible for cell proliferation and the increase in hyper-phosphorylation of Akt. To assess the role of Notch-1-mediated inhibition of PTEN in resistant cells,

we measured cell proliferation in resistant cells transfected with a scrambled control, Notch-1, and/or PTEN siRNA treated with PBS or trastuzumab (20 μg/ml) for 10 days. In the acquired resistant model (BT474TR), knockdown of Notch-1 reduced cell proliferation by more than 4 fold compared to the scrambled control (Figure 18A and B). Additionally, simultaneous knockdown of Notch-1 and PTEN rescued cell proliferation by 2-fold compared to Notch-1 knockdown alone. Furthermore, these proliferations results were similar either in the absence or presence of trastuzumab treatment. We repeated this assay using another trastuzumab resistant, HER2+ breast cancer line, HCC1954, and observed similar results (Fig 18C and D). It is important to note that combined knockdown of Notch-1 and PTEN in HCC1954 cells completely rescued cell proliferation compared to knockdown of Notch-1 alone.

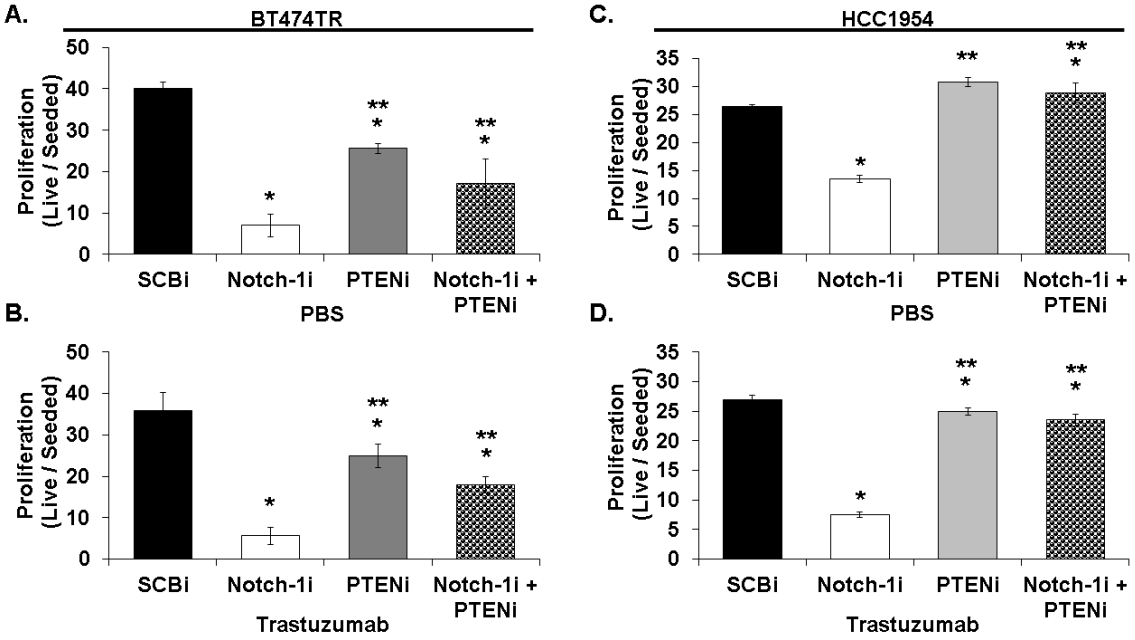

Figure 18: Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant cell proliferation.

A-B. BT474TR cells were transfected with SCBi, N-1i, and/or PTENi for 48 hours. Post transfection, the cells were harvested and seeded at 100,000 cells/ well in a 6 well plate and treated daily with 20 µg/mL of PBS (A) or trastuzumab (B) for 10 days. After treatment, the cells were harvested and counted. Mean proliferation was calculated as the number of live cells harvested / number of live cells seeded. **C-D.** HCC1954 cells were transfected with SCBi, N-1i, and/or PTENi and treated with PBS (C) or trastuzumab (D) in the same manner as A-B. Asterisk (*) denotes statistical significance compared to SCBi or (**) Notch-1i mean proliferation as calculated using a one-way ANOVA with an overall statistical significance of $p < 0.0001$ from three independent experiments.

Taken together, these results demonstrate that Notch-1-mediated repression of PTEN promotes proliferation of trastuzumab resistant cells.

Notch-1-mediated repression of PTEN is not responsible for hyper-activation of Akt. The results from the cell proliferation assays encouraged us to assess downstream PI3K/Akt pathway activity to determine if Notch-1-mediated inhibition of PTEN resulted in increased Akt activation. Western blotting was performed to assess the efficiency of Notch-1 and PTEN protein knockdown as well as to detect the activation of Akt as measured by phosphorylation of Akt at serine 473 and threonine 308. Additionally, densitometric analysis using ImageJ was used to quantitate Notch-1 and PTEN protein expression normalized to the loading control, β-actin (Rasband, 1997). This was repeated for protein levels of phosphorylated serine473 on Akt normalized to overall Akt expression (Figure 19A below blot). The results show that we were consistently able to knockdown both Notch-1 and PTEN in both the acquired [(BT474TR) left] and inherently [(HCC1954) right] resistant breast cancer cell models. Additionally, Notch-1 knockdown consistently resulted in an increase in PTEN protein expression. Interestingly, the increase in PTEN expression in response to Notch-1 knockdown either slightly increased or had little effect on the phosphorylation status of Akt. This result is contrary to our initial hypothesis as we anticipated that inhibition of Notch-1 would increase PTEN which in turn would inhibit PI3K kinase activity resulting in decreased phosphorylation of Akt. Moreover, trastuzumab treatment of acquired resistant cells (BT474TR) reduced phosphorylation of Akt yet trastuzumab had little to no effect on proliferation (Figure 19A). Similar results were seen in inherently resistant cells (HCC1954) in which Notch-1 knockdown

increased PTEN expression, yet slightly increased phosphorylation of Akt. This result was again independent of trastuzumab treatment (Figure 19B).

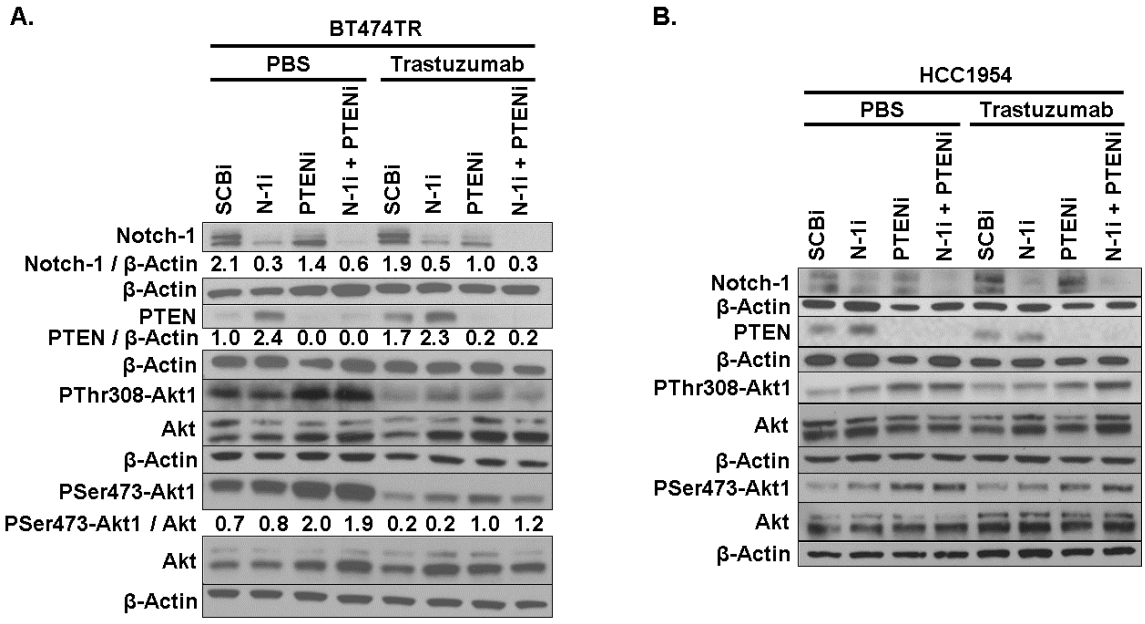

Figure 19: Notch-1 mediated inhibition of PTEN has little to no effect on phosphorylation of Akt.
A. BT474TR cells were transfected with SCBi, N-1i, and/or PTENi for 48 hours. Post transfection, total cellular Notch-1, PTEN, Akt, β-Actin, phosphorylation of Akt-1 at threonine308 (PThr308-Akt1) and serine473 (PSer473-Akt1) protein expression from the transfected cells post 48 hour PBS (left) or trastuzumab (right) treatment was analyzed by Western blot. Mean values from densitometric analysis of Notch-1, PTEN, and PSer473-Akt1 normalized to β-Actin or Akt, respectively, are displayed below their respective blots, and quantitated from three independent experiments. **B.** HCC1954 cells were transfected, treated, and analyzed by Western blot in a similar manner as in A.

Taken together, these data indicate that Notch-1-mediated repression of PTEN is not promoting trastuzumab resistance due to activation of Akt. Contrary, Notch-1-mediated repression of PTEN could be promoting cell proliferation by activating another pathway.

PTEN siRNA B inhibits trastuzumab resistant cell proliferation. We wanted to validate the results of our cell proliferation assays by using the three individual siRNAs that comprise the PTEN SmartPool siRNA. The results of our cell proliferation assays displayed a decrease in resistant cell proliferation when transfected with the PTEN SmartPool siRNA (Figure 18A and B). Therefore, we asked the question: Is one of the individual siRNA oligonucleotides in the PTEN SmartPool having an anti-proliferative effect on trastuzumab resistant cells? To address this question, we measured cell proliferation of acquired resistant cells (BT474TR) transfected with the three separate oligonucleotides (A, B, C) compared to a control siRNA and the PTEN SmartPool siRNA. The cell proliferation studies revealed that PTEN siRNA B reduced resistant cell proliferation (Figure 20A). Additionally, whole cell lysates from the cell proliferation study were used to measure PTEN protein expression by Western blotting. The three PTEN siRNAs were able to downregulate PTEN protein expression (Figure 20B). These results explain the decrease in resistant cell proliferation when transfected with the PTEN siRNA SmartPool.

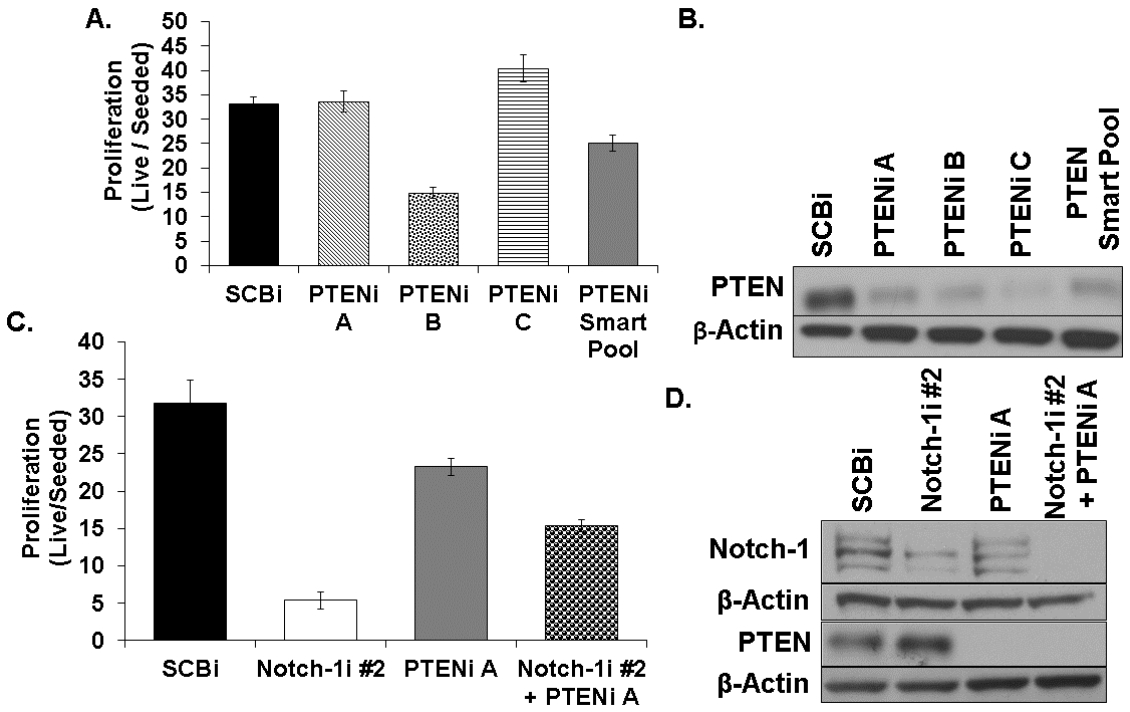

Figure 20: Distinct Notch1 and PTEN siRNAs Produce Similar Effects.
A. BT474TR cells were transfected with SCBi, PTENi-A, PTENi-B, PTENi-C, or the PTENi SmartPool for 48 hours. Post transfection, the cells were harvested and 100,000 live cells / well were plated into a 6 well plate and grown for 10 days. On day 10, the cells were harvested and counted. Mean cell proliferation was calculated as the number of live cells / number of cells seeded from triplicate wells. **B.** Total cellular PTEN and β-Actin protein expression from samples in A was analyzed by Western blot. **C.** BT474TR cells transfected with SCBi, Notch-1i #2 and/or PTENi-A for 48 hours. Post transfection, the cells were harvested and 100,000 live cells / well were seeded into 6 well plates and grown for 10 days. Mean cell proliferation was calculated from triplicate wells in the same manner as A. **D.** Total cellular Notch-1, PTEN, and β-Actin protein expression from samples in C was analyzed by Western blot.

Distinct Notch-1 and PTEN siRNAs have similar effects on the proliferation of trastuzumab resistant cells. Given the off-target effects of one of the PTEN SmartPool siRNAs, we wanted to validate the results of the Notch-1 siRNA in our cell proliferation studies. Thus, we asked the question: does a second Notch-1 siRNA yield similar results when combined with an individual PTEN siRNA? We addressed this question by performing the same cell proliferation studies in which we transfected acquired resistant cells (BT474TR) with a scrambled control, a second Notch-1 siRNA (#2), and/or a single PTEN siRNA A. The cell proliferation study yielded similar results to the initial studies in which combined knockdown of Notch-1 and PTEN partially rescued cell proliferation compared to Notch-1 knockdown alone (Figure 20C). Western blotting confirmed that the second, distinct siRNAs against Notch-1 and PTEN were efficient in decreasing protein levels (Figure 20D). These results confirm that Notch-1 is required to repress PTEN expression and this mechanism is responsible for proliferation of trastuzumab resistant, HER2+ breast cancer cells.

Notch-1 is not sufficient to induce trastuzumab resistance. Thus far, we have demonstrated that proliferation of trastuzumab resistant cells is dependent on Notch-1-mediated repression of PTEN. Previous results have shown that Notch-1 is sufficient to repress PTEN in sensitive cells. Thus, the critical aim now is to determine if Notch-1 is sufficient to induce resistance in cells that are initially sensitive to trastuzumab. To address this aim, we performed a series of cell proliferation assays in which we overexpressed NICD1 or a vector control

(pcDNA3) in trastuzumab sensitive cells and treated them with PBS or trastuzumab (20 µg/ml) for 10 days. NICD1 overexpression modestly increased cell proliferation of sensitive cells in response to trastuzumab treatment. Taken together, these results suggest that Notch-1 overexpression alone is not sufficient to promote trastuzumab resistance (Figure 21A and B). These results indicate that prolonged exposure to trastuzumab treatment is necessary for upregulation of Notch-1 and the development of resistance.

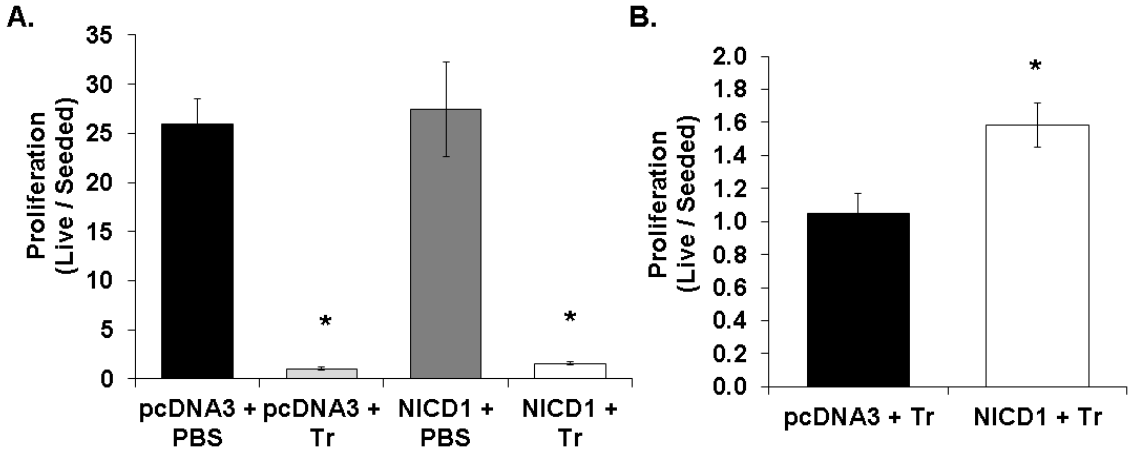

Figure 21: Notch-1 overexpression in trastuzumab sensitive cells modestly increases proliferation in response to trastuzumab treatment.
A. BT474TS cells transfected with NICD1 or pcDNA3 expression vectors for 48 hours. Post transfection, the cells were harvested and 100,000 cells were seeded / well in a 6 well plate. Cells were treated daily with 20 µg/mL trastuzumab or PBS for 10 days. On day 10, the cells were harvested and live cells were counted. **B.** Cell proliferation of BT474TS cells transfected with pcDNA or NICD1 and treated with trastuzumab from A. Mean cell proliferation rate was calculated by the number of live cells / number of cells seeded. Asterisk (*) denotes statistical significance between mean cell proliferation of PBS and trastuzumab treated cells (A) or pcDNA3 and NICD1 transfected cells under trastuzumab treatment (B) from three independent experiments using a Student's t-test.

Aim 2B: Determine the Role of the MAPK Pathway in Notch-1-Mediated Repression of PTEN in Trastuzumab Resistant Cells.

Our results demonstrate that Notch-1 inhibition resulted in little to no effect on the activation status of Akt in the resistant cell models. Previous work has demonstrated that PTEN is able to attenuate phosphorylation of ERK1/2 in a HER2-negative breast cancer cell model, MCF-7 (Chung et al., 2005b). Additionally, MAPK pathway activity is inhibited by trastuzumab and has been found to be upregulated in models of acquired resistance to trastuzumab (Chan et al., 2005). The MAPK pathway is known for controlling cell death and proliferation which leads us to hypothesize that Notch-1-mediated repression of PTEN is promoting the activation of ERK1/2 to increase cell proliferation.

Trastuzumab resistant cells have increased ERK1/2 activity compared to sensitive cells. If the ERK1/2 pathway is critical to promoting proliferation of resistant cells, then these cells should have higher expression of phosphorylated ERK1/2 than sensitive cells. To address this aim, we measured the phosphorylation status of ERK1/2 in both sensitive and resistant whole cell lysates. The results indicate that resistant cells have increased ERK1/2 phosphorylation compared to sensitive cells (Figure 22A) (Rogowski, 2011). This result suggests that proliferation of resistant cells could be dependent on increased ERK1/2 activity.

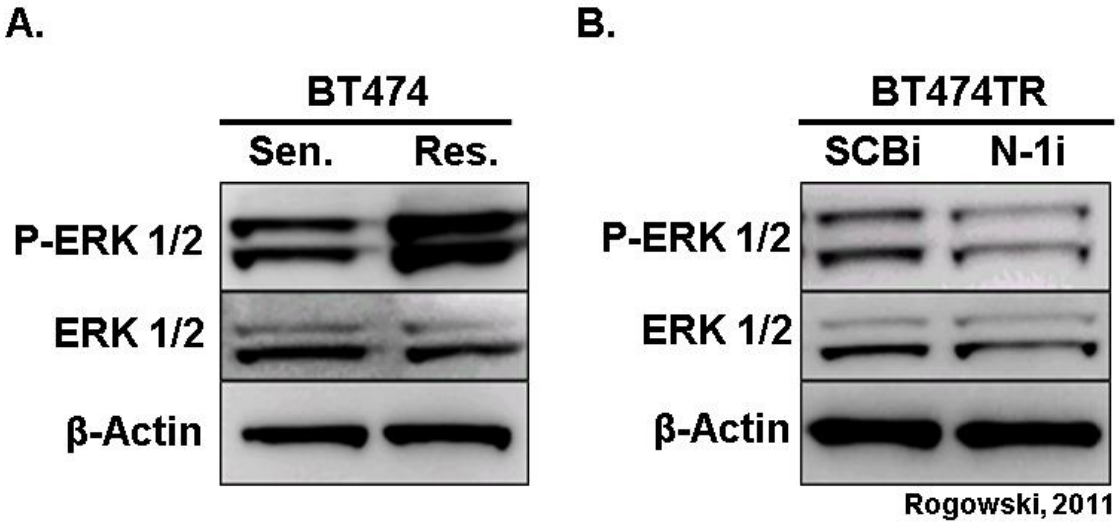

Figure 22: Trastuzumab resistant cells have increased phosphorylation of ERK1/2 mediated by Notch-1.
A. Total protein lysates from BT474TS (Sen.) and BT474TR (Res.) underwent Western blot analysis to detect P-ERK1/2, ERK1/2, and β-Actin protein expression. **B.** BT474TR cells were transfected with SCBi or N-1i for 48 hours. Post transfection, total cellular P-ERK1/2, ERK1/2, and β-Actin protein expression was analyzed by Western blot.

Notch-1 is required for ERK1/2 phosphorylation in trastuzumab resistant cells. Our data demonstrate that the ERK1/2 pathway is upregulated in trastuzumab resistant cells compared to sensitive cells, yet the role of Notch-1 in regulating the ERK1/2 pathway has yet to be assessed. Therefore, we asked the question: is expression of Notch-1 required for the increased phosphorylation of ERK1/2 in trastuzumab resistant cells? To address this question, we measured ERK1/2 phosphorylation in lysates from scrambled control or Notch-1 siRNA treated cells by Western blot analysis. The results show that ERK1/2

phosphorylation decreases upon Notch-1 knockdown compared to the scrambled control (Figure 22B).

These data suggest that Notch-1 is necessary to activate or at least maintain ERK1/2 activity in trastuzumab resistant cells.

Inhibition of the MAPK pathway reduces proliferation of trastuzumab resistant cells. Proliferation of trastuzumab resistant cells depends on Notch-1. This dependence may be due, in part, to the ability of Notch-1 to activate or maintain the ERK1/2 pathway. To determine if proliferation of resistant cells is dependent on the MEK1/2-ERK1/2 pathway, we used a MEK1/2 inhibitor, U0126, in a series of cell proliferation assays. We transfected resistant cells with a scrambled control, Notch-1, and/or PTEN siRNA and treated them with the vehicle, dimethylsulfoxide (DMSO) or U0126 for 10 days. We found that treatment of resistant cells with U0126 dramatically reduced proliferation of resistant cells (Figure 23A). Importantly, Western blot analysis of cell lysates showed that the decrease of ERK1/2 phosphorylation in response to Notch-1 knockdown was rescued by knockdown of PTEN. The U0126 treatment almost completely abrogated ERK1/2 phosphorylation under all conditions (Figure 23B).

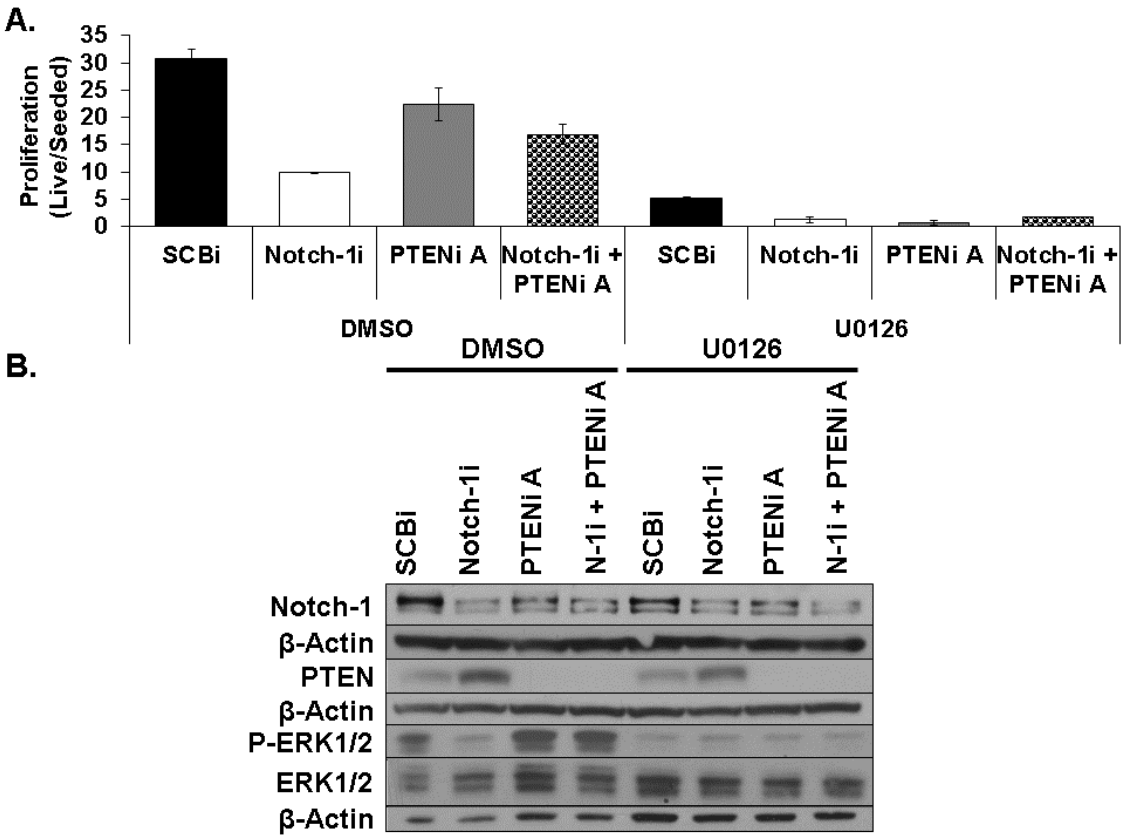

Figure 23: Notch-1 mediated repression of PTEN activates ERK1/2 which is necessary for resistant cell proliferation.
A. BT474TR cells were transfected with SCBi, N-1i, and/or PTENi-A for 48 hours. Post transfection, 100,000 cells/well were plated in a 6 well plate and treated daily with 10 µg/mL U0126 (left) or DMSO (right) for 10 days. Post treatment, the cells were harvested and counted. Mean cell proliferation was calculated as the number of live cells harvested / number of cells seeded from triplicate wells. **C.** Total cellular Notch-1, PTEN, P-ERK1/2, ERK1/2 and β-Actin protein expression from the cells in A post 48 hours of DMSO (left) or U0126 (right) was analyzed by Western blot.

Taken together, these results suggest that Notch-1-mediated repression of PTEN maintains or activates ERK1/2 phosphorylation via MEK1/2 activity to drive resistant cell proliferation.

Constitutive MEK1/2 activation rescues trastuzumab resistant cell proliferation from Notch-1 inhibition. Thus far, we have shown that MEK1/2 activity is required for proliferation of resistant cells. What is not known is whether constitutive activation of MEK1/2-ERK1/2 activity can rescue the decrease in resistant cell proliferation upon Notch-1 knockdown. To test this, we measured proliferation of resistant cells transfected with a scrambled control or a Notch-1 siRNA in combination with a vector control (pEXP) or a constitutively active mutant of MEK1/2 (pEXP-MEK1DD) (www.addgene.org/31202/). MEK1DD has been reported to maintain phosphorylation of ERK1/2 (Yang et al., 2011). Protein expression from cell lysates were measured by Western blotting. The results show that constitutive ERK1/2 phosphorylation by MEK1DD partially rescued the growth inhibitory effects mediated by Notch-1 knockdown by nearly 4-fold compared to Notch-1 knockdown alone (Figure 24A). Importantly, the decrease in ERK1/2 phosphorylation by Notch-1 knockdown was prevented when MEK1DD was expressed (Figure 24B).

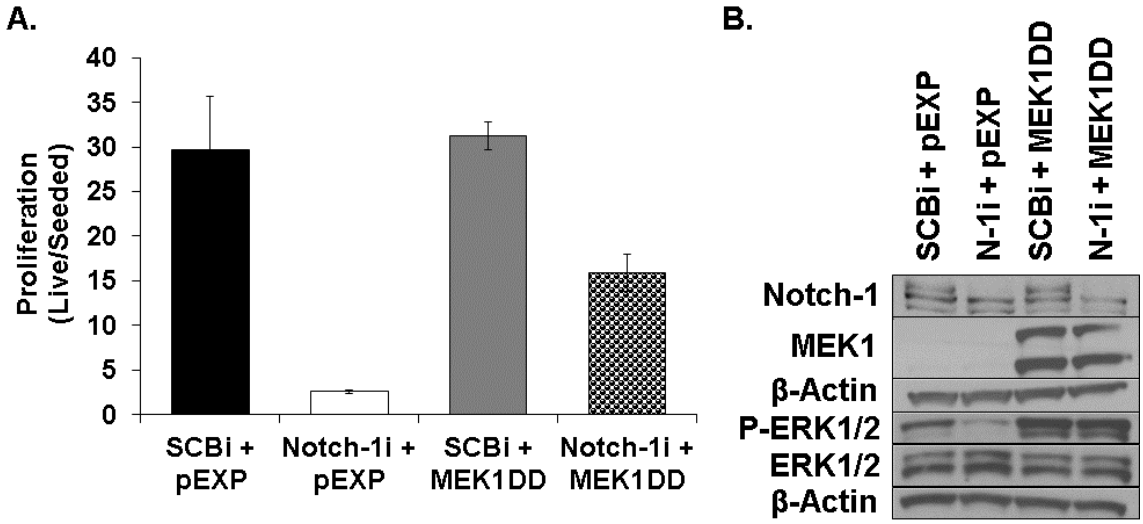

Figure 24: Notch-1 promotes ERK1/2 phosphorylation which is sufficient for resistant cell proliferation.
A. BT474TR cells were transfected with SCBi or Notch-1i and MEK1DD or pEXP expression vectors for 48 hours. Post transfection, cells were harvested and seeded at 100,000 cells / well of a six well plate. After 10 days, the cells were harvested and counted. Mean cell proliferation was calculated as number of live cells harvested / number of cells seeded from triplicate wells. **B.** Total cellular Notch-1, MEK1, P-ERK1/2, ERK1/2 and β-Actin protein expression from the cells in A post transfection was analyzed by Western blot.

Together, these results indicate a novel mechanism in which Notch-1 maintains proliferation of trastuzumab resistant, HER2+ breast cancer cells by repressing PTEN expression to increase the activity of the MEK1/2-ERK1/2 pathway.

Aim 2C: Deduce if Notch-1-Mediated Repression of PTEN Promotes Trastuzumab Resistant, Breast Cancer Stem Cell Survival and Self-Renewal.

The discovery of the cancer stem cell population has garnered much attention as it is hypothesized to promote cancer metastasis, recurrence, and drug resistance (Liu et al., 2014). Previous work has shown that HER2 (Korkaya et al.,

2014) and Notch-1 (Harrison et al., 2010) promote expansion and survival of the BCSC population. Additionally, PTEN has been shown to increase BCSC differentiation through inhibition of both PI3K/Akt and MAPK pathways (Jordan et al., 2009; Han et al., 2012). Moreover, trastuzumab resistant breast cancer cells have displayed an increase in BCSC enrichment through increased Akt pathway activity (Korkaya et al., 2008). With data presented so far, we found it important to determine the role of the Notch-1-PTEN-ERK1/2 signaling axis in survival and self-renewal of our trastuzumab resistant cells. The goal of aim 2C is to determine the effect of Notch-1-mediated activation of the downstream MAPK pathway through PTEN inhibition on the survival and self-renewal of the trastuzumab resistant BCSC population.

Trastuzumab resistant cells are enriched for BCSCs. Trastuzumab has been shown to reduce the BCSC population in trastuzumab sensitive cells (Magnifico et al., 2009). These data led us to assess the effect of trastuzumab on the BT474 sensitive BCSC population. We assessed BCSC survival of BT474 sensitive cells by using an *in vitro* mammosphere forming assay (Shaw et al., 2012) in response to a vehicle control (PBS) or trastuzumab (20 μg/ml) treatment. We found that trastuzumab treatment significantly reduced the primary percent mammosphere forming efficiency (%MFE) of the BT474 sensitive cells (Figure 25A). Moreover, we observed that the size of mammospheres from BT474 sensitive cells were smaller (<50μm) in response to trastuzumab treatment (Figure 25B). Reduced size of mammospheres and a decrease in %MFE confirmed that

trastuzumab inhibited the survival of the HER2- dependent BCSC population as previously demonstrated by Magnifico et al.

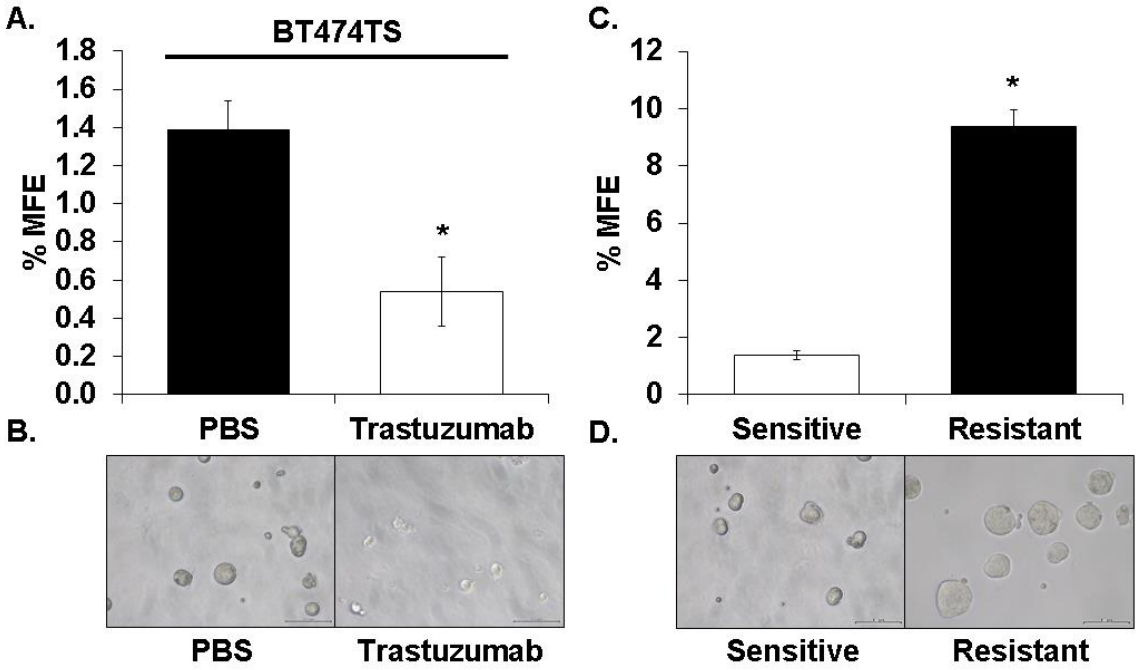

Figure 25: Breast Cancer Stem survival effects in trastuzumab sensitive and resistant cells.

A. 100,000 BT474TS cells were plated into PBS or trastuzumab (20 μg/mL) inoculated mammosphere media. Mammospheres were grown for 10 days. On day 10, mammospheres were harvested and counted to determine mean percent mammosphere forming efficiency (%MFE) which is calculated as [(number of mammospheres counted / number of cells seeded) X 100]. **B.** Representative photographs of BT474TS mammospheres from A at 20x magnification before harvest. **C.** 100,000 BT474TS or BT474TR cells were plated into mammosphere media and incubated for 10 days. On day 10, mammospheres were harvest and counted from three independent experiments in the same manner as A. **D.** Representative photographs of BT474TS and BT474TR mammospheres from C at 20x magnification before harvest. Scale bare = 50μm. Asterisk (*) denotes statistical significance between mean %MFE of PBS and trastuzumab treated cells (A) or BT474TS and BT474TR mammospheres (C) from three independent experiments using a Student's t-test.

Previously, trastuzumab resistant cells have been shown to be enriched for BCSCs compared to sensitive cells (Korkaya et al., 2008). Given this information, we wanted to assess the mammosphere forming potential of our resistant cells compared to sensitive cells to characterize the BCSC population in our cell models. We assessed %MFE of both BT474 sensitive and resistant cells and determined that %MFE of trastuzumab resistant cells was significantly higher than %MFE of trastuzumab sensitive cells by almost 8 fold (Figure 25C). It is important to note that mammospheres from resistant cells were nearly twice the size of mammospheres from sensitive cells (100μm versus 50μm) (Figure 25D).

Together, these data confirm findings from previous work and suggest that long term treatment with trastuzumab enriches for BCSCs.

Notch-1-mediated repression of PTEN is necessary for breast cancer stem cell survival and self-renewal. Notch has been shown to be a primary driver of BCSC survival and self-renewal from studies performed on breast cancer cells lines as well as patient tumor samples (Grudzien et al., 2010; Harrison et al., 2010). Yet, the mechanism by which Notch-1 promotes BCSC survival and self-renewal remains, for the most part, unknown. In order to investigate the role of Notch-1-mediated repression of PTEN in the BCSC population *in vitro*, we performed both primary and secondary mammosphere forming assays from trastuzumab resistant cells transfected with a scrambled control, Notch-1, and/or PTEN siRNAs in response to PBS or trastuzumab. Measurement of primary %MFE determines the survival of the BCSC, while secondary %MFE determines self-

renewal capacity of the BCSC (Shaw et al., 2012). The results showed that knockdown of Notch-1 significantly reduced both primary and secondary %MFE by nearly 5-fold compared to the scrambled control (Figure 26). Knockdown of PTEN had little effect on primary or secondary %MFE compared to the control (Figure 26). Importantly, knockdown of PTEN partially rescued the effects of Notch-1 knockdown by more than 2-fold compared to Notch-1 knockdown alone. These results were similar regardless of trastuzumab treatment (Figure 26, compare right panels to left panels). Mammospheres were photographed at 20x magnification with a 100μm scale bar (Figure 26A and C). Similar results were observed with the inherently resistant HCC1954 cell line. Simultaneous knockdown of Notch-1 and PTEN almost completely recovered both primary and secondary %MFE compared to Notch-1 knockdown alone (Figure 27A-D).

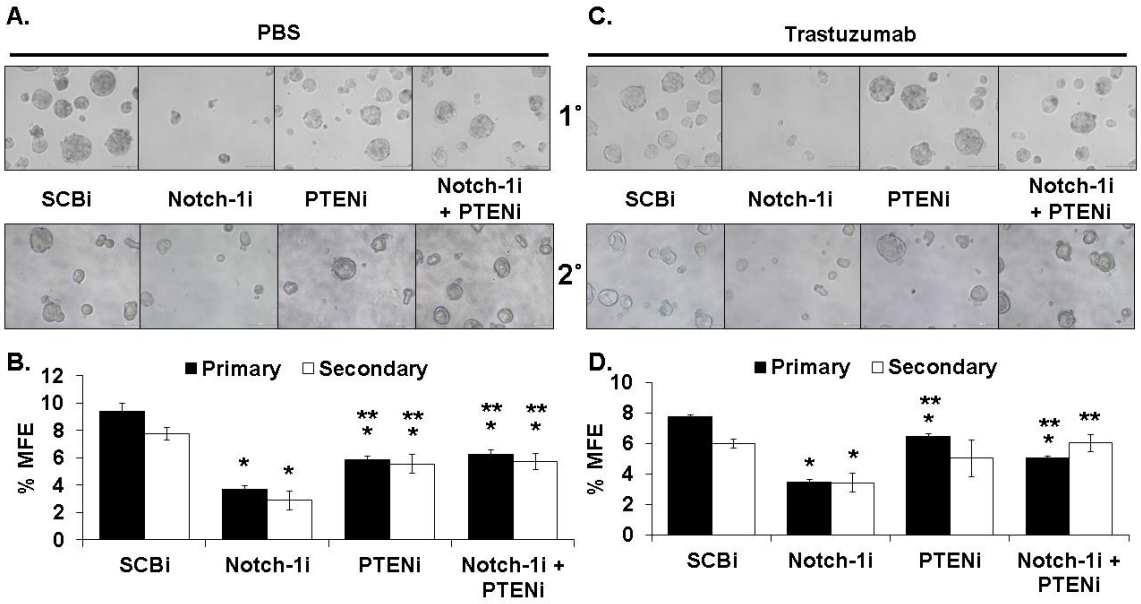

Figure 26: Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant breast cancer stem cell (BCSC) survival and self-renewal of BT474TR cells.

A and C. BT474TR cells were transfected with SCBi, Notch-1i, and/or PTENi for 48 hours. Post transfection, the cells were harvested and 100,000 cells/well were seeded into PBS (A) or trastuzumab (20 µg/mL) (C) inoculated mammosphere media in a 6 well plate. The mammospheres were incubated for 10 days to form primary (1º) mammospheres then harvested, disaggregated, and 100,000 live cells from the primary mammospheres were seeded into PBS or trastuzumab inoculated mammosphere media. The cells were incubated for 10 days to form secondary (2º) mammospheres. Before harvesting, representative photographs of primary (top) and secondary (bottom) mammosphere were taken on day 10 at 20x magnification. **B and D.** Mammospheres were harvested in PBS and mammospheres over 50 µm were counted as such. Primary percent mammosphere forming efficiency (%MFE) (black bars) was calculated as: [(number of mammospheres counted / well) / number of cell seeded X 100]. Secondary %MFE (white bars) was calculated in the same manner as primary %MFE. Asterisk (*) denotes statistical significance compared to SCBi or (**) Notch-1i mean %MFE as calculated using a one-way ANOVA with an overall statistical significance of $p < 0.01$ from three independent experiments. Scale bar = 50µm.

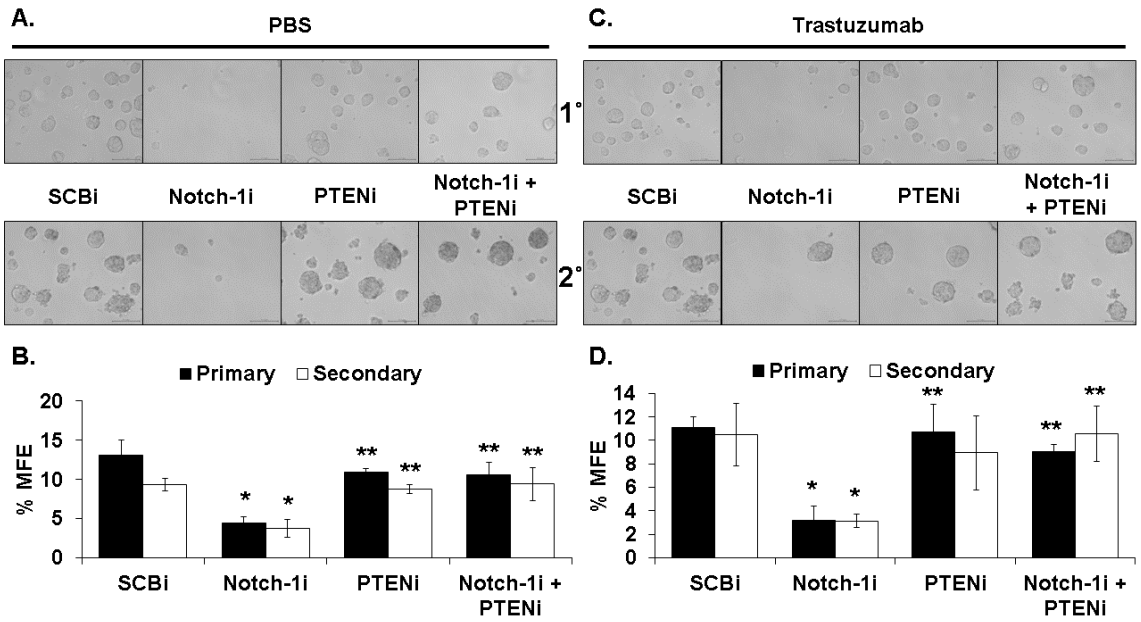

Figure 27: Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant breast cancer stem cell (BCSC) survival and self-renewal of HCC1954 cells.

A and C. HCC1954 cells were transfected with SCBi, Notch-1i, and/or PTENi for 48 hours. Post transfection, the cells were harvested and 50,000 cells/well were seeded into PBS (A) or trastuzumab (20 μg/mL) (C) inoculated mammosphere media in a 6 well plate. The mammospheres were incubated for 7 days to form primary (1º) mammospheres then harvested, disaggregated, and 50,000 live cells from the primary mammospheres were seeded into PBS or trastuzumab inoculated mammosphere media. The cells were incubated for 7 days to form secondary (2º) mammospheres. Representative photographs of primary (top) and secondary (bottom) mammosphere were taken on day 7 before harvest at 20x magnification. **B and D.** Mammospheres were harvested in PBS and mammospheres over 50 μm were counted as such. Primary percent mammosphere forming efficiency (%MFE) (black bars) was calculated as: [(number of mammospheres counted / well) / number of cell seeded X 100]. Secondary %MFE (white bars) was calculated in the same manner as primary %MFE. Asterisk (*) denotes statistical significance compared to SCBi or (**) Notch-1i mean %MFE as calculated using a one-way ANOVA with an overall statistical significance of $p < 0.01$ from three independent experiments. Scale bar = 50μm.

Taken together, these results indicate that Notch-1 is necessary for the survival and self-renewal of trastuzumab resistant BCSCs at least partially through repression of PTEN.

Inhibition of MEK1/2 (MAPKK) activity reduces trastuzumab resistant BCSC survival. We showed that activation of MEK1/2-ERK1/2 signaling rescued proliferation of bulk, trastuzumab resistant cells from knockdown of Notch-1. If the bulk, resistant cell population is dependent on MEK1/2-ERK1/2 activity for proliferation, then this pathway may be required for BCSC survival. To test this, %MFE was measured from trastuzumab resistant cells transfected with a scrambled control, Notch-1, and/or PTEN, siRNAs in response to a vehicle (DMSO) or the MEK1/2 inhibitor (10μM U0126). The results showed that %MFE of control siRNA transfected, resistant cells was decreased by nearly 3 fold in response to U0126 (Figure 28). Knockdown of Notch-1 almost completely inhibited %MFE regardless of U0126 treatment. Importantly, %MFE of cells transfected with the PTEN siRNA alone or PTEN plus Notch-1 siRNAs was inhibited to the same degree as cells transfected with the Notch-1 siRNA alone when treated with U0126 (Figure 28). These data suggest that survival of BCSCs from the trastuzumab resistant cell population are dependent on Notch-1-mediated activation of MEK1/2-ERK1/2 signaling possibly through repression of PTEN.

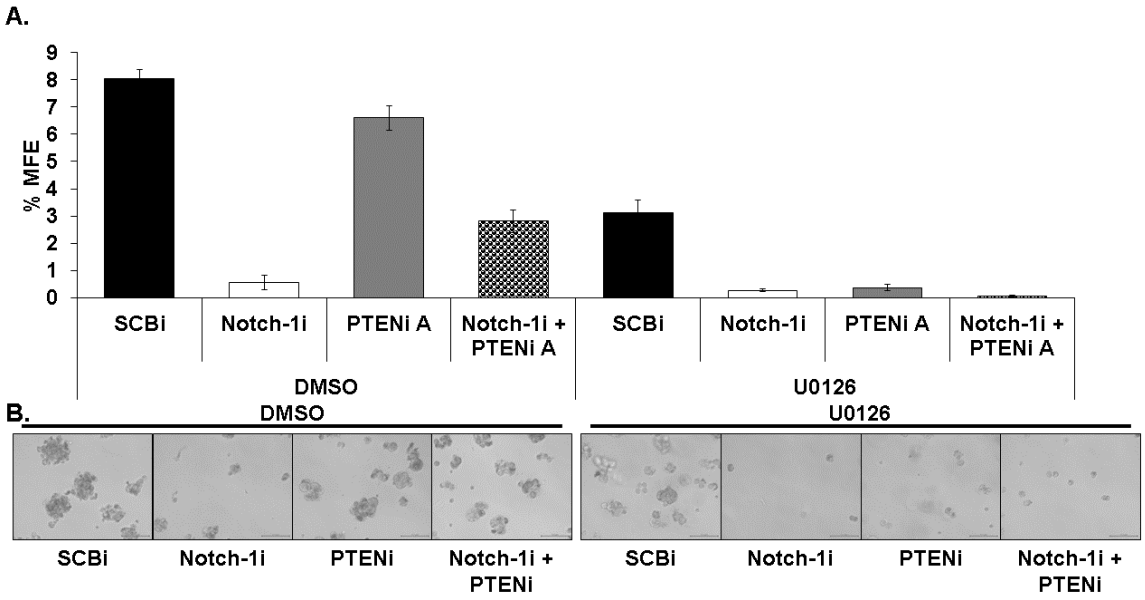

Figure 28: MEK1/2 inhibition reduces trastuzumab resistant BCSC survival. **A.** BT474TR cells were transfected with SCBi, Notch-1i, and/or PTENi-A for 48 hours. Post transfection, cells were harvested and 100,000 live cells / well were seeded into 10 µg/mL DMSO (left) or U0126 (right) inoculated mammosphere media in a 6 well plate and incubated for 10 days. On day 10, mammospheres were harvested in PBS and counted. Mean percent mammosphere forming efficiency (%MFE) which was calculated as [(number of mammospheres counted / number of cells seeded) X 100] from triplicate wells. **B.** Representative photographs of BT474TR mammospheres from A at 20x magnification before harvest. Scale bare = 50μm.

Constitutive MEK1/2 activity and ERK1/2 phosphorylation does not rescue BCSC survival of trastuzumab resistant cells from Notch-1 inhibition. Since the survival of BCSCs from the resistant cell population is dependent on MEK1/2-ERK1/2 activity, we tested whether constitutive MEK1/2-ERK1/2 activity is sufficient to rescue %MFE in response to Notch-1 knockdown. We assessed %MFE of resistant cells transfected with a scrambled control or a Notch-1 siRNA plus a vector control (pEXP) or the MEK1DD mutant. The results showed that although knockdown of Notch-1 decreased %MFE, expression of MEK1DD did not

rescue BCSCs from these inhibitory effects (Figure 29). Surprisingly, expression of MEK1DD with the control siRNA inhibited %MFE similarly to Notch-1 knockdown. One main problem with this assay could have been that expression of MEK1DD and increased ERK1/2 phosphorylation was not maintained in the mammosphere culture up to 10 days. Alternatively, hyper-activation of ERK1/2 could potentially be detrimental to the survival of BCSCs. These studies need to be repeated using stable cell lines in the future to confirm these results.

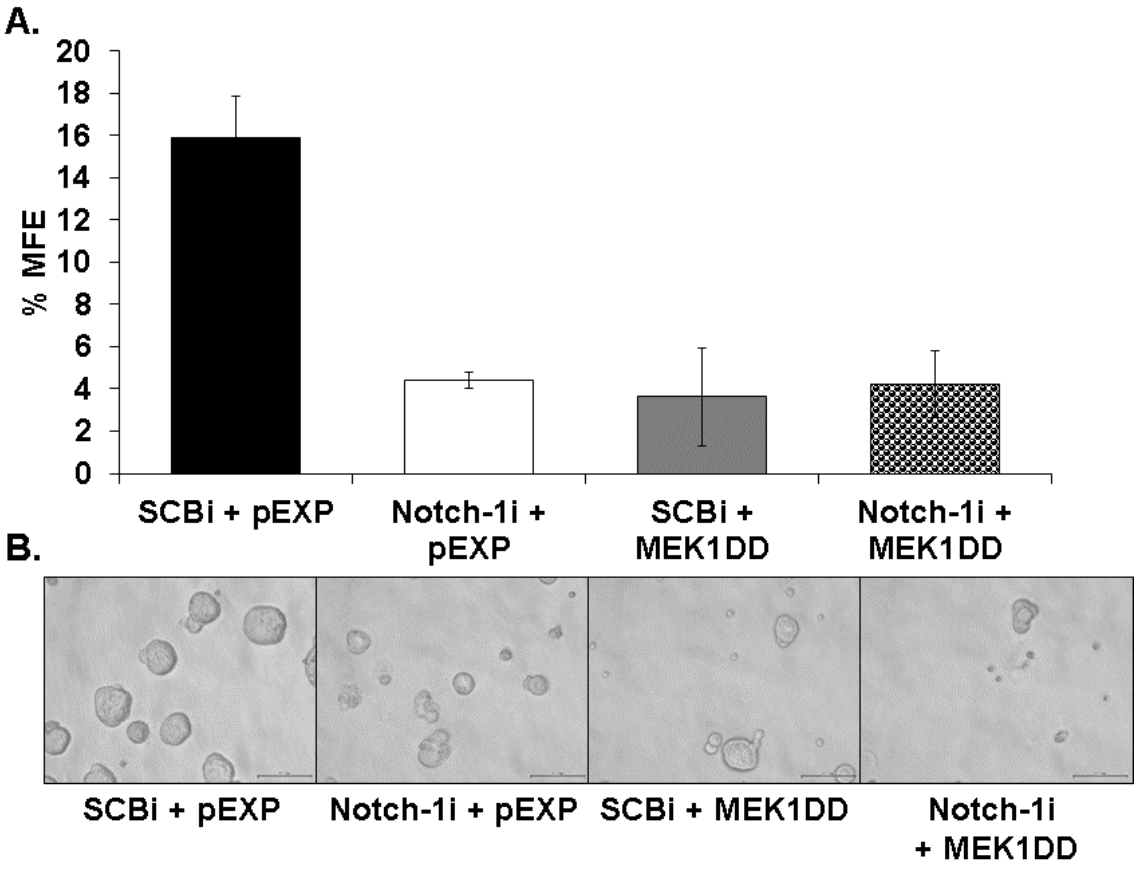

Figure 29: Constitutive activation of ERK1/2 does not promote resistant BCSC survival.
A. BT474TR cells were transfected with SCBi or Notch-1i and MEK1DD or pEXP expression vectors for 48 hours. Post transfection, cells were harvested and 100,000 live cells / well were seeded into mammosphere media in a 6 well plate and incubated for 10 days. On day 10, mammospheres were harvested in PBS and counted. Mean percent mammosphere forming efficiency (%MFE) which was calculated as [(number of mammospheres counted / number of cells seeded) X 100] from triplicate wells. **B.** Representative photographs of BT474TR mammospheres from A at 20x magnification before harvest. Scale bare = 50μm.

Notch-1 and PTEN siRNAs do not have off target effects on trastuzumab resistant BCSCs. Previous work in the resistant bulk cell population demonstrated than PTEN siRNA B has an off target effect on resistant cell proliferation. We wanted to confirm that results from the Notch-1 and PTEN siRNAs on %MFE were

not due to off target effects. To address this issue, we transfected resistant cells with a scrambled control, secondary Notch-1 (#2), and/or PTEN-A siRNAs and performed mammosphere forming assay to assess %MFE (Figure 30A and B). The %MFE results were similar to the previous results using distinct siRNAs (Figure 30). These data confirm that Notch-1- mediated inhibition of PTEN is necessary to promote survival and self-renewal of BCSCs from trastuzumab resistant breast cancer cells.

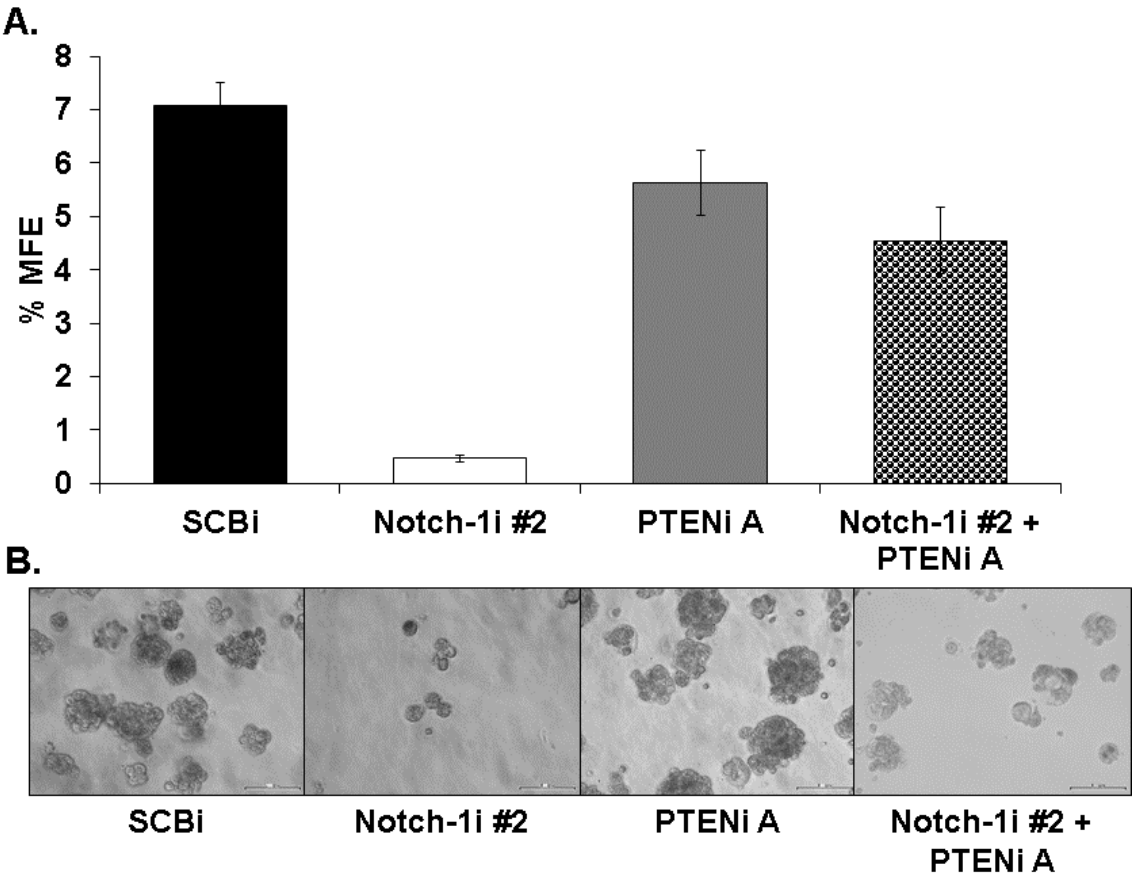

Figure 30: Distinct Notch1 and PTEN siRNAs Produce Similar Effects in BCSCs.

A. BT474TR cells were transfected with SCBi, secondary Notch-1 siRNA (Notch-1 #2) and/or PTENi-A for 48 hours. Post transfection, 100,000 live cells were seeded / well into mammosphere media and incubated for 10 days. % MFE was calculated as [(number of mammospheres counted / number of cells seeded) X 100] from triplicate wells. **B.** Representative photographs of BT474TR mammospheres from E at 20x magnification before harvest. Scale bare = 50μm.

Specific Aim 3. Determine if Notch-1-mediated repression of PTEN is necessary for trastuzumab resistant tumor initiating potential *in vivo*.

The goal of specific aim 3 is to test the following hypotheses:

Aim 3A: Determine if Notch-1-mediated inhibition of PTEN is necessary for tumor initiation.

Aim 3B: Discern the co-expression profile of Notch-1 and PTEN and if this co-expression predicts outcome in patients with breast cancer.

Aim 3A: Determine if Notch-1-Mediated Inhibition of PTEN is Necessary For Tumor Initiation.

Rationale. The data from the mammosphere assays demonstrate that the survival and self-renewal of resistant BCSCs are dependent on Notch-1-mediated repression of PTEN. These results encouraged us to determine if Notch-1-mediated inhibition of PTEN was necessary for tumor formation in a mouse tumor xenograft model. Due to a lack of pharmacological means to specifically inhibit Notch-1 and PTEN, we inhibited Notch-1 and PTEN using siRNAs as in our *in vitro* studies which have demonstrated the ability to persistently inhibit target gene expression for an extended period of time. Pandya et al. showed that GSI can reduce BT474TR tumor xenograft growth and recurrence (Pandya et al., 2011). However, GSI is a pan Notch inhibitor, and bulk BT474TR cells were used to create resistant tumor xenografts in a nude mouse model. However, the specific role and contribution of Notch-1 on trastuzumab resistant BCSC tumor xenografts remained unknown. The goal of aim 3A is to determine if specific inhibition of Notch-1 can

reduce BCSC tumor xenograft formation and if this inhibition can be recovered by combined knockdown of Notch-1 and PTEN *in vivo*.

Notch-1-mediated inhibition of PTEN promotes trastuzumab resistant breast cancer stem cell tumor formation *in vivo.* Breast tumor xenografts were generated from resistant BCSCs. BT474TR cells were transfected with a scrambled control, Notch-1, and/or PTEN siRNAs and incubated in mammosphere media for 10 days. The primary mammospheres were harvested and disaggregated into single cells. 10,000 live, primary mammosphere-derived cells in PBS were mixed at a 1:1 ratio with matrigel and subcutaneously injected into the mammary fat pads of FOXN1-null, athymic, nude mice. Injection of the cells was followed by implantation of an estrogen capsule to provide a constant release of estrogen as BT474 cells express moderate levels of ERα and require estrogen for optimal growth. Tumors were allowed to grow for 12 weeks at which time some tumors had reached the maximum cross sectional area ($0.5cm^2$) allowed and all of the tumors were harvested. The results demonstrate that Notch-1 knockdown prevented tumor formation while the combined Notch-1 and PTEN knockdown rescued tumor formation compared to Notch-1 or PTEN siRNA (Figure 31A – C). Additionally, tumor growth rates and volumes were similar in the control, PTEN, and combined Notch-1 and PTEN siRNA tumors (Figure 32A and B).

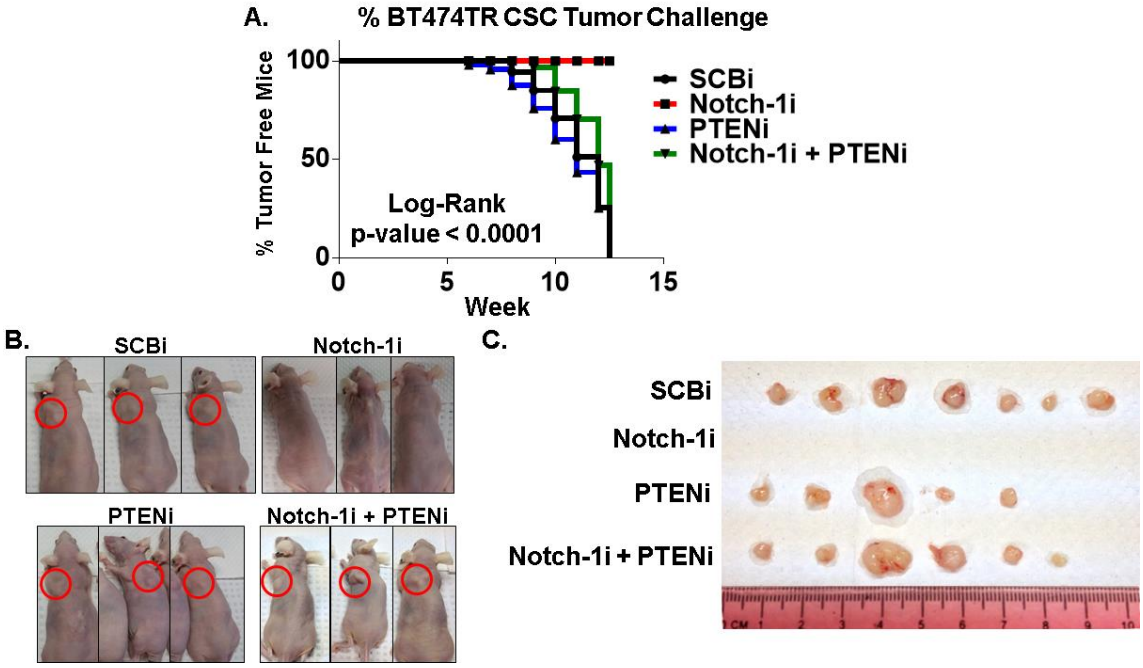

Figure 31: Notch-1 mediated inhibition of PTEN promotes trastuzumab resistant breast cancer stem cell xenograft tumor formation *in vivo*.
A. BT474TR cells were transfected with SCBi, N-1i, and/or PTENi for 48 hours. Post transfection, the cells were harvested and seeded in mammosphere media at 100,000 cells/ well in a 6 well plate for 10 days. After 10 days, the primary mammospheres were harvested and trypsinized into single cells. 10,000 live cells in 50 µl of PBS were mixed with 50 µl of Matrigel and subcutaneously injected bilaterally into the mammary fat pads of athymic nude mice along with a 17β-estradiol capsule. Mice were monitored for tumors weekly for 12 weeks. The Kaplan-Meier curves and log rank test (<0.0001) displays percent tumor free mice in SCBi, Notch-1i, PTENi, and Notch-1i combined with PTENi tumor-bearing mice. **B.** Photographs of SCBi, Notch-1i and/or PTENi treated BCSC tumor formation (red circles) in nude mice. **C.** SCBi, Notch-1i and/or PTENi treated BCSC tumors excised from nude mice.

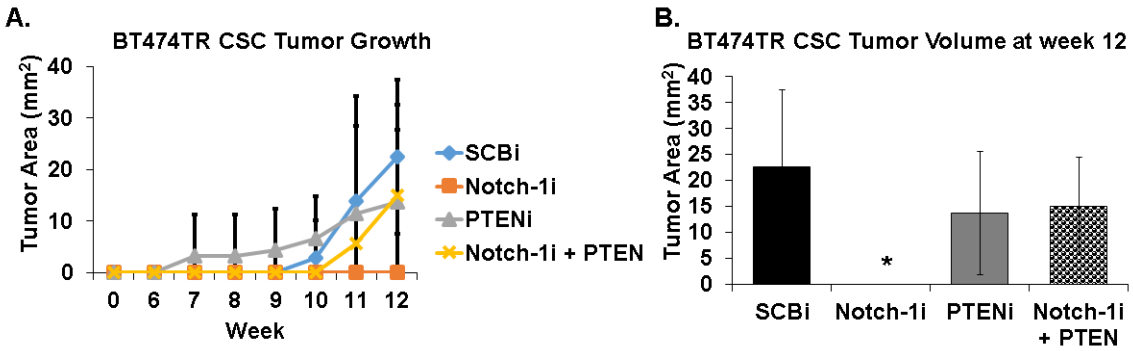

Figure 32: Notch-1 is required for Trastuzumab resistant breast cancer stem cell xenograft tumor growth *in vivo*.
A. BT474TR cells were transfected with SCBi, N-1i, and/or PTENi for 48 hours. Post transfection, the cells were harvested and seeded in mammosphere media at 100,000 cells/ well in a 6 well plate for 10 days. After 10 days, the primary mammospheres were harvested and trypsinized into single cells. 10,000 live cells in 50 µl of PBS were mixed with 50 µl of Matrigel and subcutaneously injected bilaterally into the mammary fat pads of athymic nude mice along with a 17β-estradiol capsule. Tumors in mice were measured (l X w) weekly for 12 weeks to determine average tumor volume for each treatment group weekly. **B.** Final tumor sizes at week 12 for each treatment group. Asterisk (*) denotes statistical significance compared to SCBi mean tumor volume as calculated using a one-way ANOVA with an overall statistical significance of $p < 0.06$.

Taken together, these results demonstrate that Notch-1 is required for trastuzumab resistant tumor initiation and BCSC self-renewal *in vivo*. Furthermore, Notch-1-mediated repression of PTEN is necessary for self-renewal of trastuzumab resistant BCSCs.

Aim 3B: Discern the Co-Expression Profile of Notch-1 and PTEN and if this Co-Expression Predicts Outcome in Women With Breast Cancer.

The data thus far suggest that an inverse relationship between Notch-1 and PTEN expression may increase trastuzumab resistance as well as promote a worse outcome for women with HER2+ breast cancer. We used the Oncomine database (oncomine.org) to find three cohorts of microarray data sets that

assessed Notch-1 and PTEN RNA transcript expression in human breast tumor samples (Curtis et al., 2012; Bonnefoi et al., 2007; Hatzis et al., 2011). Analysis of Notch-1 and PTEN co-expression as well as their effect on patient survival time was performed by Dr. Jun Li from the University of Notre Dame. The goal of aim 3B is to determine if an inverse relationship between Notch-1 and PTEN expression exists in breast tumors and if this relationship has an effect on overall survival outcome in patients with breast cancer.

There is a potential inverse relationship between Notch-1 and PTEN co-expression in breast cancer patient tumors. RNA can be extracted from core biopsies of breast cancer patient tumors and analyzed by microarray screening. Analysis of microarray data can determine variations in gene expression between patient samples. This high-throughput screening technology can determine differences in gene expression between multiple patient samples. For instance, gene expression profiling by microarray screening can determine gene alterations in patients responding to a treatment compared to patients that aren't responding to a treatment. These data can determine if particular genes are up or downregulated to induce resistance to a particular cancer treatment regimen. Here, we identified three cohorts of microarray data sets (Curtis, Bonnefoi, and Hatzis) that expressed Notch-1 and PTEN (Curtis et al., 2012; Bonnefoi et al., 2007; Hatzis et al., 2011). First, Dr. Li used Spearman's correlation coefficient to assess if there is a relationship between Notch-1 and PTEN expression in each cohort. Results of the analysis show that an inverse relationship between Notch-1

and PTEN exists in the Bonnefoi cohort (Table 3, top). Conversely, there appears to be no relationship between Notch-1 and PTEN in the Curtis or Hatzis cohorts as this analysis did not reach statistical significance. It is important to note that these cohorts contain both HER2-positive and HER2-negative patient samples. The HER2+ patient samples were analyzed in the largest cohort (Curtis) but the number of HER2+ tumors was very small and did not produce statistically significant results.

Co-Expression Correlations of Notch-1 and PTEN		
	Rank Correlation	p-value
Curtis	0.0141	0.515
Bonnefoi	-0.1537	0.052
Hatzis	-0.03651	0.412
Cox Proportional Hazards Model		
	Hazard Function Estimate	p-value
Notch-1 Coeffecient	1.112	0.0636
PTEN Coeffecient	-0.227	0.0038

Table 3: Inverse expression of Notch-1 and PTEN in breast cancer patient tumors is associated with poorer outcome.
Top, correlation of Notch-1 and PTEN co-expression in each of the three breast microarray datasets as calculated by Spearman's correlation coefficient. Bottom, co-expression of high Notch-1 and low PTEN correlates with an overall increase in patient survival time in the Curtis Breast dataset as determined by the Cox proportional hazards model. The log-rank test p-value = 0.001262.

Co-expression of high Notch-1 and low PTEN predicts poorer breast cancer patient survival outcome. If there is an inverse relationship between Notch-1 and PTEN occurring one cohort of breast tumors, then this inverse relationship may negatively affect patient survival time. To this end, the effect of Notch-1 and PTEN expression on patient survival time was assessed using a Cox

proportional hazards model in the largest cohort, the Curtis dataset. To this end, we found that co-expression of high Notch-1 and low PTEN in patient tumors significantly reduced survival time (p-value = 0.001262 by a log-rank test) (Table 3, bottom).

Overall, these data indicate that an inverse relationship between Notch-1 and PTEN occurs in 1 out of 3 microarray data cohorts. The inverse relationship between Notch-1 and PTEN may be associated with decreased overall patient survival time but we cannot say for sure as we would need further statistical analysis of microarray cohorts. Further analysis may need to include another variable such as P-ERK1/2, HER2+, or trastuzumab treatment.

CHAPTER 5

DISCUSSION

Trastuzumab has improved the treatment of HER2+ breast cancer by specifically blocking HER2 receptor activity. Trastuzumab combined with chemotherapy has been shown to reduce HER2-driven tumor growth. Unfortunately, resistance to trastuzumab treatment remains a major clinical problem. Determining the mechanism of trastuzumab resistance could improve trastuzumab-based therapies, avoid development of resistance, and may shed some light on mechanisms of resistance in other cancers. Here, we have shown a novel mechanism for Notch-1 as it is directly recruited to the PTEN promoter which correlates with reduced PTEN expression at the transcriptional level. Notch-1-mediated inhibition of PTEN reduces dephosphorylation of ERK1/2 resulting in increase MAPK pathway activity in resistant cells. The Notch-1-PTEN-ERK1/2 signaling axis promotes bulk cell proliferation as well as BCSC survival and self-renewal. Notch-1-mediated inhibition of PTEN was shown to promote tumor initiation of resistant BCSCs while inhibition of Notch-1 alone blocked tumor formation in a mouse tumor xenograft model. Together these data present a new role for Notch-1 in which Notch-1 directly binds to the PTEN promoter potentially to inhibit PTEN transcript expression and promote trastuzumab resistant, HER2+ breast cancer stem cell survival and self-renewal through MEK1/2-ERK1/2 pathway activation.

The transcription of Notch target genes is facilitated by the formation of a Notch transcriptional complex (NTC). CBF-1 is a critical repressor of Notch target genes when NICD is not present. CBF-1 binds to a number of CBF-1 binding sites in the promoter of Notch target genes. Promoter bound CBF-1 recruits negative co-regulators such as SHARP and CtBP to block gene transcription in the absence of NICD (Oswald et al., 2005). Binding of NICD to CBF-1 allows the release of the negative co-regulators and the recruitment of co-activators to form the NTC. Proper NTC formation begins transcription of the Notch target gene. Our data shows that Notch-1 is both necessary and sufficient to inhibit PTEN transcript RNA levels possibly by directly binding to regions containing CBF-1 elements near the PTEN promoter. Notch-1 enrichment on the PTEN promoter may inhibit transcription in a number of ways. For instance, formation of the NTC may be incomplete at the PTEN promoter. As such, the CBF-1:NICD1 complex may fail to completely release all negative co-regulators or recruit all co-activators to initiate transcription. NTC formation begins with MAML-1 which is the lynch pin to formation of the NTC (Reviewed in Gordon et al., 2008). Previous work has shown that recruitment of MAML-1 alone to the CBF-1:NICD1 complex is a potent inhibitor of gene transcription in T-ALL (Weng et al., 2003). Additionally, failure to recruit the histone acetyltransferase, p300, would result in nearby histones remaining in a closed conformation with DNA, thereby blocking access of RNA polymerase II to the initiation start site. Moreover, competition between transcription factors for co-activators has been shown to occur as some co-activators are in limited amounts

in the nucleus. Previous work has shown that MAML-1 is necessary for the proper formation of several transcription complexes such as p53 (Zhao et al., 2007) and β-catenin (Alves-Guerra et al., 2007). These data allow us to form several hypotheses as to how NICD1 could be involved in the inhibition of PTEN transcription, all of which would require further investigation.

Notch-1-mediated inhibition of PTEN may be caused by interactions with transcription factors that regulate transcription of PTEN. For instance, previous work has shown that p53 can bind to MAML-1 in the NTC (Yun et al., 2015) resulting in inhibition of Notch transcriptional activity in an *in vitro* breast cancer model. By this rationale, an increase in p53 expression may increase recruitment of p53 to the NTC thereby inhibiting NICD1 from activating transcription of PTEN. In agreement with this scenario, it was shown that co-expression of HER2 and p53 correlated with higher rates of cancer recurrence and poorer overall survival (Yamashita et al., 2003). Additionally, the cell models used here, BT474 and HCC1954 have Y163C and E285K missense mutations, respectively, that have been shown to increase the stability of p53 resulting in accumulation of a putative active form of p53 in the nucleus (www.atcc.org).

Notch paralogues may compete to form NTCs and this competition could regulate PTEN transcription. Our lab has observed that both Notch-3 and Notch-4 expression are increased in resistant cells upon HER2 blockade using trastuzumab (Data generated by Dr. Kinnari Pandya). Presumably, the four Notch paralogues form similar NTCs and may compete for CBF-1 binding as well as the

recruitment of the co-activators needed for proper NTC formation. Competition for p300 has been seen among different Notch paralogues and mutant forms of the Notch receptor (Oswald et al., 2001) as well as between NFκB and estrogen receptors in heart smooth muscle cells (Speir et al., 2000). Additionally, previous research has demonstrated transcriptional inhibition among Notch paralogues through cooperative binding between two CBF-1-NICD-MAML1 complexes. The orientation of CBF-1-NICD-MAML complexes on the promoter determines their ability to initiate transcription (Nam et al., 2007). But, inhibition of PTEN transcription by Notch-1 has been previously described by Graziani et al. in which NICD1 is directly recruited to the PTEN promoter to inhibit its transcription in a NSCLC model (Graziani et al., 2008). Together these data indicate that NICD1 may inhibit transcription of PTEN through multiple possible mechanisms.

NICD1 overexpression in trastuzumab sensitive cells results in minimal reduction of PTEN transcript expression indicating that the transcription of PTEN in sensitive cells differs from transcription of PTEN in resistant cells. As the sensitive cells are treated with trastuzumab over an extended period of time, we presume they acquire changes in their genomic landscape that may alter how PTEN transcription is activated or repressed. By this hypothesis, the promoter of PTEN may be crowded with transcriptional activators that keep PTEN expression high in sensitive cells. In sensitive cells, Tremendous over expression of Notch-1 may be able to interact with PTEN transcription factors on the PTEN promoter by displacing or counter acting transcriptional activators of PTEN. The PTEN

promoter in the resistant cell may lack some of the transcriptional activators at the PTEN promoter compared to the sensitive cell or may have room for Notch-1 to bind to and counteract these activators or actively repress PTEN transcript expression in resistant cells. Regardless, thorough analysis or both sensitive and resistant PTEN promoters and the effects of NICD1 on PTEN transcriptional activity should be done to address these hypotheses.

Indirect inhibition of PTEN by Notch-1 has been shown to occur in several types of cancer. PTEN inhibition by Notch-1 has been shown to occur through Notch-1-mediated upregulation of the transcriptional repressor, HES-1 (Palomero et al., 2007). Aberrant Notch-1 activity in T-ALL cells, due to mutations, results in increased expression of HES-1, a direct Notch target gene. HES-1 is directly recruited to the PTEN promoter to inhibit transcription of PTEN. Such is not the case in trastuzumab resistant cells, as knockdown of HES-1 had no effect on PTEN RNA transcript levels (Figure 33). Indirect inhibition of PTEN by Notch-1 has been shown to occur through regulation of the NFκB pathway. Work by Du et al. has shown that the p65 (RelA), a subunit of NFKB, increases PTEN transcription in trastuzumab resistant cells (Du et al., 2014). In this work, Notch-1 was shown to sequester p65 in the cytoplasm thereby inhibiting its ability to enter the nucleus and initiate transcription of PTEN. Moreover, Notch-1 has been shown to upregulate the NFkB pathway in TNBC (Li et al., 2016) and in HER2-negative BCSCs (Mao et al., 2013) suggesting a possible feedback mechanism between Notch-1 and NFκB in these breast cancer models. Although the conclusions of

these data do not speak to direct inhibition of PTEN by Notch-1, indirect inhibition of PTEN may also occur in our resistant cells to perpetuate PTEN downregulation.

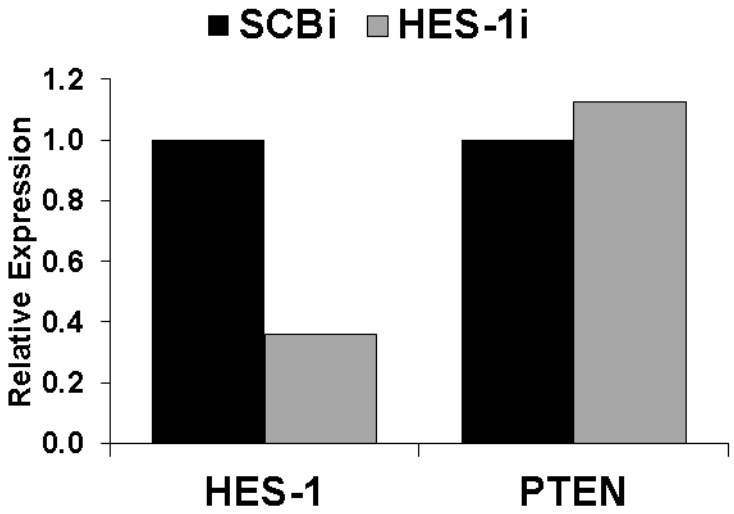

Figure 33: HES-1 has no effect on PTEN RNA transcript expression.
BT474TR cells were transfected with SCBi or HES-1i for 48 hours. Post transfection, relative expression of HES-1 (left) and PTEN (right) was measured by RT-PCR.

Other than direct transcriptional repression of PTEN, inhibition of PTEN can be facilitated by modification of the PTEN protein to block its phosphatase activity. Post-translational modification of PTEN activity is exemplified by its acetylation. Acetylation of PTEN promotes binding to PCAF which facilitates its entry into the nucleus, loss of PI3K regulation, and an increase in cell cycling (Okumura et al., 2006). Silent mating type information regulation 2 homolog 1 (SIRT1) has been shown to facilitate inhibition of PTEN nuclear translocation by deacetylation of the PTEN PDZ domain (Ikenoue et al., 2008) (Figure 9). Deacetylation of PTEN by SIRT1 inhibits its nuclear activity such as dephosphorylation of MAPK effectors. Co-expression of high Notch-1 and low SIRT1 correlates with shorter overall

survival and DFS in breast cancer patients (Cao et al., 2014). These results suggest that crosstalk between Notch-1 and SIRT1 may occur to regulate PTEN protein activity as well in resistant cells.

The role of the Akt pathway in the trastuzumab resistant model presented here remains controversial. Our results show that Notch-1-mediated inhibition of PTEN has little to no effect on the phosphorylation status of Akt. This finding was observed in two distinct models of trastuzumab resistant breast cancer cells, BT474 and HCC1954. This finding is counterintuitive to the lipid phosphatase activity of PTEN on PIP_3. These results suggest that PTEN may have lost the ability to dephosphorylate PIP_3, PIP_3 is not responsive to PTEN phosphatase activity, or that Notch-1 may be regulating phosphorylation of Akt through other proteins capable of changing the phosphorylation status of Akt. One other possible explanation is that the resistant cells used in these experiments have a H1047R or K111N *PIK3CA* missense mutation (www.atcc.org) resulting in increased catalytic activity of the p110 subunit of PI3K that can counter act PTEN-mediated inhibition of PI3K activity.

Characterization of our acquired resistant cells shows that these cells express markers of trastuzumab resistance: increased IGF-1R and decreased $p27^{Kip1}$ compared to sensitive cells (Personal Communication with Dr. Pandya). Additionally, the resistant cells express more Hrg-2 and less HER4 protein compared to sensitive cells. Hrg-2 and HER4 are two novel markers of trastuzumab resistance found by our laboratory (Reviewed in Figure 34). Previous

work has shown that the expression of Hrg-1 is upregulated by an increase in ectodomain shedding of nascent Hrg-1 by ADAM10 (Ebbing et al., 2016). Upregulation of Hrg-1 results in increased activation of HER3 in acquired trastuzumab resistant esophageal cancer. Analysis of our acquired trastuzumab resistant HER2+ breast cancer cells showed an increase in Hrg-2 over Hrg-1 compared to sensitive cells hence Hrg-2 may be contributing to trastuzumab resistance in our cell model (Data generated by Dr. Kinnari Pandya).

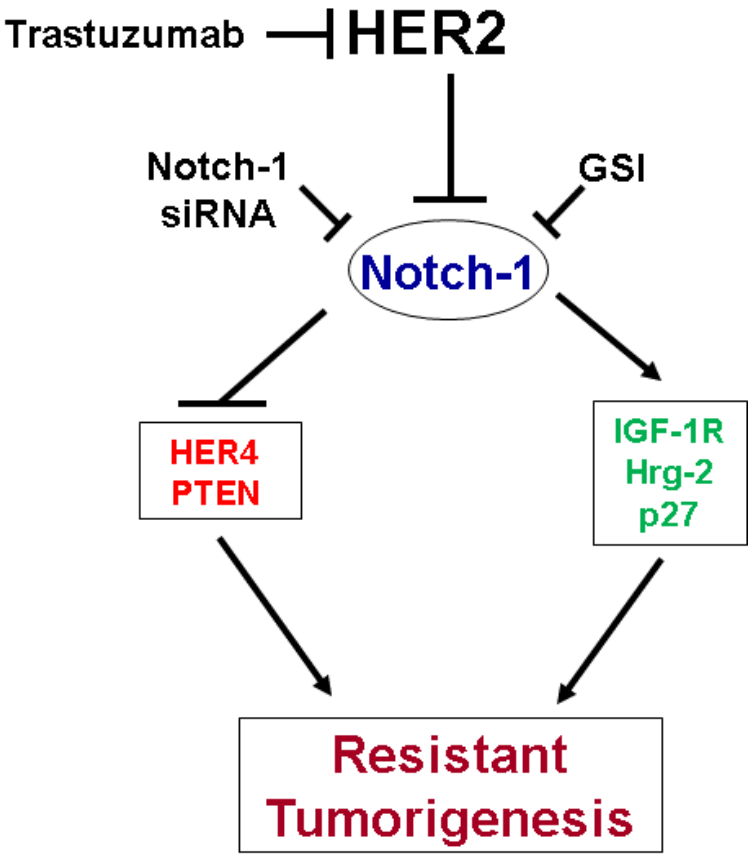

Figure 34: Notch-1 is a master regulator of IGF-1R, $p27^{Kip1}$, HER4, PTEN, and Hrg-2 in trastuzumab resistant cells.

Notch-1 knockdown reverses the expression of proteins and RNA transcripts associated with trastuzumab resistance (Personal Communication with Dr. Pandya). This suggests that Notch-1 is potentially regulating many of the markers of trastuzumab resistance. Furthermore, preliminary ChIP assays of NICD1 enrichment at CBF-1 sites on the HER4, IGF-1R, and Hrg-2 promoters show an increase in endogenous NICD1 enrichment in resistant compared to sensitive cells as well as in sensitive cells overexpressing NICD1 compared to the pcDNA3 vector control (Figure 36A-F). Regulation of trastuzumab resistant

markers by Notch-1 may affect downstream phosphorylation of Akt. One such Notch-1 target, HER4, is downregulated in resistant cells. Much like PTEN, knockdown of Notch-1 increased HER4 protein and RNA expression (Personal Communication with Dr. Pandya). Similarly, overexpression of NICD1 reduced expression of HER4 RNA transcripts (Figure 35). HER4, like HER2, can increase downstream phosphorylation of Akt. Notch-1-mediated inhibition of HER4 and PTEN could potentially have compensatory effects on the phosphorylation status of Akt. For example, Notch-1 knockdown would increase HER4 expression to sustain PI3K activation and thus maintain Akt activity regardless of upregulation of PTEN. As such, PTEN attenuates PI3K-mediated phosphorylation of Akt which can be negated by HER4-mediated PI3K activation thereby limiting the overall effect of Notch-1 on the phosphorylation of Akt and Akt activity. Taken together, these data indicate that Notch-1 may act as a master regulator of several markers of trastuzumab resistance. As such, Notch-1 may have little to no overall effect on the phosphorylation of Akt and Akt pathway activity in HER2+, trastuzumab resistant breast cancer.

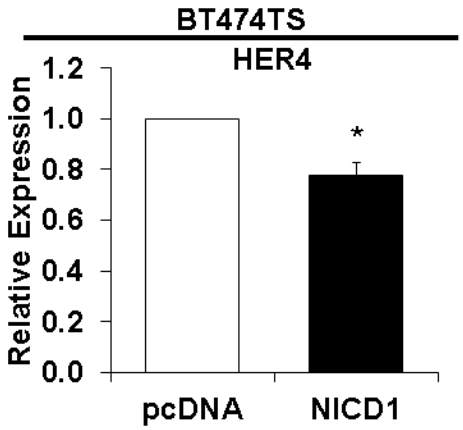

Figure 35: Notch-1 overexpression reduces HER4 transcript expression. BT474TS cells were transfected with NICD1 or pcDNA3 expression vectors for 48 hours. Post transfection, relative expression of HER4 was measured by RT-PCR.

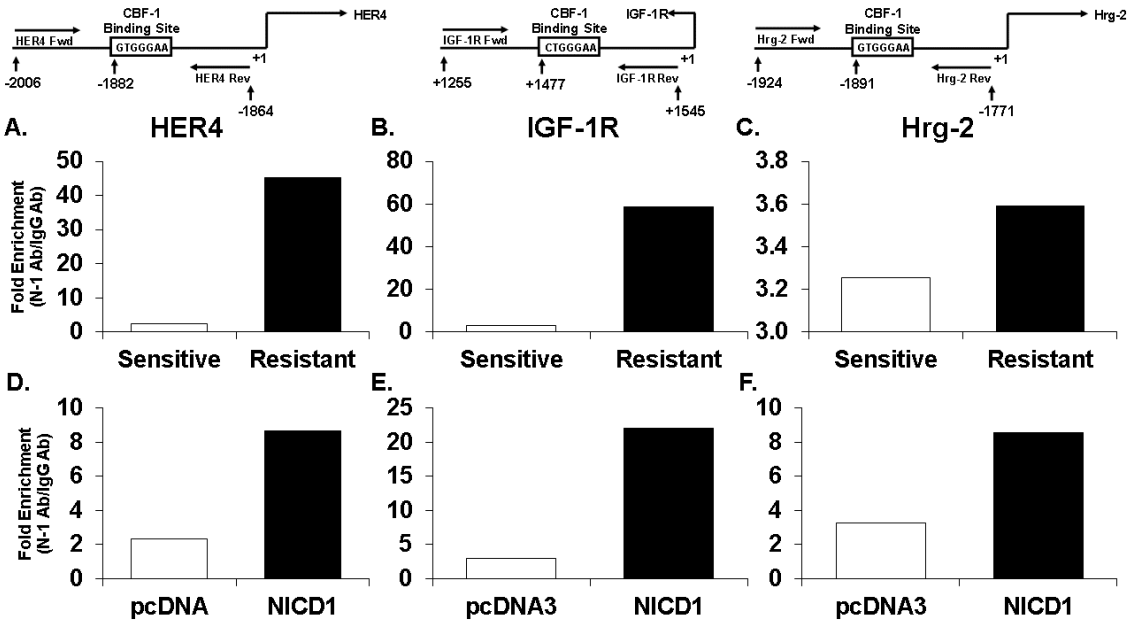

Figure 36: Notch-1 is directly recruited to the HER4, IGF-1R, and Hrg-2 promoter.

A-C. BT474TR and BT474TS cells were fixed, fragmented, and chromatin was immunoprecipitated by a C-terminal Notch-1 (Santa Cruz Notch-1 Ab) or control IgG antibody. Purified DNA from Notch-1 or IgG immunoprecipitated chromatin was used to quantify NICD1 enrichment of HER4 (A), IGF-1R (B), or Hrg-2 (C) promoters by qPCR using primers specific to a CBF-1 binding site in the HER-4, IGF-1R, or Hrg-2 promoter (maps above bar graphs). **D-F.** BT474TS cells were transfected with NICD1 or pcDNA3 expression vectors for 48 hours. Post transfection, cells were harvested, fixed, fragmented, immunoprecipitated by Notch-1 or IgG antibodies, and NICD1 enrichment of HER4 (D), IGF-1R (E), or Hrg-2 (F) promoters was quantified by qPCR using the same HER-4, IGF-1R, or Hrg-2 primers as in A-C.

Notch-1 has been shown to regulate downstream effectors of the Akt pathway. Previous work has shown that Notch-1 can upregulate cyclin D1 which results in activation of CDK 4/6 to increase proliferation of T-ALL cells (Choi et al., 2012). A similar increase of cyclin D1 has been observed in resistant breast cancer, yet the role of Notch-1-mediated upregulation of cyclin D1 has yet to be elucidated (Witkiewicz et al., 2014). Previous characterization of acquired resistant

cells showed that expression of $p27^{Kip1}$ is decreased (Nahta et al., 2004). Our resistant cells display a decrease in $p27^{Kip1}$ protein expression compared to sensitive cells (Personal Communication with Dr. Pandya). More importantly, Notch-1 knockdown caused an increase in $p27^{Kip1}$ expression in resistant cells suggesting that Notch-1 may regulate downstream $p27^{Kip1}$ expression (Personal Communication with Dr. Pandya). $p27^{Kip1}$ downregulation may occur through Notch-1-mediated upregulation of IGF-1R which has been shown to decrease downstream $p27^{Kip1}$ expression in trastuzumab resistant, SkBr3 cells (Lu et al., 2004). These data suggest that Notch-1 may regulate proteins downstream of the Akt pathway to contribute to resistant cell growth.

Specifically targeting Notch-1 in the resistant cell model may affect the expression of other proteins in an effort to adapt to the loss of Notch-1 expression. As seen in the development of trastuzumab resistance, HER2+ cancer cells can adapt to specifically targeting the HER2 receptor through upregulation of Notch-1. Similarly, T-ALL cells can adapt to GSI treatment by upregulating downstream Akt pathway activity through inhibition of PTEN. Cancer cells take advantage of the number of redundancies that control cell proliferation and survival in order to continue to survive under a multitude of treatment conditions. By this hypothesis, cancer cells can continue to grow and adapt to changing environmental conditions. As such, specifically targeting Notch-1 may alter the expression of other proteins in an effort to maintain the tumorigenicity of the trastuzumab resistant cell.

The role of the MAPK-ERK1/2 pathway in trastuzumab resistant bulk breast cancer cells may differ from its role in the resistant BCSCs. Our data show that inhibition of ERK1/2 phosphorylation by treatment with the MEK1/2 inhibitor, U0126 reduces both bulk (Figure 23A) and BCSC (Figure 28A and B) proliferation and survival, respectively. Constitutive expression of an active MEK1 (MEK1DD) rescues bulk resistant cell proliferation from Notch-1 inhibition (Figure 24A and B) but does not have the same effect on BCSC survival (Figure 29A and B). These data present a disconnect between MAPK activity in resistant bulk and BCSCs in which MAPK activity is required to promote bulk cell proliferation but not BCSC survival. It is important to note that the experiments using the MEK1DD expression vector were done in triplicate. Also, the Notch-1i/MEK1DD transfected cells used in the bulk cell proliferation assay were the same cells used in the mammosphere assay thereby reducing technical mistakes between the two experiments. None the less, expression of the MEK1DD plasmid may not have been able to maintain phosphorylation of ERK1/2 in the mammosphere culture for 10 days. Alternatively, hyper-activation of ERK1/2 could potentially be detrimental to the survival of BCSCs. These studies need to be repeated using constitutively active MEK1DD stable cell lines to confirm these results.

Differences in the effect of constitutive MEK1 activity in resistant bulk and BCSCs may occur for several different reasons. One reason may be that the mammosphere assay is unable to assess changes in the mesenchymal stem cell population. The mammosphere assay is the standard experiment for determining

changes in the epithelial stem cell population through the formation of mammospheres. FACS analysis can allow us to sort and analyze specific cell populations such as the epithelial and mesenchymal BCSC subpopulations or the bulk and BCSC subpopulations. Further assessment of the resistant BCSC population may reveal Notch-1-mediating downstream pathways to promote enrichment of the BCSC population, proliferation of stem cell-like populations, or potentially dedifferentiating or transdifferentiating subpopulation of cells that are in between stem and differentiated states. Additionally, we can determine the effect of Notch-1 and/or PTEN knockdown on the different subpopulations within our resistant cell models. Assessment of the role Notch-1 plays in augmenting the stem-like state of resistant cells can be addressed in future studies.

The results presented here focus on downstream activity of the PI3K/Akt pathway in bulk cells but not in the BCSC population. Preliminary data demonstrated the ability of constitutively activated Akt (Myr-Akt) to partially rescue trastuzumab resistant bulk cell proliferation from Notch-1 inhibition (Figure 37A-C). One explanation for this effect is that any stimulation of a downstream pathway that promotes cell survival will be taken advantage of by the cancer cell. Another possible explanation is that the Akt pathway is used by the BCSCs to promote differentiation and bulk cell proliferation. The Akt pathway has been implicated in promoting enrichment of the BCSC population and drug resistance (Berns et al., 2007). Here, resistant cells may be dependent on Akt activity in some capacity. The development of trastuzumab resistance and the increase in the resistant

BCSC population may have initially been mediated through the Akt pathway. Initial Akt pathway activation could be mediated through repression of PTEN, or one of the many other proteins regulated by Notch-1, enabling the cell to evade and survive under constant trastuzumab treatment.

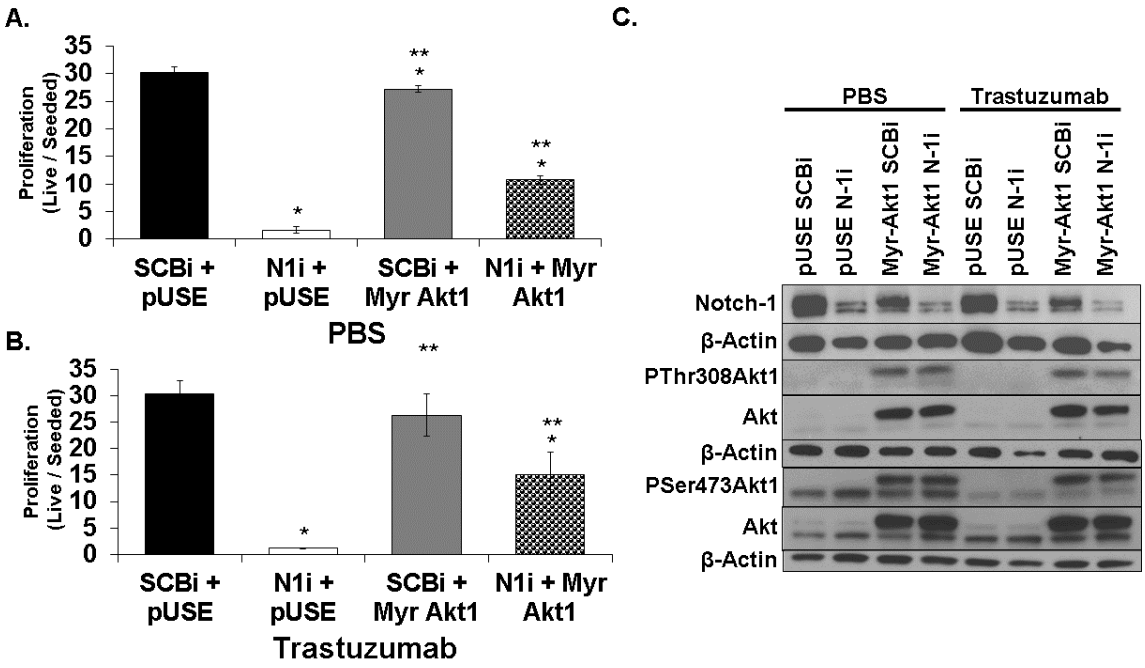

Figure 37: Constitutive activation of Akt promotes trastuzumab resistant cell proliferation.

A-B. BT474TR cells were transfected with SCBi or Notch-1i and Myr-Akt1 or pUSE expression vectors for 48 hours. Post transfection, cells were harvested and seeded at 100,000 cells / well of a six well plate and treated daily with 20 µg/mL of PBS (A) or trastuzumab (B) for 10 days. After treatment, the cells were harvested and counted. Mean proliferation was calculated as the number of live cells harvested / number of live cells seeded. Asterisk (*) denotes statistical significance compared to SCBi or (**) Notch-1i mean proliferation as calculated using a one-way ANOVA with an overall statistical significance of $p < 0.0001$ from three independent experiments. **C.** Total cellular Notch-1, Akt, β-Actin, phosphorylation of Akt-1 at threonine308 (PThr308-Akt1) and serine473 (PSer473-Akt1) protein expression from the transfected cells post 48 hour PBS (left) or trastuzumab (right) treatment was analyzed by Western blot.

Recent research has shown that a loss of PTEN can promote dependency on the MAPK pathway as inhibition of PTEN was found to be required for MEK1 signaling in a HER2+ breast cancer model (Ebbesen et al., 2016). Initial dependence of the resistant cell on Notch-1 mediated activation of the Akt pathway through inhibition of PTEN could have switched to activation of the MAPK pathway to promote bulk cell proliferation but not BCSC survival. Downstream pathway activation may be dependent on the receptors available to dimerize with HER2. It has been shown that HER2 homodimers increase downstream MAPK signaling while HER2 heterodimers increase downstream Akt signaling (Reviewed in Yarden and Sliwkowski, 2001) (Chan et al., 2005). In contrast, the BCSC population is dependent on Akt pathway activity while the bulk cells are dependent on MAPK activity. Moreover, Notch-1 may increase ERK1/2 phosphorylation by non-canonical means as previous students in our lab have shown that membrane bound Notch-1 may be required for increased ERK1/2 phosphorylation and MAPK activation *in vitro*. Further assessment of the Akt and MAPK pathways during the development of trastuzumab resistance *in vitro* may shed some light onto the pathway used by bulk or BCSCs as resistance to trastuzumab treatment occurs.

Conclusion.

The crux of the research presented here demonstrates that trastuzumab resistant, HER2+ breast cancer cells have shifted their growth dependence from HER2 to Notch-1. Specifically targeting Notch-1 in resistant cell models inhibits their ability to proliferate and form tumors through upregulation of PTEN. In the

work presented here we describe an important mechanism by which Notch-1 promotes trastuzumab resistant, HER2+ breast cancer bulk cell proliferation and BCSC survival and self-renewal by repressing PTEN to maintain MAPK-ERK1/2 activity (Summarized in Figure 12). We have set forth data that show the adverse effect of Notch-1 and PTEN co-expression on the survival time of breast cancer patients. Together, these data make the case for an investigation to determine the importance of Notch-1 and PTEN co-expression status as predictive markers for trastuzumab sensitivity in women with HER2+ breast cancer. Currently, clinical trials using the Notch-1 specific antibody [OMP-52M51 (Brontictuzumab)] are underway and should yield results soon (ACC trial, 2015). Furthermore, PTEN and Notch-1 expression status could deliver information for a clinical trial to validate a combinatorial approach using a Notch-1 biologic with a MEK-ERK inhibitor to prevent or treat trastuzumab resistance.

CHAPTER 6

FUTURE INVESTIGATION

Our research demonstrates that Notch-1 represses PTEN in HER2+ breast cancer cells. Inhibition of PTEN results in an increase in phosphorylation of MEK1-ERK1/2 to promote resistant cell proliferation, BCSC survival and self-renewal, as well as contributing to tumor initiating potential in a mouse tumor xenograft model.

An area of controversy in these data is that constitutive activation of MEK1 in resistant BCSCs is not sufficient to rescue BCSC survival from inhibition of Notch-1. We would like to affirm these results by repeating this experiment using a resistant cell line with stable expression of constitutively active MEK1. Constitutive MEK1 activity will increase ERK1/2 phosphorylation and maintain MAPK pathway activity in the cell. We propose to use the MEK1 stable cell line with or without Notch-1 knockdown in primary, and possibly secondary, mammosphere assays. These assays would determine if constitutive activation of MEK1-ERK1/2 is sufficient to promote BCSC survival and self-renewal compared to Notch-1 knockdown. These mammosphere experiments could provide some insight into the dependency of the resistant BCSCs on Notch-1-mediated activation of the MAPK pathway.

These data presented here show that overexpression of NICD1 in sensitive cells was not able to induce trastuzumab resistance. These studies could be complimented with the creation of a stable sensitive cell line constitutively expressing activated MEK1. Previous work has shown that an increase in Notch-1 and Ras-MAPK pathway activity is necessary for TNBC BCSC survival and possibly drug resistance. By this hypothesis, our sensitive cells may require an increase in both Notch-1 and MAPK pathway activity to become resistant to trastuzumab treatment. Under this assumption, constitutively active MEK1-expressing sensitive cells can be transfected with NICD1 and treated with trastuzumab to determine if they lose sensitivity to trastuzumab treatment. Additionally, sensitive cells can be simultaneously transfected with NICD1 and PTEN siRNA in an attempt increase downstream MAPK activity and induce trastuzumab resistance. These experiments may determine the pathways necessary for sensitive cells to acquire resistance to trastuzumab treatment.

PTEN phosphatase activity is dependent on its location within the cell. As previous work has shown, cytoplasmic PTEN reverses the effects of PI3K on Akt activity and promotes cell death whereas nuclear PTEN attenuates ERK1/2-MAPK activity and promotes cell quiescence. Here, Notch-1 is shown to inhibit PTEN resulting in increased phosphorylation of ERK1/2 to promote resistant cell proliferation and possibly tumor initiating potential. This hypothesis presumes that PTEN is dephosphorylating ERK1/2 in the nucleus to induce resistant cell quiescence. This hypothesis can be tested by determining the location of PTEN in

the resistant cells and its phosphatase activity in the nucleus or cytoplasm. To this end, we can determine if PTEN nuclear localization drives dephosphorylation of ERK1/2 in resistant cells using resistant cells that stably express NLS mutant PTEN constructs that inhibit PTEN nuclear localization. Similar to previous work done by Chung et al., resistant cells stably expressing the NLS mutant PTEN construct will have PTEN localized to the cytoplasm while wild-type PTEN is able to enter the nucleus. Additionally, the PTEN mutants are tetracycline inducible meaning that we can control PTEN nuclear entry with tetracycline treatment. We can assess the effects of cytoplasmic or nuclear PTEN by cell fractionation and Western blot analysis of nuclear and cytoplasmic cell fractions to determine the effect of PTEN on downstream Akt and ERK1/2 phosphorylation. Additionally, we can fluorescently label PTEN, Notch-1, Akt, and ERK1/2 to visualize where PTEN is localized in the cell in relation to Notch-1 and effectors of the Akt and MAPK pathways by fluorescent microscopy. Furthermore, we can assess the apoptotic or quiescent state of the resistant cells under Notch-1 and/or PTEN knockdown as well as cells expressing NLS mutant PTEN by propidium iodide staining for FACS analysis or terminal deoxynucelotide transferase dUTP nick end labeling (TUNEL) for fluorescent microscopy. Together, these data will determine if the location of PTEN effects its phosphatase activity as well as the state of resistant cells after Notch-1 and/or PTEN inhibition.

Further assessment of NICD1 enrichment at gene promoters would be necessary to better understand the role of Notch-1 in promoting resistant cell

proliferation, BCSC survival, self-renewal, and tumor initiating potential. To further assess the role of Notch-1 in trastuzumab resistance, we would do an unbiased ChIP-on-ChIP microarray, and/or ChIP sequencing, to determine where on the human genome, NICD1 is binding to in our resistant cells compared to our sensitive cells. Additionally, we can use resistant cells with Notch-1 knockdown and sensitive cells with NICD1 overexpression as controls in the ChIP microarrays for comparison purposes. We have performed extensive ChIP studies that demonstrate that increased Notch-1 recruitment to several different gene promoters correlates with inhibiton of transcript expression of these genes in our resistant cells. We can perform the Notch-1 ChIP-ChIP microarray with other members of the NTC such as CBF-1, MAML-1, p300, or RNA polymerase II to determine if the DNA enriched by NICD1 pull-down also contain members of the NTC complex. In addition, we can perform luciferase assays to determine the effect of Notch-1 on transcription of PTEN as well as use the Crisper-cas system to mutate endogenous CBF-1 binding sites to confirm the role of Notch-1 in PTEN transcription.

Additional *in vivo* studies would allow us to determine the effects Notch-1 and/or ERK1/2 inhibitors on growth of resistant tumor xenografts. Using the Notch-1 antibody, Brontictuzumab, as well as an ERK1/2 inhibitor (PD 098059 or U0126) *in vivo* could give us clinical insight into the effects of these drugs on resistant tumor growth and recurrence. We would use a resistant tumor xenograft model similar to the resistant BCSC tumor xenograft model used to generate data

presented here. We would treat the mice with a Notch-1 antibody with or without a MEK1/2 inhibitor to determine their effects on tumor growth. Additionally, we can remove the treatments if complete regression is observed, to see if the tumors recur. If the tumors recur upon treatment removal, we can resume the treatments to assess their effect on reducing recurring tumors. Moreover, we can grow sensitive and resistant patient derived tumor xenografts (PDX) to evaluate the effects of Notch-1 and/or MEK-1 inhibition on patient tumors. These *in vivo* experiments will give us an idea of the efficacy of these inhibitors on reducing resistant tumor growth and recurrence as well as their potential for success in a clinical setting.

An interesting result in the data presented here is that resistant cells depend on activation of the MAPK pathway through inhibition of PTEN to promote bulk cell proliferation and BCSC survival. This is in contrast to a number of publications that show that an increase in Akt pathway activity, through the loss of PTEN, can drive trastuzumab resistance as well as enrichment of the BCSC population. Our results are more in line with current publications which state that the loss of PTEN is necessary for MEK1-mediated activation of the MAPK pathway in HER2+ breast cancer cells. The ability of PTEN to attenuate both Akt and MAPK pathways can have many effects on cancer development and resistance. One possibility is that inhibition of PTEN facilitates a shift from the Akt to the MAPK pathway as the cells become resistant to trastuzumab treatment. Another possibility is that different resistant cell subpopulations, such as bulk and BCSC populations, are dependent

on either the Akt or the MAPK pathway. Further assessment of differences between bulk and BCSC subpopulations could determine if Notch-1-mediated inhibition of PTEN is driving dedifferentiation of bulk cells into BCSCs or keeping cells in a stem-like state to increase the BCSC population (Figure 38A).

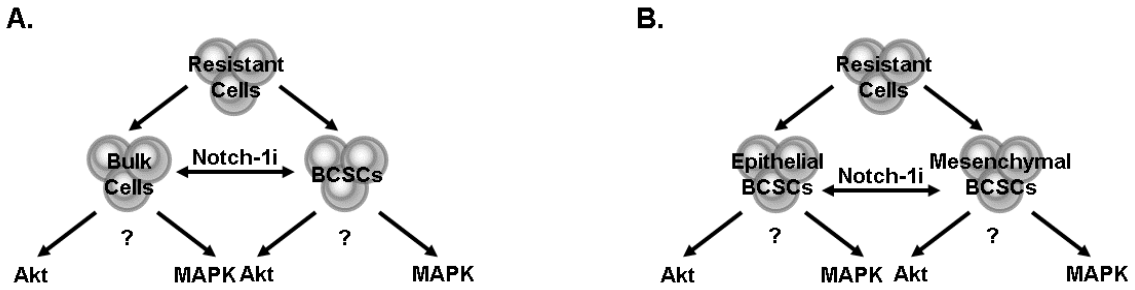

Figure 38: Sorting of BT474TR cells.
Resistant BT474 cells can be sorted into bulk and BCSC (A) or epithelial and mesenchymal (B) BCSC subpopulations to discern differences in Akt and/or MAPK pathway activity. Inhibition of Notch-1 will allow us to determine if Notch-1 can regulate the number cells in each subpopulation.

We can further assess resistant cell subpopulations to determine if the BCSCs are in more epithelial or mesenchymal cell states and how this effects resistance and tumor growth. Previous work by Sun et al. demonstrated that BT474TS and BT474TR PTEN deficient cells display epithelial (ALDH+) and mesenchymal ($CD44^+/CD24^-$) morphology, respectively. They generated trastuzumab resistant cells by knockdown of PTEN combined with long term trastuzumab treatment (BT474-PTEN-LTT). Trastuzumab treatment combined with PTEN deficiency is proposed to transition epithelial stem cells into mesenchymal stem cells ultimately transforming the phenotype of trastuzumab resistant HER2+ cells to resemble that of TNBCs. We may be able to build from

this work by proposing that Notch-1-mediated regulation of PTEN and downstream activation of ERK1/2-MAPK may regulate EMT/MET in the resistant BCSC population and how this effects BCSC survival, self-renewal, and potentially tumorigenesis (Figure 38B). Sorting of resistant cells into subpopulations to determine how the Notch-1-PTEN-ERK1/2 signaling pathway effects their survival and/or proliferation would be important for future studies.

Development of the trastuzumab resistant model has been a source of intrigue as it would be interesting to see how the cells adapt to anti-HER2 treatment over time. Using the trastuzumab resistant cell model as an example, we would develop two acquired resistant cell models from BT474 and SkBr3 cell lines. In addition to these cell lines, we can acquire trastuzumab sensitive patient samples to assess changes in these cells as they are consistently exposed to anti-HER2 treatment. We can use intrinsically resistant cell lines, such as the HCC1954 cells and resistant patient samples, as positive controls for resistance. The use of patient derived cells may give us a translational view of how cells from a patient can develop resistance to anti-HER2 treatments. Mimicking the development of the trastuzumab resistant cells, we will treat the sensitive cells with incrementally increasing doses of trastuzumab, pertuzumab, lapatinib, or TDM-1 over a 6-month period until they are resistant to high doses of the anti-HER2 treatment (Figure 39 top). Over the course of the treatment, we will take samples from the cells on a monthly basis. These cells will be lysed and reverse transcribed into cDNA for assessment of protein and RNA expression as the cells become resistant to their

prescribed treatment. Additionally, we will freeze down a number of cells for future assessment if need be. We can determine changes in Notch-1, PTEN, P-Akt, and P-ERK expression in the cells as they become resistant to the anti-HER2 treatment. Moreover, we can sort the BCSC population from the bulk cell population to determine changes in these subpopulations over prolonged exposure to treatment. Together these data will determine if Notch-1 upregulation is the preferred compensatory pathway in cells that acquire resistance to anti-HER2 therapies.

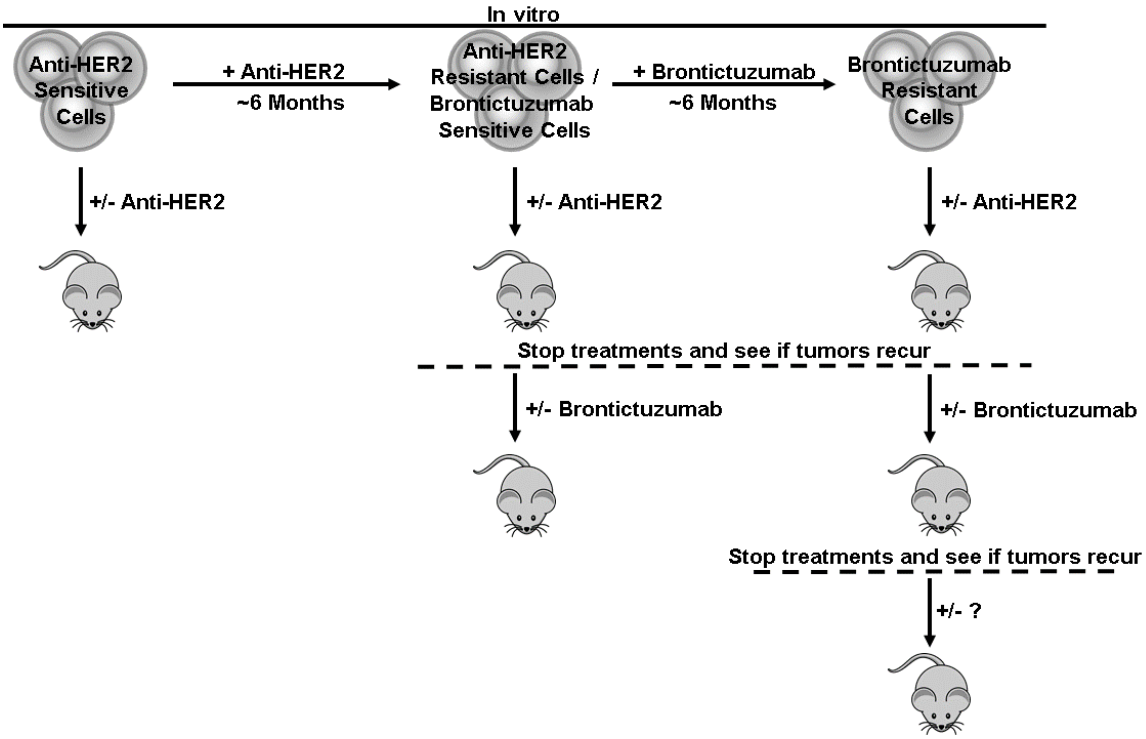

Figure 39: Experiment flow chart to determine efficacy of Anti-HER2 and Anti-Notch-1 targeted treatments

Flow chart for the development of acquired resistant cells to anti-HER2 and anti-Notch-1 (Brontictuzumab) targeted treatments *in vitro* (top). Sensitive and resistant cells will be injected into mice (bottom) to form tumor xenografts that are sensitive or resistant to their respective treatment. The tumors will be treated with the drugs they showed sensitivity/resistance to *in vitro* thereby demonstrating tumor sensitivity/resistance to the targeted treatment. Treatments can be stopped to assess recurrence and altered to determine efficacy of changing treatments to ablate tumor growth and/or formation.

Assessment of the cells as they become resistant may be better facilitated by microarrays to analyze changes in the whole genome, transcriptome, and proteome to give us an overall view of what is happening in the cell. Moreover, we can sort cells by high Notch-1 and low PTEN to determine when these changes start to occur as well as the effect of PTEN loss on the Akt or MAPK pathway.

Sequentially sampling cells as they become resistant to targeted treatment over time may allow us to pinpoint the time when cells acquire resistance.

If HER2+ cells can become resistant to targeted treatment of the HER2 receptor, then they can also acquire resistance to targeted treatment of the Notch-1 receptor. In accordance with this hypothesis, we would also like to look at the effect of persistent anti-Notch-1 (Brontictuzumab) treatment on trastuzumab resistant cells. If resistant cells become dependent on Notch-1 then resistant cells must become dependent on another receptor(s) or protein(s) when consistently treated with Brontictuzumab over time. Similar to the development of GSI resistance in T-ALL cells, we can assess our trastuzumab resistant cells for resistance to Brontictuzumab therapy *in vitro*. Additionally, the Brontictuzumab resistant cells may upregulate downstream Akt pathway activity to compensate for the loss of Notch-1 as previously seen in GSI resistant T-ALL cells. Comparable to the development and testing of trastuzumab resistant cell lines, we can also develop Brontictuzumab resistant cell lines from our trastuzumab resistant cells in an effort to predict how trastuzumab resistant HER2+ cancer may react to anti-Notch-1 therapy (Figure 39 top).

Once we complete evaluation of acquired drug resistance to targeted treatments in a cell model, we can attempt to recreate resistant tumors in an *in vivo* mouse model. We will implant sensitive and resistant cells into mice to form tumor xenografts. The sensitive and resistant tumor xenografts can be treated to stop or not stop tumor growth thereby showing sensitivity and resistance to the

prescribed treatment. The treatment of resistant tumors can be stopped and started again to see if the treatment effects tumor recurrence. This method of treating resistant tumors can be done for several different treatments in an effort to mimic the *in vitro* course of acquiring resistance to targeted treatments. For example, we could implant several different sensitive and resistant cells into mice that resemble how the cells become resistant *in vitro* such as trastuzumab sensitive and resistant cells as well as Brontictuzumab sensitive and resistant cells (Figure 39). We would begin by treating the tumors with their respective drugs to show sensitivity and resistance to the initial treatments. As such, we can treat the trastuzumab resistant tumors with Brontictuzumab and the Brontictuzumab resistant tumors with the treatment found to stop their proliferation *in vitro*. Together, these data will give us insight into how resistance is developed *in vitro* in an *in vivo* model that we hope will translate into patients.

Recreating acquired resistant cells will allow us to track the changes that occur in cells as they become resistant to targeted treatments. Changes in protein and gene expression can be used to create a panel of genes that are altered to predict the acquisition of resistance in a patient. This panel could begin with changes in protein expression commonly observed at the first treatment and end with protein expression at the last treatment when the cells have acquired resistance. It can present the changes in proteins/genes over time to indicate if resistance is, or is not, occurring. Tendencies towards resistance or treatment ineffectiveness can have alternative treatment options and similar panels can be

produced to help the clinician determine if an alternative treatment is having an effect or not. Creating a flow chart for how to tell if a treatment is working or if resistance is occurring may help both the patient and the doctor react to developing refractory disease.

Development of the trastuzumab resistant model has been a source of intrigue as it would be interesting to see how the cells adapt to trastuzumab treatment over time. The continued development of treatments that specifically target one receptor or one protein may be promoting resistance in cancer. Cancer cells seem to take advantage of redundancies in the signal transduction pathways enabling them to grow and survive in a multitude of different treatments. By targeting certain proteins in cancer cells we may be motivating the cells to become more adaptive to targeted treatments. Like insects that are exposed to a number of different pesticides, or bacteria that are exposed to a number of different antibiotics, we may be creating cancer cells that are resistant to a number of different therapies. By this hypothesis, we could attempt to track the changes in the sensitive cell as they acquire resistance to trastuzumab, lapatinib, pertuzumab, TDM-1, chemotherapy, or any other therapies used to treat HER2+ breast cancer in the clinic today.

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VITA

Andrew Baker was born in Rochester, New York to Thomas and Jana Baker. He currently resides in Forest Park, Illinois with his beloved wife Anastasia Mousouli whom he married on November 15, 2015.

Andrew began his graduate research in the lab of Dr. Seiser at Roosevelt University. He completed his Master's thesis on implementation of the BioBrick Program at Roosevelt University where he completed his Masters in Biotechnology. He went on to work at Valent Biosciences as well as Loyola University as a lab technician for the Campbell lab. Under Dr. Campbell he aided in the construction of the fluorescent tagged TRIM expression vector library.

In August of 2011, Andrew joined the Ph.D. program in the Cell Biology, Neurology, and Anatomy (CBNA) department of Loyola University, Chicago. Andrew joined the laboratory of Dr. Osipo where he studied the role of Notch-1 in trastuzumab resistant HER2+ breast cancer. He won the 2015 Senior Research Award for the Oncology Research Institute and Infectious Disease and Immunology Research Institute poster session at the Stritch School of medicine. He was awarded the Arthur J. Schmitt Dissertation Fellowship in 2016. He has had the opportunity to publish reviews, lecture classes, and aid in the completion of a number of papers. He has also acted as a research mentor for high school, undergraduate, graduate, and medical students.

Andrew Baker has accepted a post-doctoral position at Harvard University in the Lab of Dr. Rueda where he will continue to perform research on endometrial cancer. He is looking forward to applying the methods and techniques he has learned about drug resistance in breast cancer and expounding upon them in a different cancer type.