LOYOLA UNIVERSITY CHICAGO

INVESTIGATING NOVEL ROLES OF NOTCH-1 SIGNALING IN ESTROGEN RECEPTOR-α POSITIVE BREAST CANCER

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“The first principle is that you must not fool yourself—

And you are the easiest person to fool.”

Richard Feynman

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ABSTRACT

Breast cancer is a highly prevalent and life-threatening malignancy. Though therapies exist to treat breast cancer, advances in the field are necessary in order to overcome clinical hurdles such drug toxicity and cancer recurrence. Several signaling pathways are known to be dysregulated in breast cancer, and this thesis aims to describe novel crosstalk mechanisms between three distinct pathways: Estrogen Receptor-α (ERα), Notch, and Extracellular Regulated Kinase 1 and 2 (ERK1/2). High ERα expression is associated with 75% of all breast cancer cases. Anti-estrogen therapies are part of the standard of care for ERα positive breast cancer. We model anti-estrogen therapy by depriving cell cultures of estrogens in order to investigate the effect of these estrogens on Notch and ERK1/2 signaling. The Notch signaling pathway is commonly dysregulated in breast cancer. Drugs that target the Notch signaling pathway exist making it an attractive mode of investigation in breast cancer.

Classical Notch signaling is carried out by a series of proteolytic cleavages. Notably, the γ-secretase complex cleaves the membranous Notch receptor yielding a diffusible Notch intracellular domain (NICD). After cleavage, NICD translocates to the nucleus where it modulates the expression of specific target genes. Inhibitors of the γ-secretase complex can effectively attenuate this canonical Notch signaling paradigm. However, mounting evidence suggests Notch signaling functions that occur independently of this canonical pathway. Little is known about Notch signaling mechanisms that may cause Notch to behave independently of its ability to act as a transcription factor. Others have demonstrated crosstalk between the Notch, ERα and ERK1/2 signaling pathways. However, some of the observations made remain unexplained by the traditional Notch signaling paradigm. This work seeks to identify and

characterize signaling crosstalk mechanisms that occur independently of the ability of Notch to act as a transcription factor. In order to distinguish between canonical and non-canonical signaling, a scheme was devised to specifically inhibit either the canonical mechanism or the non-canonical mechanism. Cellular proliferation was used to compare the effects of γ-secretase inhibition (targeting the canonical pathway) to siRNA ablation of Notch1 (targeting both canonical and non-canonical pathways) in the presence or absence of estrogen. The results from these preliminary experiments as well as the conclusions drawn from previous work suggest that a non-canonical function of Notch1 is required for cellular proliferation in an ERK1/2 and ERα dependent manner. The hypothesis drawn to describe these findings is that: **the membrane-bound Notch1 receptor is required for growth and survival of MCF-7 cells in an estrogen dependent manner by activating the ERK1/2 signaling pathway.** Two aims will be employed in order to test these hypotheses.

Specific Aim 1: Determine whether the membrane-bound Notch1 receptor is necessary and sufficient for ERK1/2 activation. Our preliminary data suggest that in the presence of estrogen, non-canonical Notch1 signaling is required for proliferation of MCF-7 cells, but in the absence of estrogen, the canonical function of Notch1 is required for cell proliferation. Previous published work by Rizzo, et al. demonstrated that in the presence of estrogen, Notch1 is sequestered at the cell membrane, but in the absence of estrogen, Notch1 is cleaved and activated by the γ-secretase complex. Taken together, these findings suggest a signaling role for the membrane-bound Notch1 receptor. Work performed previously in our lab by Allison Rogowski shows that Notch1 is required for ERK1/2 activation. This aim seeks to bridge these correlations and demonstrate that the membrane-bound Notch1 receptor is required for the activation of ERK1/2.

Specific Aim 2: Determine whether the Notch1-dependent effects on MCF-7 cell proliferation and survival are due to ERK1/2 activation.

The data put forth suggesting a link between Notch1 and ERK1/2 and proliferation suggests a correlation between these signaling pathways and a biological output. If a constitutively active ERK1 or ERK2 can rescue the proliferation phenotype under Notch1 knockdown conditions, then this would provide strong evidence that a crosstalk mechanism between Notch1 and ERK1/2 controls proliferation in these cells.

CHAPTER 1

INTRODUCTION

BREAST CANCER

Breast cancer is one of the most frequently occurring cancer types. Women have a one in eight lifetime risk of being diagnosed with breast cancer[1]. Breast cancer is the second most prevalent type of cancer in women with over 300,000 cases, including both invasive and non-invasive cancer types, being diagnosed per year in the United States[1]. Breast cancer is the second leading cause of cancer-related mortality in women accounting for over 40,000 deaths per year in the United States[1].

Breast tumors usually arise from the tissues that make up the milk-producing mammary ducts and lobules[2]. Homeostasis of mammary tissue is a highly dynamic and tightly controlled process. Shifts in mammary homeostasis occur in response to various physiological events such as puberty, pregnancy, and menopause. During these phases, the cells comprising the mammary tissue undergo various degrees of cell proliferation, quiescence, and apoptosis in response to endocrine, paracrine, and autocrine factors[3]. For example, during pregnancy, prolonged levels of elevated progesterone and estrogen induce rapid cell proliferation and stabilization of the mammary tissue in order to support milk production[4]. This phenomenon requires signaling pathways, which coordinate processes such as mitosis, tissue patterning, and survival under high metabolic demands[3]. Though these processes are usually controlled with high fidelity, genetic errors such as point mutations and gene duplications sometimes occur[5]. The dynamic nature of mammary tissue makes this tissue type prone to genetic errors[5]. If enough of these errors accumulate over time, then a breast tumor may develop.

The breast is comprised of a wide variety of tissue types[3]. Adipose makes up the bulk of breast tissue, and the main function of breast adipose is to provide nourishment to mammary tissue during milk production. Additionally, adipose tissue secretes proteins that make up the connective tissue that makes up the breast stroma. Furthermore, breast adipose tissue is a source of some types of estrogens, which are proposed to be important for estrogen receptor positive breast cancer in post-menopausal women[6].

Perhaps the most important tissue type in the breast with regards to breast cancer is the epithelial tissue that makes up the mammary duct and gland system. The mammary tissue is a highly branched system of tubes that originates at the nipple and projects into the breast adipose and stroma[3]. The tubes that make up the mammary duct are composed of two different cell types: basal epithelial (also known as myoepithelial) and luminal epithelial[3]. The basal epithelial cells line the outside of the duct. Basal epithelial cells secrete extracellular matrix components conferring mechanical stability to the duct[7]. Additionally, the basal epithelium serves a contractile function, which is important for the excretion of milk. The luminal epithelium lines the interior of the mammary duct and is responsible for the production of milk. The luminal epithelial cells express high levels of the hormone receptor estrogen receptor α (ERα) and progesterone receptor (PR).

ERα and PR are important molecular markers in breast cancer. Tumors deemed ER+ comprise up to 84% of all breast cancers. ERα function is typically required for survival of ER+ breast cancer cells, and drugs designed to inhibit ERα function are the clinical standard of care for this subtype. ER+ breast cancer can be further subdivided based on the overexpression of the epidermal growth factor receptor, HER2. Breast cancers that are ER+/HER2+ comprise about 10% of all breast cancer cases. Though less common, some breast cancers can overexpress

HER2 but be negative for ERα or PR. The HER2 only subtype makes up about 4% of all breast cancer cases. Finally, a particularly aggressive form of breast cancer exists which lacks all three of these molecular markers. As such, this type of breast cancer is termed triple negative and makes up about 15% of all breast cancers. Of note, PR may be absent or present in any of these subtypes except for the triple negative breast cancer. Drugs targeting PR do not usually yield an advantage over targeting of ERα or HER2, but the presence of PR can help to predict how a patient will respond to other targeted therapies. For instance, patients who are ER+/PR+ tend to have a more favorable prognosis compared to ER+/PR- patients (purdie and quinlan). The molecular features of these four different types of breast cancer (ER+/HER2-, ER+/HER2+, ER-/HER2+, Triple negative) correspond with histological descriptions that are also commonly used to characterize breast cancer (summarized in Table1-1).

Histological Classification	Molecular Classification	Prevalence[1]	Targeted Therapies
Luminal A	ER+, PR+/-, HER2-	74%	Hormone Therapy
Luminal B	ER+, PR+/-, HER2+	10%	Hormone Therapy and HER2 Targeted Therapy
HER2 Type	ER-, PR-, HER2+	4%	HER2 Targeted Therapy
Basal	ER-,PR-,HER2- (Triple Negative)	12%	No Standard Targeted Therapy

Table 1-1. Breast Cancer Subtypes. The four most common types of breast cancer can be subdivided based on histological and molecular characteristics. Hormone therapy such as Tamoxifen and Letrozole are commonly administered for ER+ breast cancers. Drugs such as Trastuzumab have been developed to specifically target cancer cells aberrantly overexpressing the HER2 receptor. No targeted therapy currently exists as a standard of care for patients with triple negative breast cancer.

ESTROGEN SIGNALING

A hormone is defined as a molecule, which is produced in a specialized organ or gland and is secreted into the blood allowing it to elicit physiological effects on distant organs. Hormones affect a vast array of physiological processes such as growth and development, appetite, sleep/wake cycles, mood, and heart rate just to name a few. Most hormones are made up of either protein or lipid biomolecules. Estrogen itself is a steroidal hormone so we turn our attention to the synthesis pathway that produces estrogen.

Like all steroidal hormones, the estrogen molecule is derived via a series of enzymatic reactions, which uses cholesterol as its initial substrate. A number of different but similar molecules are produced which collectively are called estrogen. However, the most prevalent and potent of the estrogens is 17β estradiol. Estrogen has several physiological impacts on the female body mainly tied to the ability to reproduce and support offspring. Estrogen synthesis, as it pertains to the menstrual cycle and maintenance of pregnancy, occurs primarily in the ovaries. However, other organs such as the liver, adrenal cortex, and adipose tissue can produce estrogens as well. Ovarian estrogen production is first observed at menarche. Two main tissue types exist that possess the enzymes necessary to produce estrogen within the ovaries:

One of the most prevalent molecular markers of invasive breast cancer is the nuclear hormone receptor, Estrogen Receptor α (ERα). Elevated ERα expression is observed in as many as 75% of all invasive breast cancer cases. ERα is an intracellular protein, which belongs to a broad class of proteins known as the nuclear receptors. There are four functionally distinct types of nuclear receptors. However, all nuclear receptors are known to bind to DNA and regulate transcription subsequent to ligand binding. ERα belongs to the TypeI family of nuclear receptors. In the absence of an activating ligand, ERα adopts a conformation, which remains bound to Heat

Shock Protein 90 and is sequestered in the cytoplasm. Upon binding to its ligand, estrogen, ERα undergoes a conformational change allowing it to dissociate from HSP90. HSP90 dissociation leads to homodimerization of the ERα receptors whereupon it translocates to the nucleus. Once localized to the nucleus, ERα binds to a specific DNA consensus sequence known as an Estrogen Response Element (ERE).

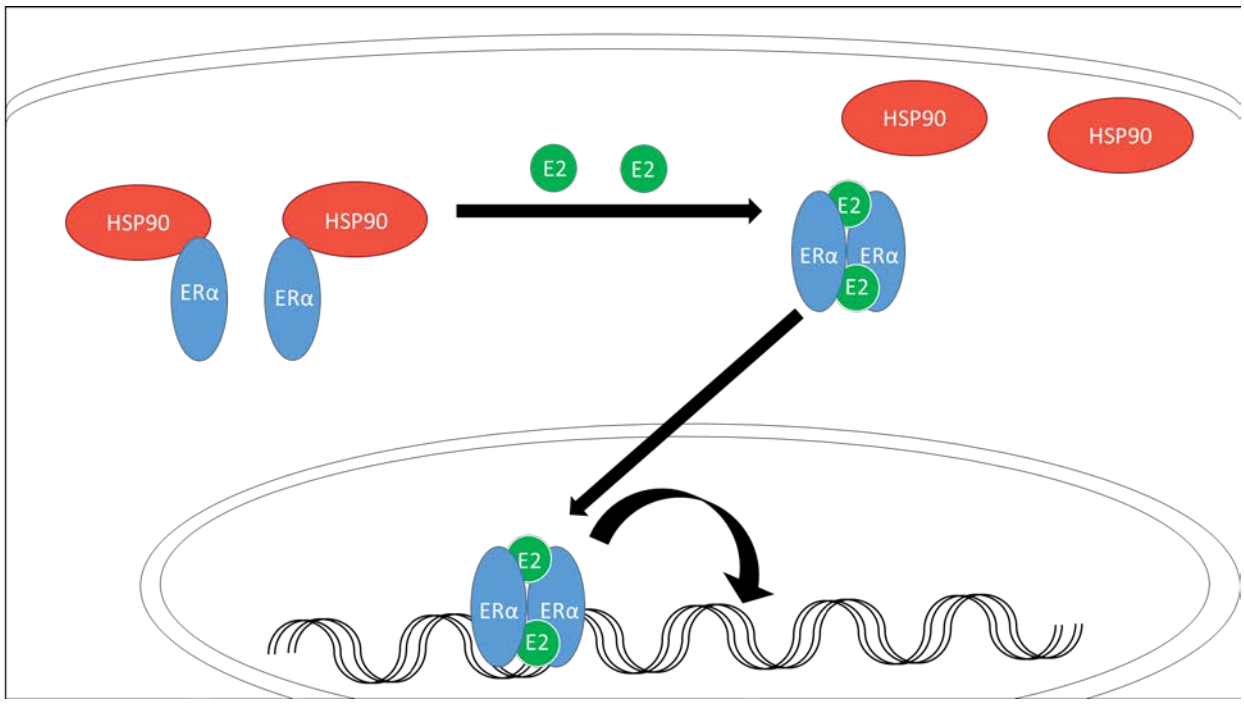


Figure 1-1. Classical Estrogen Signaling. ERα is a nuclear hormone receptor whose activity is largely determined by its subcellular localization. In the absence of an activating hormone such as 17-β Estradiol, ERα is sequestered in the cytoplasm via interaction with HSP90. Whenever estrogen binds to ERα, it undergoes a conformational shift allowing it to dissociate from HSP90 and dimerize with a second ERα protein. The ERα dimer translocates to the nucleus and binds to specific DNA sequences called estrogen response elements located within promoter and/or enhancer regions of target genes. ERα activity can be modulated by several factors including posttranslational modifications on ERα itself or the presence of other transcriptional modulators proximal to the ERE. Depending on the other modifications in place, ERα may be either a transcriptional activator or repressor.

NOTCH SINGALING

Notch signaling is a highly conserved signaling pathway that is crucial for proper development[8]. Notch has a role in multiple developmental processes such as embryogenesis[9], tissue patterning[10], organogenesis[11], angiogenesis[12], wound healing[13], cell differentiation[14], and stem cell renewal[15]. Notch signaling can be briefly summarized beginning with post-translational Golgi processing[16,17]. Notch is glycosylated and cleaved in the Golgi apparatus and exists on the cell membrane as a heterodimer of the two cleavage products. Notch is recognized by one of its ligands, which is expressed on an opposing cell[18]. Ligand binding triggers cleavage by an ADAM/TACE family proteinase[19]. This newly truncated Notch is recognized and cleaved further by the γ-secretase complex[20]. Cleavage by γ-secretase releases the Notch intracellular domain allowing it to translocate to the nucleus where it acts as a transcriptional activator of numerous target genes[20].

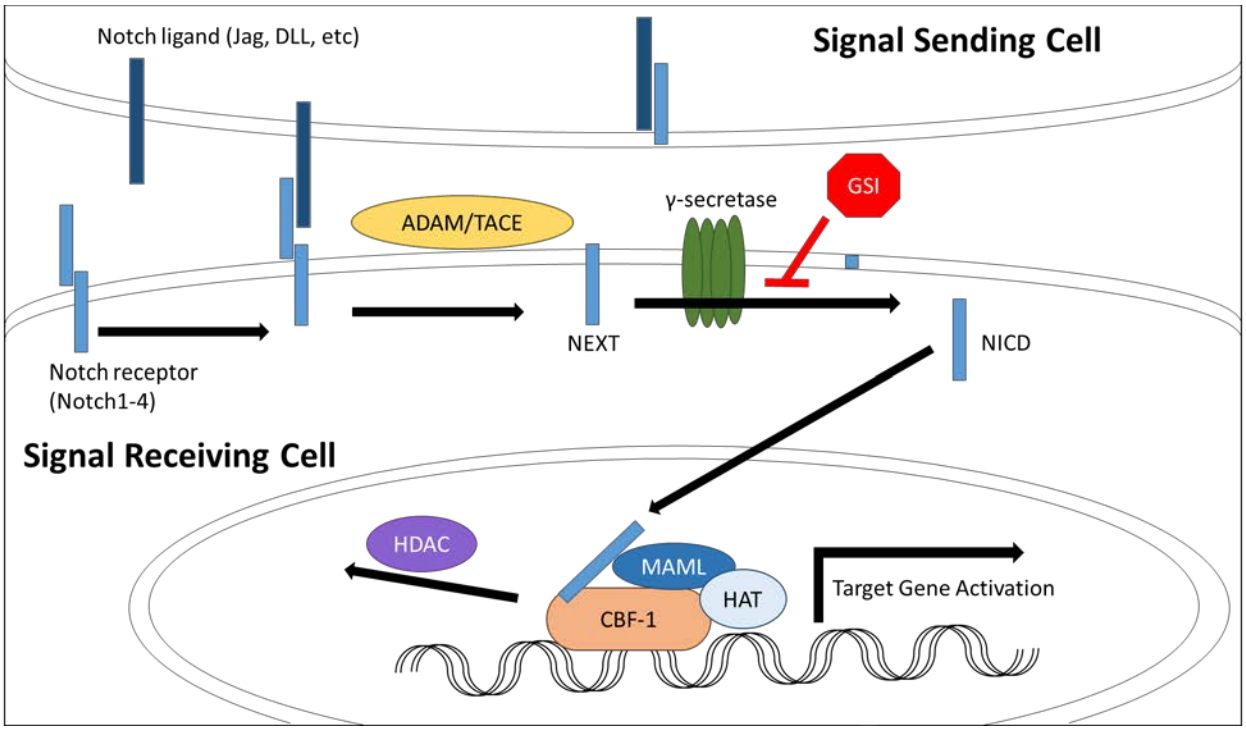


Figure 1-2. Canonical Notch Signaling. The Notch signaling pathway is activated when a membrane receptor of the Jagged/DLL family binds to one of the four paralogous Notch receptors. Ligand binding triggers an internalization of the ligand into the signal sending cell. This puts mechanical stress on the Notch receptor revealing a cleavage site that is recognized by members of the ADAM/TACE family metalloproteinases. Cleavage by ADAM/TACE leaves a membrane-bound Notch lacking its extracellular domain. This so-called NEXT domain is a substrate for proteolytic cleavage by the γ-secretase complex. Once cleaved by the γ-secretase complex, Notch intracellular domain (NICD) translocates to the nucleus where it interacts with transcriptional co-activators MAML and histone acetyltransferases such as p300. By displacing transcriptional repressors and recruiting transcriptional activators, NICD activates the transcription of putative target genes such as HES1 and HEY1. A common mode of inhibiting this pathway is to administer a γ-secretase inhibitor which decreases the amount of NICD produced.

The human genome contains four NOTCH genes (NOTCH1, NOTCH2, NOTCH3, and NOTCH4), and each is encoded on a different chromosome[21]. After translation of a NOTCH gene, the nascent protein is transported from the endoplasmic reticulum (ER) to the trans-Golgi where it undergoes two critical post-translational modifications: glycosylation and proteolytic cleavage[16,22]. One feature common to all of the Notch paralogs is cleavage of the nascent Notch protein into a dimer by a Furin convertase proteinase[16]. Furin cleavage, termed S1 cleavage, is necessary for activation of the Notch receptor[23]. The two cleavage products which include the extracellular portion and the transmembrane portion are held in a heterodimeric form by non-covalent interactions which require an interposed Ca2+ cation[17]. All four Notch proteins have an extracellular domain containing anywhere from 29 to 36 EGF-like domains[24]. Glycosylation of the EGF repeats located on both the Notch ligands and receptors is necessary for their mutual interaction[24]. The extracellular domain of Notch features three repeating Lin12 motifs which protect the Notch receptor from premature cleavage[25]. The interface between the two fucose-generated cleavage products is present on the extracellular region of the protein[23]. After processing in the Golgi, Notch is transported via vesicle trafficking to the plasma membrane where it awaits ligand interaction[16].

Glycosylation of Notch is necessary for proper signal transduction. Notch glycosylation is carried out in two sequential steps by two different proteins[22]. O-Fucosyltransferase 1 (OFUT1) carries out the first step in the ER[26]. The substrate for this enzyme is a conserved C-X-X-X-X-S/T-C motif located within the EGF domains[22]. OFUT1 catalyzes the formation of an O-glycosidic linkage between the conserved serine or threonine residue and O-fucose using GDP-fucose as a saccharide donor[22]. Therefore, OFUT1 primes the EGF repeats for further polyglycosylation to be carried out in the Golgi by the Fringe family of glycosyltransferases[22].

The Fringe family is made up of three enzymes: Lunatic Fringe (LFNG), Manic Fringe (MFNG) and Radical Fringe (RFNG)[27]. Fringe elongates the O-fucosyl serine/threonine via β-1,3-N-acetylglucosaminyltransferase activity[28]. LFNG activity increases the affinity of Delta-Like Notch ligands for Notch receptors at the expense of Jagged Notch ligands[27]. Recent reports have shown that LFNG is a tumor suppressor in the mammary gland[29].

The Notch receptors can be recognized by one of five members of the Delta/Serrate/LAG (DSL) family transmembrane glycoproteins termed Delta-Like1, Delta-Like3, Delta-Like4, Jagged1, and Jagged2[18]. Binding of the ligand to receptor is followed by endocytosis of the extracellular portion of receptor into the signal-sending cell[30]. The pulling force on the Notch receptor generated by endocytosis enables the LAG-12 domain of the Notch receptor to unfold[30]. This exposes the S2 cleavage site making it vulnerable to proteolysis by either ADAM-10 or ADAM-17, member of the A disintegrin and metalloproteinase (ADAM) family proteinases[19]. The S2 cleavage yields two fragments: the N-terminal fragment (NECD) and the C-terminal fragment (NEXT)[19]. The N-terminal fragment is internalized into the signal sending cell bound to the ligand[18]. The C-terminal fragment, NEXT domain, awaits S3/4 cleavage by the γ-secretase complex[31].

For many proteins, the transmembrane domain is viewed as an inert linker between extracellular and intracellular domains. However, this is not the case for substrates of the γ-secretase complex, including Notch, for which the transmembrane domain serves as a substrate for a third proteolytic cleavage[31]. The γ-secretase is a multi-pass transmembrane complex composed of four subunits: presenilin, nicastrin, APH1, and PEN2[32]. Presenilin, the catalytic subunit of the complex, is an aspartyl proteinase which has a vast number of transmembrane protein substrates[33]. Though the other members of the γ-secretase complex lack an enzyme active

site, they are critical for enzyme activity and substrate specificity[34]. Nicastrin is able to specifically interact with the N-terminal cleavage product formed after S2 cleavage[35]. Nicastrin binding brings the NEXT domain into position within the γ-secretase complex allowing presenilin to perform cleavage at S3 in the NEXT domain[35]. Nicastrin has been suggested as a therapeutic target due to the ability of an anti-Nicastrin antibody, A5226A, to have anti-tumor effects on lung adenocarcinoma xenografts.

Cleavage caused by γ-secretase releases the Notch intracellular domain (NICD) into the cytoplasm enabling NICD to translocate to the nucleus and carry out its function as a transcriptional activator. Of all the possible nodes that could be targeted along the Notch pathway, the majority of anti-Notch drugs aim to inhibit the release of NICD from the membrane. Most attention regarding targeting of Notch focuses on γ-secretase inhibitors. However, other attractive targets such as the ADAM proteinases as well as the receptors and ligands themselves have been investigated for targeted anti-Notch therapy[36].

Up to this point in the cascade, all four of the Notch paralogs are strikingly similar. However, the cytoplasmic portions of the four Notch intracellular domains (NICDs) differ in terms of their structure and function. These different structures confer diversity and complexity to the Notch signaling pathway. All of the Notch paralogs harbor three nuclear localization signals, which facilitate translocation to the nucleus after S3 cleavage[37]. Once in the nucleus, Notch facilitates the formation of an active transcription complex, which is centered about the CSL (CBF-1, Suppressor of Hairless, Lag-2 after its mammalian, Drosophila, and Caenorhabditis elegans orthologues respectively) DNA-binding protein[20,37]. In the absence of nuclear NICD, CSL inhibits transcription by recruiting transcriptional repressors such as NCoR or SMRT. Upon NICD recruitment to CSL, NICD displaces the transcriptional repressors and

recruits transcriptional co-activators such as the p300/CAF histone acetyltransferase. The NICD/CSL interaction is stabilized by another protein called Mastermind Like-1 (MAML-1), a factor that is necessary for recruitment of the co-activators. The domains required for these protein-protein interactions have been described in detail by Bray et al.[38]. The N-terminus of NICD harbors the RAM domain, which is the site responsible for the NICD/CSL interaction[37]. MAML binds to Notch at the ankyrin repeat domain, which is made up of seven highly conserved ankyrin motifs[39]. Targeting transcription factors or activators has been challenging for drug discovery. The structural identification of the Notch1/MAML1 interface has allowed for development of novel MAML1-specific inhibitors[39-41].

Notch facilitates conversion of the CSL transcriptional repressor to a transcriptional activator and causes a complex cascade of events including transcription of the primary Notch target genes HES (Hairy Enhancer of Split) and Hey (Hairy/Enhancer-Of-Split Related With YRPW Motif Protein)[42]. In turn, Notch signaling regulates a number of genes. The most notable of these genes include p21, c-Myc, cyclinD1, p27cip1, Slug, and NF-kB[43]. Some of the Notch target genes are implicated in targeted combination therapy of several different types of cancer including breast cancer[44,45].

Notch signaling performs in a synergistic fashion with a number of different oncogenes. Notch1 is required for the transforming activity of H-RAS[46]. and TGF-α during pancreatic tumorigenesis[47]. The formation of adenocarcinomas and their metastases in transgenic mouse models demonstrates the synergistic effects between Notch and MYC[48]. Notch 1 is involved in crosstalk with PI3K-AKT as demonstrated by Guo et al.[49]. It has been shown that malignant transformation by Notch requires signals from the Erk/MAP kinase and PI-3 kinase pathways downstream of Ras[50]. Cross-talk between Notch and the estrogen receptor in breast cancer also

has been extensively investigated as it was shown that estrogen inhibits Notch signaling while estrogen deprivation reactivates the Notch pathway[51-53]. Another important crosstalk between Notch and HER2 receptors has been shown using a breast cancer model[52]. HER2 overexpression inhibits Notch signaling while repression of HER2 transcription or HER2 pharmacological inhibition causes increased Notch signaling[54]. Also of significance is the correlation between Notch1, Notch4, and PEA3[55]. PEA3 is a transcription factor whose expression has been connected with tumorigenesis possibly through activation of Notch1 and Notch4[56-58].

An important function of Notch signaling is its ability to stimulate stem cell survival, differentiation, and self-renewal[59]. The ability of Notch to promote cancer stem cell maintenance, EMT regulation, and drug resistance can occur through regulated expression of Hes1, a canonical Notch target gene[60].

THERAPIES TARGETING NOTCH IN BREAST CANCER

The γ-secretase inhibitors (GSIs) are the most extensively studied modulators of Notch signaling. Excellent reviews exist that discuss the pharmacology and medicinal chemistry of the molecules designed for γ-secretase inhibition [61,62]. Furthermore, advances in the structural biology of the γ-secretase components provide a fresh perspective of these inhibitors. In 2013, an excellent article detailing the structure of an archaeal homolog of presenilin known as presenilin/SPP homolog (PSH) was published [63]. PSH bears a high degree of sequence homology to the mammalian presenilin proteins [63]. At the time of this publication, resolution of the PSH structure provided the best picture of how an aspartic proteinase could cleave a peptide bond buried within the cell membrane. Similar to presenilin, PSH has nine transmembrane alpha helices [63]. The two required catalytic aspartate residues are located on separate helices and come

together to form an acidic pocket [63]. The enzymatic mechanism of aspartic proteinases requires a water molecule. However, how the water molecule reaches the catalytic site was previously subject to speculation. By solving this complex structure, the authors demonstrate the formation of a hydrophilic pore that can shield the necessary water molecule as it diffuses from the cytoplasm to the active site [63].

Resolution of the PSH crystal structure was an important first step in advancing our knowledge of the γ-secretase complex. However, PSH does not operate in the context of a multi-subunit complex as γ-secretase [63]. In 2014 and 2015, shortly after the publication of the PSH structure, two articles were published by members of the same group detailing a structure for the complete γ-secretase heterotetrameric complex [32,64]. The proposed γ-secretase structure demonstrates the juxtaposition of the two aspartate residues as well as the hydrophilic pore discussed for the PSH structure [32,64]. The co-advancement of structural biology computer-aided modeling has deep implications regarding drug discovery. Elucidation of the γ-secretase complex allows for a more thorough understanding of drug/protein interactions of compounds that already exist. More importantly, solving the γ-secretase structure takes the predictive power of drug design to a higher level. The elucidation of the γ-secretase structure may provide an important missing link for *in silico* modeling.

γ-secretase is a promiscuous enzyme complex, and GSIs are not typically developed strictly with the purpose of targeting the Notch pathway [33,62]. Activity of the γ-secretase complex is strongly implicated in the etiology and pathology of Alzheimer's disease [65]. Alzheimer's is characterized by an accumulation of the amyloid-β peptide in the affected areas of the brain [65]. Amyloid-β accumulates as a result of γ-secretase dependent cleavage of the amyloid precursor protein (APP) [65]. As a result of the efforts of those seeking to inhibit the cleavage of APP, the

majority of our knowledge pertaining to GSIs comes from the study of Alzheimer's disease [62]. As such, many of the GSIs were originally designed with the intent of inhibiting γ-secretase-mediated cleavage of APP [66]. One of the greatest clinical hurdles associated with GSI therapy is overcoming aberrant on-target effects of GSIs [67]. That is, GSIs specifically inhibit the target enzyme, but since the target enzyme has many substrates, unintended pathways are often affected. The most conspicuous side effect of GSI treatment is severe diarrhea, an effect attributed to the role of Notch in the intestinal epithelium [68]. Whereas Alzheimer's researchers are interested in developing GSIs that leave Notch signaling intact, cancer researchers seek a drug that would target the Notch pathway but only in cancer cells. Numerous clinical trials have been conducted in patients with a wide variety of cancer diagnoses. At present, the only option for overcoming intestinal toxicity when using a GSI is to control dosing schedules [69].

The drugs used in clinical trials to target the γ-secretase complex mainly fall under three classes: protein isosteres, azepines, and sulfonamides [33]. These drugs can be further subdivided into transition state analogs and non-transition state analogs, which may act as competitive or allosteric inhibitors, respectively. All of the protein isostere GSIs can be classified as transition state analogs. The prevailing strategy in creating an inert peptide mimetic is to remove the amide bond that is typically cleaved by presenilin and replace it with a non-hydrolyzable group such as hydroxyeltylene [70]. The γ-secretase complex prefers to cleave the peptide bond between two amino acids with small and/or aliphatic side chains such as valine, leucine, isoleucine, and glycine [20]. When designing protein isosteres as transition state analogs, it seems logical to mimic the natural substrate as closely as possible. Therefore, scientists studying the inhibition of APP targeted cleavage originally created isosteres of valine/isoleucine and adenine/threonine [70]. However, these peptide mimetics exhibited no GSI activity [70]. In contrast, dipeptide isosteres

mimicking phenylalanine/phenylalanine were shown to have significant potency in inhibiting γ-secretase [70].

The limited clinical application of peptide isostere GSIs has led to the search for drugs with improved pharmacological profiles. Modifications to the seven-member azepine ring system yield GSIs with more desirable qualities. Azepine derivatives may be either transition state analogs or non-transition state analogs as defined by their ability or inability to displace known competitive inhibitors such as protein isosteres.

Recent attention has turned to specific Notch-targeted therapies that could provide fewer on-target side effects. Techniques for developing and employing humanized monoclonal antibodies are constantly improving. Antibodies allow targeting of virtually any unique epitope located outside of the cell. In fact, antibodies can be so specific as to distinguish between each of the four different Notch paralogs [71]. The ability to distinguish between the different Notch receptors has important implications in circumventing intestinal toxicity [71]. This implication is based on the finding that defects in intestinal cell differentiation only occur if both Notch1 and Notch2 are simultaneously inhibited [71,72]. In the intestine, Notch1 and Notch2 have redundant functions, but in tumors, the interdependency of Notch1 and Notch2 is not as straightforward. For example in colorectal cancer, Notch1 is an oncogene, but some have suggested that Notch2 has a tumor suppressor role [72,73]. Thus, antibody developers hypothesize that inhibition of either Notch1 or Notch2 can give the desired therapeutic effect while avoiding the side effects seen with pan-Notch inhibitors [74].

Aveo Pharmaceuticals has developed a humanized monoclonal antibody called 23814, which targets the ligand-binding domain of Notch1 [74]. Anti-tumor activity is observed in preclinical studies with 23814 in mice, and intestinal toxicity was not detected [74]. A phase1a trial

for an antibody against the Notch ligand DLL4, Enoticumab from Regeneron Pharmaceuticals, was concluded in 2014 [75]. Enoticumab was tolerated well, and of the 53 patients enrolled, two exhibited partial tumor response, and 16 showed disease stability [75]. OncoMed Pharmaceuticals currently have four monoclonal antibodies in their pipeline that target multiple components of the Notch signaling pathway. Two of their antibodies target the Notch ligand DLL4. The anti-DLL4 antibody, Demcizumab, is labeled as an anti-cancer stem cell drug [76]. Demcizumab is currently undergoing phase1b (active) and phase 2 (recruiting) clinical trials as a combination therapy for treating non-small cell lung, pancreatic, and ovarian cancers [77-81]. Results from the phase1b trial demonstrate that 89% of pancreatic cancer patients exhibited clinical benefit (50% partial response and 39% disease stability) from the Demzizumab+gemcitabine +Abraxane phase1b trial [76]. These patients exhibited improved survival metrics over those who receive gemcitabine+Abraxane alone [76]. Due to the promising pre-clinical investigation, patients with solid tumors are being recruited for a phase1a trial of the other anti-DLL4 antibody OMP-305B83 [82]. Tarextumab has similar anti-tumor effects but targets the receptors, Notch2 and Notch3, instead of the DLL4 ligand [83]. Results from the phase 1b clinical trial of Tarextumab demonstrate tolerability as well as desirable anti-tumor effects [83]. The fourth antibody from OncoMed, OMP-52M51, targets Notch1 and is currently recruiting for phase1a trials in solid and lymphoid malignancies [84,85]

MITOGEN ACTIVATED PROTEIN KINASE SIGNALING

Members of the Mitogen Activated Protein (MAP) kinase signaling pathway are among the most frequently mutated genes in cancer. The MAP kinases lie downstream of a wide variety of effectors including receptor tyrosine kinases[86], G-protein coupled receptors[87], receptor serine/threonine kinases[88], Src family kinases[89], and cytokine receptors[90] to name a few.

Activation of these effectors is typically induced by a ligand-binding event which initiates a cascade of enzymatic reactions. For instance, binding of Epidermal Growth Factor (EGF) to one of its cognate receptors causes the receptor to dimerize with another identical or closely related EGF receptor. Upon dimerization, the intracellular tyrosine kinase domains of these receptors phosphorylate each other[91]. This creates phosphotyrosine sites which can be recognized by the SH2 domain of the scaffolding protein GRB2. Next, GRB2 recruits the guanine nucleotide exchange factor called Son of Sevenless (SoS) via SH3 domain interaction, which effectively localizes SoS to the cell membrane[92]. Membrane localization brings SoS into proximity of its target, Ras. The Ras family members are GTPases which are inactive when bound to GDP and active when bound to GTP[92]. SoS activates Ras by exchanging its bound GDP for a molecule of GTP. The GTP-bound Ras adopts an active conformation which can in turn bind to and induce conformational activity of its target, the MAP kinase kinase kinase Raf. Raf phosphorylation initiates a cascade of phosphorylation events wherein Raf activates the MAP kinase kinase MEK, and MEK activates the MAP kinases ERK1 and ERK2[93]. ERK1/2 in turn phosphorylates a number of substrates that generally act to promote cell survival and proliferation. One well-described substrate of ERK1/2 is c-fos[94]. C-fos is a member of the AP-1 transcription factor. ERK1/2 phosphorylates c-fos thereby stabilizing it and encouraging dimerization with other AP-1 family members[94]. The activated AP-1 dimer binds to a consensus DNA sequence proximal to genes which generally favor survival and proliferation phenotypes. In a classic example of AP-1 mediated control of proliferation, AP-1 activates the transcription of the CyclinD1, which in turn activates CyclinD1 promotes cell cycle progression[94].

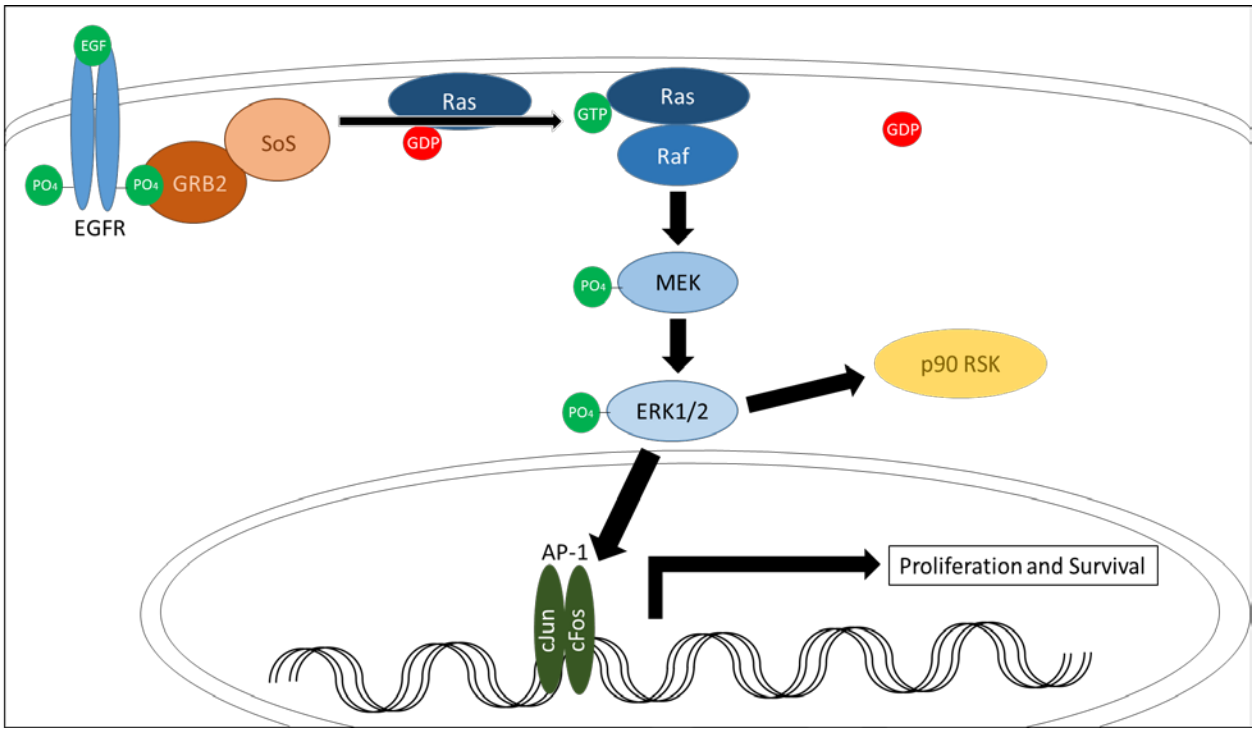


Figure 1-3. Classical MAP Kinase Activation. The MAP kinase cascade lies downstream of multiple activating receptors including TGF-β receptors, G-protein coupled receptors, etc. One of the most thoroughly described modes of activation of the MAP kinase pathway is via receptor tyrosine kinases such as the EGF Receptor depicted here. When the EGF ligand binds to its cognate receptor, EGFR, the receptors dimerize. The proximity induced by ligand binding allows the intrinsic tyrosine kinase activity of the receptors to cross-phosphorylate. The phosphorylated EGFR is recognized and bound by the SH2 domain of the GRB2 scaffolding protein. GRB2 then binds to the guanosine nucleotide exchange factor, Son of Sevenless via SH3 domain interaction. Allocation of SoS in proximity to the cell membrane allows it to exchange the bound GDP on Ras with an activating GTP molecule. The GTP-bound Ras undergoes a conformational change which binds to and activates Raf. Raf, in turn, phosphorylates MEK which then phosphorylates ERK1/2. ERK1/2 phosphorylates a number of substrates such as the AP-1 member, cFos and the Ribosome S6 Kinase, p90RSK. ERK1/2 activity typically correlates with pro-survival and proliferation phenotypes.

The previous paragraph describes canonical signaling through the ERK1/2 pathway. However, variations in the MAP kinase signaling cascade exist. Generally speaking, the MAP kinase cascades fall within four main columns: p38, Jun Kinase (JNK), ERK/big MAP kinase1 (BMK1), and ERK1/2[95]. The pathways are named for the terminal MAP kinase in their respective cascades, but they are further distinguishable by the upstream kinases that are characteristically activated within each cascade. For instance, whereas the Ras→Raf→ERK1/2 cascade is the accepted mode of ERK1/2 activation, the typical mode of JNK activation is TAK1→MKK4→JNK[95]. Though these putative pathways have been described, crosstalk between the cascades exist. For example, if TAK1 activates MKK3/6 instead of MKK4, then p38 MAP kinase will be the preferred terminal kinase. Activation of members of the AP-1 transcription factors is one of the most salient features of MAP kinase signaling[94]. However, numerous other substrates of the MAP kinases have been documented and described. Other substrates of ERK1/2 that are inherent to this thesis include Estrogen Receptor α and p90RSK[96]

CHAPTER 2

PRELIMINARY DATA, HYPOTHESIS, AND SPECIFIC AIMS

PRELIMINARY DATA

Expression of Notch1 in breast cancer correlates with poor prognosis due to its oncogenic potential. The Notch signaling pathway is known to promote proliferation, migration, invasion, epithelial-mesenchymal transition, and self-renewal[97,98]. Canonical Notch signaling is a highly conserved process whereby ligand/receptor interaction leads to a series of proteolytic cleavages resulting in the release of a cytoplasmic Notch domain[99]. This Notch intracellular domain (NICD) subsequently translocates to the nucleus where it binds to a transcriptional repressor known as CBF-1. Once bound to CBF-1, NICD facilitates the release of corepressors and recruits coactivators such as MAML1 and p300 to CBF-1 response elements on target genes[100]. The aims presented will seek to further investigate the diverse signaling functions of Notch in breast cancer. Our preliminary work as well as recent publications points to signaling functions of Notch that is independent of this canonical paradigm[101].

The proposed aims are influenced by the work of Rizzo et al.[52]. The authors used several breast cancer cell lines to demonstrate that in spite of increased Notch1 expression, Notch transcriptional activity decreases in the presence of estrogen[52]. Anti-estrogen drugs or estrogen depletion reverse these effects[52]. However, the mechanism whereby Notch1 promotes proliferation in the presence of estrogen remains elusive and is left open to further investigation. The aims put forth in this thesis seek to provide evidence in support of a novel mechanism to describe the relationship between Notch1 and estrogen signaling. Our preliminary work suggests that the estrogen-dependent Notch1 signaling effects may be due to non-canonical functions that

are independent of the ability of Notch1 to be cleaved and subsequently to act as a transcription factor.

In order to garner preliminary evidence for non-canonical Notch signaling, an approach to delineate canonical versus non-canonical signaling is needed. The canonical Notch pathway is dependent upon intracellular Notch release by the γ-secretase complex. Small molecule inhibition of the γ-secretase complex prevents Notch cleavage, release, and subsequent transcriptional activation of putative target genes[102]. However, preventing the release of intracellular Notch would not necessarily attenuate a non-canonical function because Notch may still be able to participate in signaling from its membrane-bound state. Therefore, in order to garner support for a non-canonical role for membrane-bound Notch, a siRNA knockdown approach was employed to attenuate all possible signaling functions of Notch1. Cellular proliferation was used to compare the effects of γ-secretase inhibition versus siRNA ablation of Notch1. This work was performed with the ERα positive breast cancer cell line MCF-7. Rizzo, et al. showed that in MCF-7 cells, Notch1 is highly expressed at the cell membrane under estrogen positive growth conditions compared to estrogen-deprived conditions[52].

Our goal is to elucidate the mechanistic underpinnings between estrogen signaling and Notch signaling. The model presented in Figure 2-1 is designed to depict the findings put forth by Rizzo et al. From this model, we draw the predictions for how estrogen and Notch1 may interact to affect MCF-7 cell proliferation. In summary, if estrogen is present, no effect on cell proliferation is expected because estrogen is thought to promote cell growth while simultaneously inhibiting Notch1 signaling. One major criticism that exists for using a GSI in order to investigate Notch signaling is the breadth of targets that exist for these drugs. Besides the four distinct Notch paralogs, the γ-secretase complex is known to have multiple membrane

substrates such as APP and Receptor Tyrosine Kinases. Using either a GSI or a Notch1 knockdown and combining the two treatments together can delineate the Notch1-specific effects of the GSI. The results obtained from these experiments are surprising. As expected, the GSI does not affect cell proliferation. However, knocking down Notch1 yields a significant decrease in cell number eight days after plating. This suggests that Notch1 is required for proliferation in a manner that occurs independently of its cleavage-mediated mechanism.

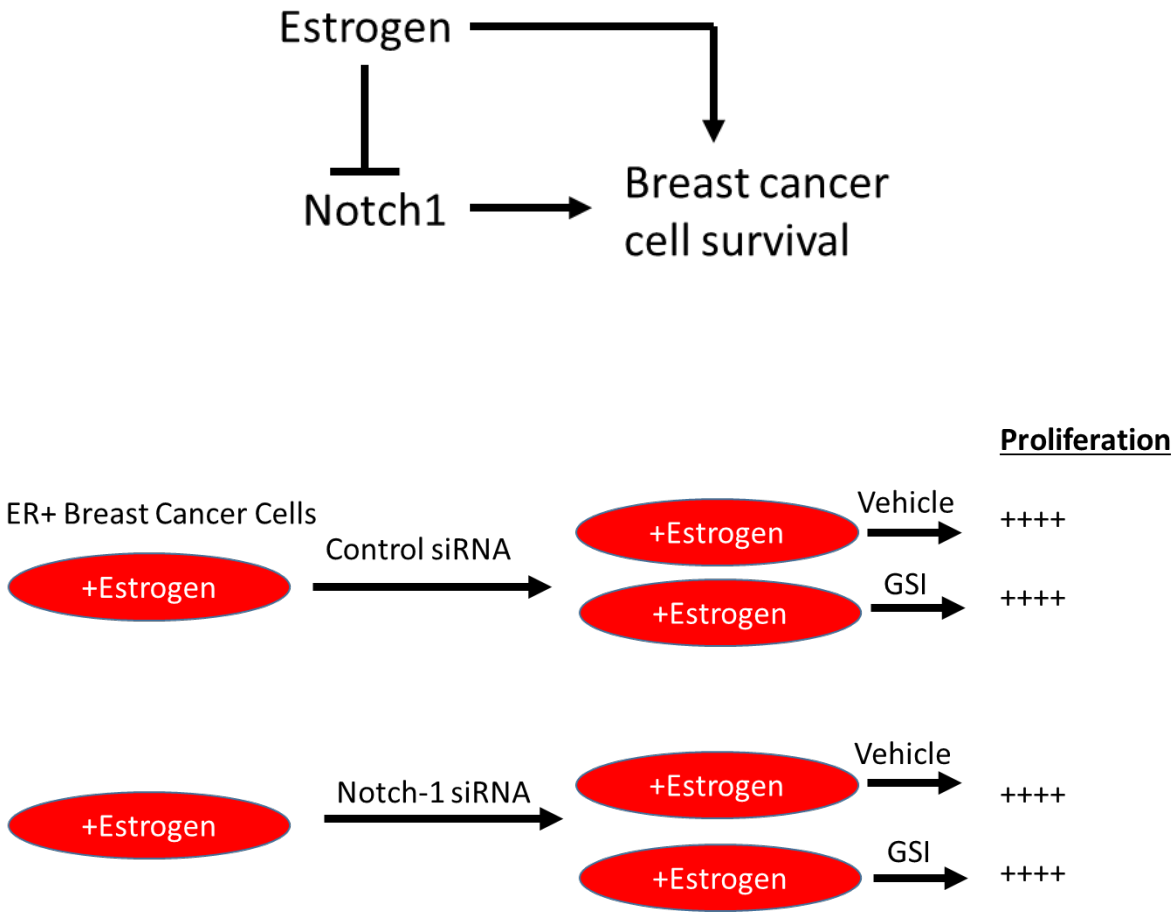


Figure 2-1. A strategy for determining Notch1-specific effects on proliferation. Based on the proposed model derived by data put forth by Rizzo, et al., no effect on MCF-7 cell proliferation is expected when cells are cultured in the presence of estrogen.

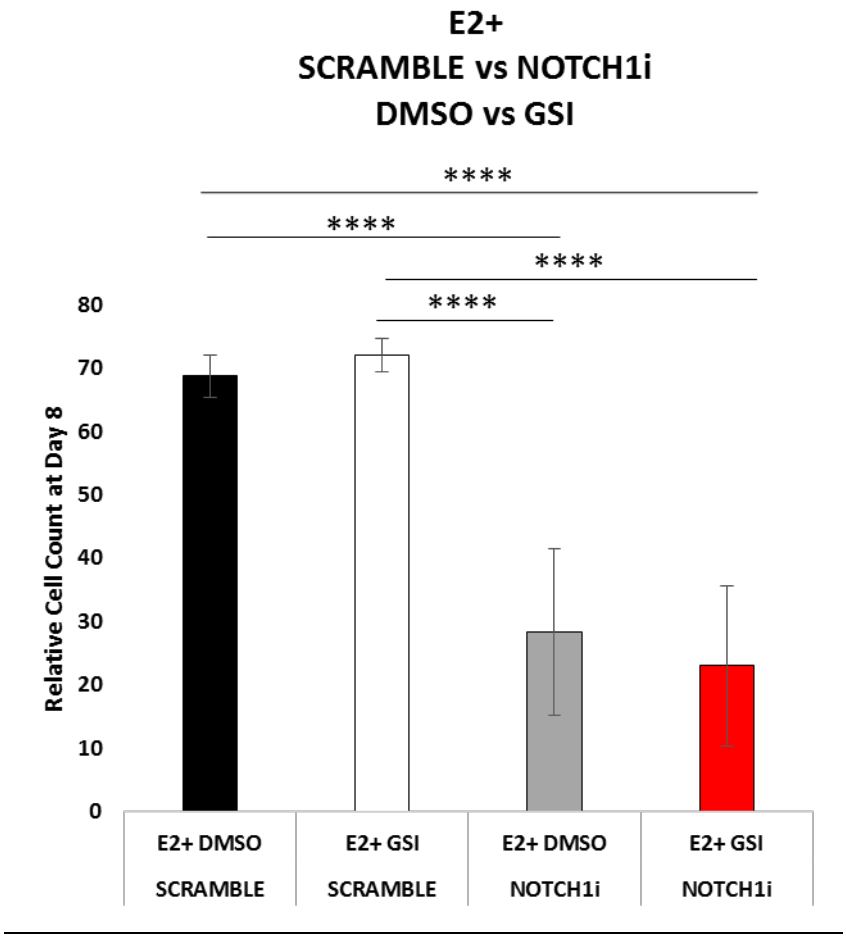


Figure 2-2. The effects of Notch1 knockdown and γ-secretase inhibition on MCF-7 proliferation in the presence of estrogen. Proliferation of cells grown in the presence of estrogen is unaffected by GSI administration. Cell proliferation is significantly reduced upon Notch1 knockdown. Data are presented as mean ± standard deviation for three biological replicates. Statistical significance was determined via 2-way ANOVA where **** signifies $p<0.0001$.

Anti-estrogen therapy is the standard of care for ER+ breast cancer. Since anti-Notch therapies, such as GSIs, have been proposed as means to circumvent tumor recurrence in these patients, it is critical to understand the interactions between anti-estrogen therapy and Notch signaling. Rizzo et al. showed that upon estrogen deprivation, tamoxifen, or fulvestrant (anti-estrogens) administration, Notch1 expression levels and membrane association decrease, and Notch1 cleavage rates increase. This suggests that estrogen inhibits Notch1 activity (Fig. 2-1) Thus, if Notch1 signaling is attenuated under estrogen-deprived conditions, then a negative effect ton proliferation is expected. Figure 2-3 presents the expectations for the effect of Notch1 inhibition under estrogen deprived growth conditions. In summary, estrogen deprivation is known to inhibit the growth of MCF-7 cells. If Notch1 is required for cell survival under estrogen-deprived growth conditions, then γ-secretase inhibition or Notch1 knockdown will yield a decrease in cell number in the absence of estrogen. Our results from the eight-day proliferation assay support our expectation. When the GSI is administered, a decrease in cell number is observed at the eighth day after plating the cells compared to the vehicle-treated control. Notch1 knockdown also yields a decrease in cell number suggesting that the effects of the GSI are at least in part due to its Notch1-inhibitory effect. Interestingly, when the GSI is administered under Notch1 knockdown conditions, an even further growth inhibitory effect is observed compared to either treatment alone.

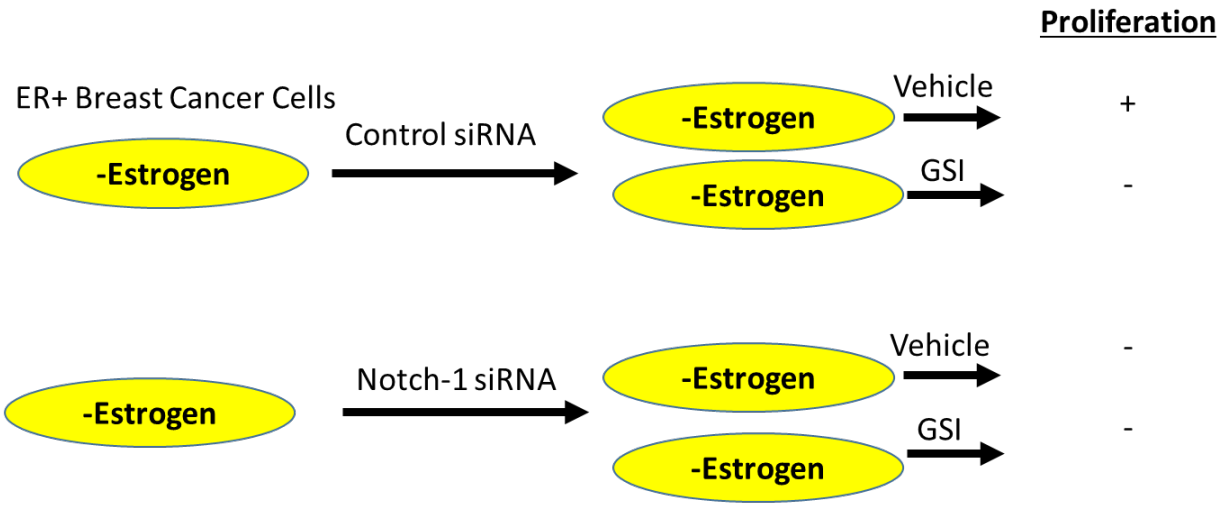


Figure 2-3. A strategy for determining Notch1-specific effects on proliferation. According to the model proposed in Figure 2-1, Notch signaling will be activated upon estrogen deprivation. Therefore, both GSI and Notch1 knockdown are expected to have a negative effect upon cell proliferation.

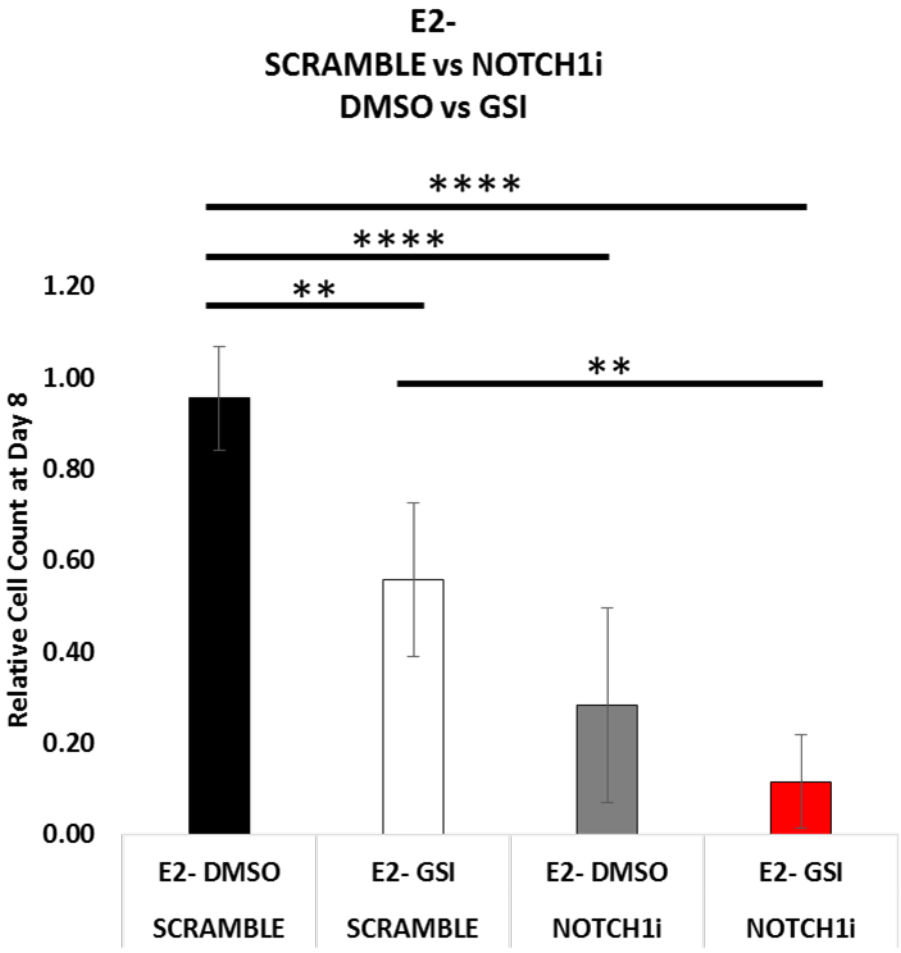


Figure 2-4. The effects of Notch1 knockdown and γ-secretase inhibition on MCF-7 proliferation in the absence of estrogen. As expected, cell proliferation is significantly decreased when cells are treated with GSI under estrogen-deprived growth conditions. An even further decrease in survival is observed under Notch1 knockdown and/or GSI administration. Data are presented as mean ± standard deviation of three biological replicates. Statistical significance was determined via 2-way ANOVA where ** signifies $p<0.01$, and **** signifies $p<0.0001$.

In order to gain a further understanding of the mechanism of Notch1 action under the GSI and Notch1 knockdown conditions, mRNA was harvested from the cells subsequent to counting for the proliferation assay. In order to verify Notch1 transcriptional activity, relative mRNA levels of the classical Notch1 target genes HES1 and HEY1 were determined via qRT PCR. As our model predicts, Notch1 target expression is low when cells are grown in the presence of estrogen. Therefore, neither Notch1 knockdown nor GSI administration had an effect on HES1 or HEY1 expression. Under the absence of estrogen, a robust induction of Notch target gene expression is observed. Both Notch1 knockdown and GSI administration inhibit this induction but do not decrease mRNA levels to those observed under the presence of estrogen. This suggests that Notch1 is partially required for HES1 and HEY1 induction in the absence of estrogen. Furthermore, γ-secretase activity is partially required for HES1 and HEY1 induction in the absence of estrogen. However, since Notch1 knockdown and GSI administration only partially inhibit HES1 and HEY1, it is possible that other Notch paralogs or transcription factors influence the expression of these genes.

The qRT PCR data are further valuable because they provide clues to the role that Notch1 plays in the presence of estrogen. Our data from the proliferation assays suggest that Notch1 is required for proliferation when estrogen is present, but Notch1 cleavage is not required for proliferation. The qRT PCR data suggests that putative Notch1 target genes are not activated in the presence of estrogen. Therefore, these data further support the hypothesis that Notch1 is required for proliferation in a γ-secretase cleavage-independent manner.

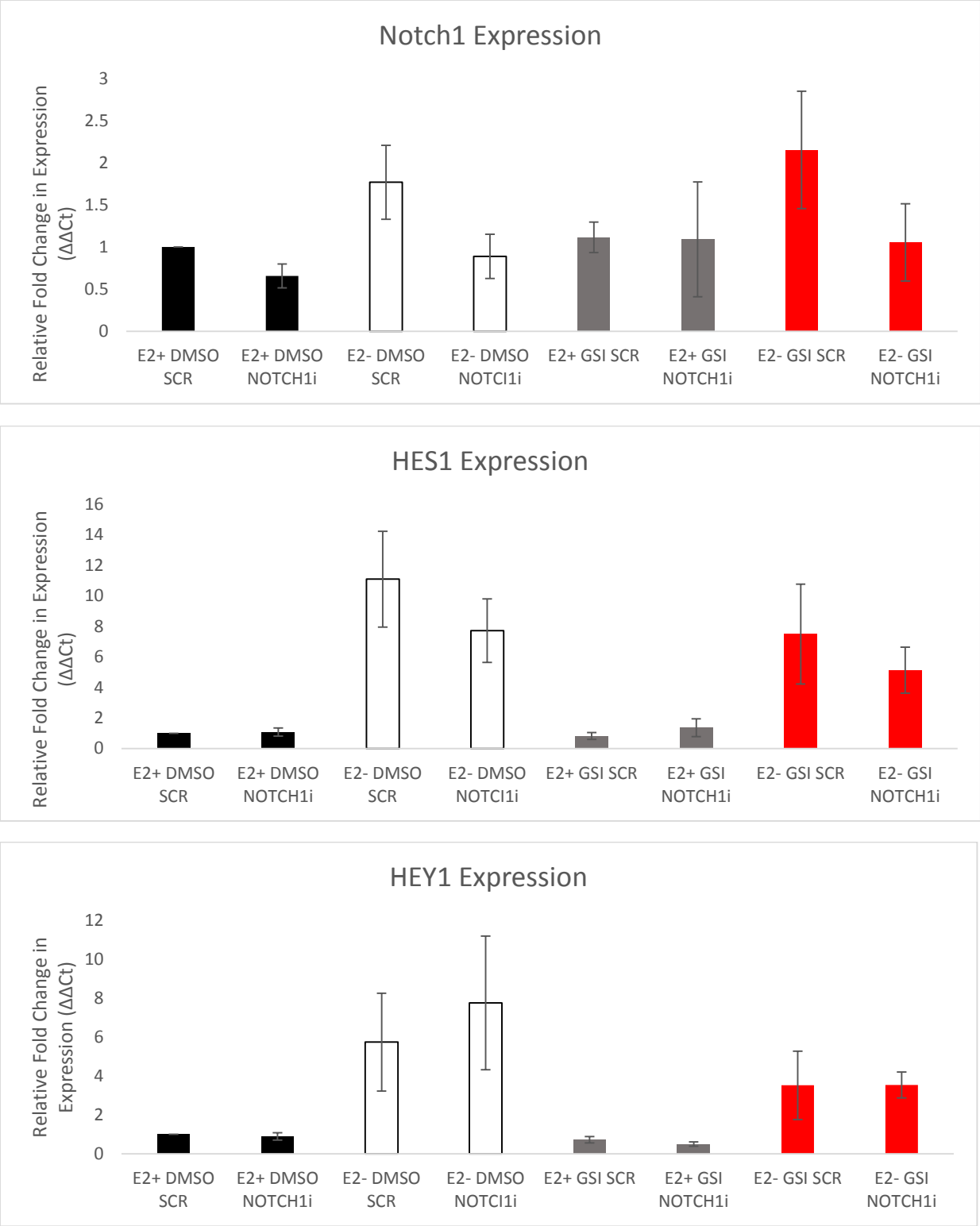


Figure 2-5. Effects of Notch1 knockdown, GSI administration, and estrogen deprivation upon Notch1 and Notch target gene expression. Though estrogen deprivation causes an increase in Notch target gene expression, Notch1 knockdown has no effect on target gene expression.

In order to move forward with the preliminary data presented thus far, a mechanism by which Notch1 is required for proliferation and survival from a membrane-bound state must be proposed. In order to do so, the data presented here must be supplemented by the work performed by Allison Rogowski. In her Master's Thesis, Rogowski reported that Notch1 is required for the phosphorylation of ERK1/2 in a variety of cell lines including MCF-7. ERK1/2 is a well-known promoter of cell survival and proliferation. Rogowski proposed that Notch1 acts on ERK1/2 by controlling the expression or activity of known ERK1/2 activators or inhibitors. MAP Kinase Phosphatases (MKPs) are known to inhibit ERK1/2 activity by dephosphorylating the active ERK1/2 protein. If Notch1 knockdown causes an increase in MKP expression then this could provide a reasonable mechanism for Notch1-mediated activity of ERK1/2. However, Notch1 knockdown caused a decrease in MKP protein levels in MCF-7 cells. Furthermore, when Notch1 and MKP1 were knocked down concomitantly, no rescue of ERK1/2 phosphorylation was observed. Thus, the hypothesis that ERK1/2 activity is mediated by Notch1 control over MKP was dismissed.

Rogowski suggested a second hypothesis in order to determine whether Notch1 activated ERK1/2 by controlling its upstream activators. A phosphor recteptor tyrosine kinase assay was performed under Notch1 knockdown and NICD1 overexpression conditions. If Notch1 is required for the activation of upstream receptor tyrosine kinases, then the knockdown of Notch1 will cause a decrease in RTK phosphorylation. If Notch1 is sufficient for upstream activation of RTKs, then expression of NICD1 will increase RTK phosphorylation. In MCF-7 cells, a decrease in VEGF R3 phosphorylation is observed upon Notch1 knockdown. However, no change in phosphorylation is observed whenever NICD1 is expressed. Since NICD1 is unable to induce

ERK1/2 phosphorylation, the hypothesis that Notch1 causes phosphorylation of ERK1/2 by activating upstream activators was dismissed.

The hypotheses put forth by Rogowski depend upon the ability of Notch1 to act in a γ-secretase cleavage-dependent manner. The experiments performed by Rogowski did not involve the use of a GSI. Thus, it is not known whether the observed effects upon ERK1/2 were due to cleaved or membrane-bound Notch1. Importantly, if ERK1/2 is required for survival and proliferation in MCF-7 cells, then the effects observed on proliferation correspond to the ERK1/2 data presented by Rogowski. The next chapter will seek to articulate testable hypotheses that can be drawn from the findings presented in these preliminary data. First, we will seek to determine whether membrane-bound Notch1 is required for ERK1/2 phosphorylation in an estrogen-dependent manner. Second, we will seek to determine whether ERK activity is required for MCF-7 proliferation and survival in a Notch1-dependent manner.

HYPOTHESIS

Based on our preliminary data, **I hypothesize that the membrane-bound Notch1 receptor is required for growth and survival of MCF-7 cells in an estrogen dependent manner by activating the ERK1/2 signaling pathway.**

SPECIFIC AIMS

Specific Aim 1: Determine whether the membrane-bound Notch1 receptor is required for ERK1/2 activation. Our preliminary data demonstrate that in the presence of estrogen, siRNA ablation of Notch1 inhibits proliferation of MCF-7 cells. In contrast, inhibiting the cleavage of Notch1 with the GSI has no effect on proliferation. This observation suggests that Notch1 promotes proliferation in a manner that is independent of its cleaved form (ICN). Moreover, in the absence of estrogen, both Notch1 knockdown and the GSI have negative effects on MCF-7

cell proliferation. Since estrogen has been shown to suppress Notch1 signaling activity and promote its localization to the cell membrane, the divergent effects of siNotch1 and GSI treatments may be due to an unidentified signaling function of the membrane-bound Notch1 receptor. Previous work performed in our lab shows that Notch1 is required for the activation of ERK1/2. However, a mechanism explaining Notch1-mediated ERK1/2 activation has yet to be described (A Rogowski, 2011, unpublished data). **I hypothesize that the membrane-bound Notch1 receptor is necessary and sufficient for ERK1/2 activation.**

Aim 1A) Determine whether membrane-bound Notch1 is necessary for ERK1/2 activation in an estrogen-dependent manner. Our preliminary data suggest that Notch1 expression is required for the proliferation of MCF-7 cells in the presence of estrogen. However, γ-secretase-dependent function of Notch1 is not required for cell proliferation in the presence of estrogen. Others have shown that in the presence of estrogen, Notch1 is localized to the membrane of MCF-7 cells. When grown in the absence of estrogen, membrane localization of Notch1 is decreased and Notch target gene activation is observed. Interestingly, in the absence of estrogen, both Notch1 knockdown and GSI treatment have a negative effect on proliferation and survival, but Notch1 knockdown has a more drastic effect than GSI treatment. This suggests that even though the canonical function of Notch1 may be able to support survival upon estrogen deprivation, cell survival via a non-canonical function of Notch1 may still be present, which would only be revealed by knocking down the Notch1 gene.

These observations suggest that in the presence of estrogen, the membrane-bound Notch1 receptor has a signaling role that is required for cell proliferation. In the absence of estrogen, the membrane-bound Notch1 receptor is less abundant, and the canonical, cleavage-mediated

response of Notch1 is required for proliferation and survival. In order to address a signaling mechanism that may be responsible for these effects, we turn to the work performed be Allison Rogowski wherein it was clearly demonstrated that Notch1 is required for ERK1/2 phosphorylation. Since ERK1/2 is closely associated with cell proliferation and survival, we first set out to determine whether the membrane-bound Notch1 receptor is required for ERK1/2 proliferation. Rogowski showed that ERK1/2 phosphorylation is diminished upon Notch1 knockdown, but this does not address the membrane-bound form specifically. In order to determine whether membrane-bound Notch1 is required for ERK1/2 phosphorylation, a knockdown versus GSI approach will be used. If ERK1/2 depletion is observed upon Notch1 knockdown and not upon GSI administration then this would suggest that the membrane-bound Notch1 receptor is required for ERK1/2 phosphorylation but that the cleaved NICD is not required.

E2 Content	**Notch1 membrane localization**	**Notch target gene activation**	**Effect of GSI on proliferation**	**Effect of siNotch1**	**Possible conclusion**
E2 present	high	low	no effect	50% decrease in cell number	Membrane bound Notch1 is required for proliferation.
E2 absent	low	high	40% decrease in number	80% decrease in cell number	Both membrane bound and cleaved Notch1 are required for survival.

Table 2-1. Summary of observations necessary for Aim 1A.

Aim 1B) Determine whether membrane-bound Notch1 is sufficient for ERK1/2 activation in an estrogen-dependent manner.

By inhibiting Notch1 function as proposed in Aim 1A, we will be able to determine whether membrane-bound Notch1 is required for ERK1/2 phosphorylation. To provide further evidence that Notch1 promotes ERK1/2 phosphorylation, we will seek to determine whether membrane bound Notch1 is sufficient for ERK1/2 phosphorylation. This will be achieved by ectopically overexpressing a membrane-bound Notch1 and blotting for phosphorylated ERK1/2. Our lab has an expression plasmid containing the membrane bound Notch1 receptor. However, this expression construct lacks the extracellular domain of Notch1 making it an ideal γ-secretase substrate. In order to observe the effects of the membrane-bound Notch1, a GSI will be administered along with the ectopically expressed, membrane-bound Notch1 (Notch1 ΔE) . If Notch1 ΔE expression in combination with GSI administration is able to induce ERK1/2 phosphorylation, then Notch1 may be deemed sufficient to activate ERK1/2.

Specific Aim 2: Determine whether the Notch1-dependent effects on MCF-7 cell proliferation and survival are due to ERK1/2 activation. Notch1 is required for ERK1/2 activation, and ERK1/2 is known to be required for cell proliferation and survival. However, the preliminary data merely suggests a correlation between ERK1/2 activation and cell proliferation/survival in a Notch1-dependent manner. In order to demonstrate a cause-and-effect relationship between ERK1/2 phosphorylation and MCF-7 proliferation and survival, a rescue experiment must be performed. In order to successfully demonstrate rescue of cell proliferation, we need to be able to constitutively activate ERK1 in a Notch1 independent manner. This will be achieved by expressing a mutated form of ERK1, which has reported constitutive activity, under

Notch1 knockdown conditions. **If Notch1 knockdown causes a decrease in proliferation/survival in an ERK1 activation-dependent manner, then constitutive ERK1 activation will rescue proliferation/survival even in the absence of Notch1**.

CHAPTER 3

MATERIALS AND METHODS

Cells and Cell Culture

MCF-7 ws8 cells were obtained from American Type Culture Collection (ATCC). Cells were maintained at 37°C in 95% humidified air supplemented with 5% CO_2. MCF-7 cells were maintained in Roswell Park Memorial Institute (RPMI 1640) medium (Corning 10-040-CV) supplemented with 10% fetal bovine serum, 1X Non-Essential Amino Acids (Life Technologies 11140-050), and 1X L-glutamine (GE Healthcare Life Sciences SH30034.01). Passage was performed by rinsing in 1X phosphate buffered saline (Corning 21-040-CV) and suspending cells in 2X Trypsin (GE Healthcare SV30037.01). Cells were passaged upon reaching 70%-90% confluence. Charcoal stripping was performed by removing complete RPMI 1640, rinsing cells with 1X PBS, and adding Charcoal Stripped RPMI 1640 (Corning 17-105-CV) supplemented with 10% charcoal-stripped fetal bovine serum, 1X non-essential amino acids, and 1X L-glutamine. Cryo-preserved cell stocks were prepared by trypsinizing and pelleting cells and resuspending the cells in a freezing media composed of 10% dimethyl sulfoxide (Fisher BP231) and 90% fetal bovine serum. Cells suspended in freezing media were stored overnight at -80°C in a cryo-freezing container. After freezing, cells were transferred to the fifth shelf of the liquid nitrogen cryogenic tank located in Cancer Center Room 221. U0126 (Cell Signaling Technologh 9903) was purchased from Cell Signaling.

Expression Plasmids and Transfection

Expression constructs bearing Notch1 ΔE GVP were previously described in by Karlstrom, et al. pcDNA3.1 was obtained from Thermo Fisher Scientific (Fisher V790-20).

NICD1. pET32 ERK1 WT and pET32 ERK1 R84S were generously donated by Natalie Ahn from the University of Colorado.

The standard transfection protocol is as follows. Transfections are performed by adding fresh growth media to adherent cells at 60%-70% confluence. In separate 15mL conical tubes, the plasmid DNA and Lipofectamine are diluted in Opti-MEM minimal media (Life Technologies 31985-070). Plasmid DNA and Lipofectamine were diluted in separate tubes and incubated for five minutes. The DNA/Lipofectamine are mixed and incubated for ten minutes. Then, the DNA/Lipofectamine/Opti-MEM is added to the growth media of the target cells. The standard DNA:Lipofectamine ratio is 1:3.

siRNA Transfection

Gene knock down was achieved using small interfering RNA (siRNA) designed to specifically target Notch1 (Santa Cruz Biotechnology sc-36095) mRNA. siRNA were reconstituted to a stock concentration of 10μM by adding 330μL of provided diluent. Transfections were performed using Lipofectamine 2000 RNAimax (Thermo Fisher 13778150) transfection reagent at a 1:1 Lipofectamine:siRNA ratio in Opti-Mem (Thermo Fisher 51985034).

The standard siRNA transfection protocol was carried out as follows. Fresh growth media is added to cells at 60%-70% confluence. In 15mL sterile conical tubes, the siRNA and Lipofectamine are diluted separately in Opti-MEM and incubated for three minutes. The siRNA/Lipofectamine were mixed and incubated for ten minutes. Then, the siRNA/Lipofectamine/Opti-Mem is added to the growth media of the target cells.

Primers for Reverse Transcription Polymerase Chain Reaction

Primers for quantitative Reverse Transcriptase Polymerase Chain Reactions (qRT-PCR) were designed using the NCBI Primer Blast application. PCR product sizes were specified to be between 70 and 150 nucleotides. Primers are designed to span exon-exon junctions in order to increase specificity for mRNA transcripts. Custom primers were supplied by Life Technologies. The lyophilized DNA was resuspended in DNase/RNase-free water to a stock concentration of 50μM.

PRIMER	**SEQUENCE**
HPRT FORWARD	5' ATGAACCAGGTTATGACCCCGAT 3'
HPRT REVERSE	5' CCTGTTGACTGGTCATTACAATA 3'
NOTCH1 FORWARD	5' ATCAACGCCGTAGATGACC 3'
NOTCH1 REVERSE	5' TTGTTAGCCCCGTTCTTCAG 3'
HES1 FORWARD	5' CGGACATTCTGGAAATGACA 3'
HES1 REVERSE	5' CATTGATCTGGGTCATGCAG 3'
HEY1 FORWARD	5' TCATTTGGAGTGTTGGTGGA 3'
HEY1 REVERSE	5' CTCGCACACCATGATCACTT 3'

Table 3-1. Primers used for RT PCR reactions.

Quantitative Real Time PCR

For quantitative RT-PCR (qRT-PCR), cells can be either trypsinized or harvested directly to the culture well by aspirating the culture media and adding TRI Reagent (Life Technologies 9738G) directly to cells and homogenizing by pipetting up and down approximately twenty times. Lysates may be stored at -80°C or placed on ice to proceed with RNA isolation using the RiboPure kit (Life Technologies AM1924). Add $1/10^{th}$ volume of 1-bromo 3-chloropropane (Sigma B9673) to TRI/lysates, vortex for fifteen seconds and incubate on ice for five minutes. Centrifuge at 14,000 RPM at 4°C for fifteen minutes. Using a pipet, transfer the top, clear aqueous layer to a clean 1.5mL centrifuge tube. Add ½ volume of 100% ethanol (Sigma E7023) to the aqueous sample and immediately vortex for five seconds. Transfer the sample to a centrifuge column and centrifuge at 10,000 RPM for thirty seconds at room temperature. Discard the flow-through and wash the column two times by centrifuging 500µL of wash buffer through the column being sure to completely remove the wash buffer after the final wash. Add 30µL of provided elution buffer to the column and incubate for one minute. Elute the column by centrifuging for one minute at 10,000 RPM. RNA concentration is determined using the NanoDrop spectrophotometer.

Reverse transcription PCR is performed to generate cDNA from the isolated RNA. RNA concentrations are normalized by diluting the RNA in DNase/RNase-free water (Life Technologies AM9937) to a concentration of 25ng/µL. For a 50µL PCR reaction yielding 250ng/µL cDNA, mix 19.25µL (12.5ng) of purified RNA to 30.75µL master mix in a PCR reaction tube. The PCR reaction is performed by going through one cycle outlined in Figure 3-1. The PCR reaction yields 25ng/µL cDNA. Quantification of the relative amounts of mRNA transcripts is carried out by amplifying the cDNA targets in the presence of the SYBR Green

fluorescent dye. The qRT-PCR reaction is carried out in technical duplicates of each sample containing primers amplifying the cDNA target of interest. Each reaction contains 2.5 µL of 25ng/µL cDNA, 0.5µL each of 50µM forward and reverse primers, 12.5µL SYBR Green Mastermix (BioRad 720001566), and 9µL DNase/RNase free water.

Components	Final concentration	Vol(ul)/tube
10X RT buffer (Life Technologies 4486220)	1X	5
25mM MgCl2 (Life Technologies 4486624)	5.5mM	11
dNTPs (Life Technologies 362275)	500uM/Dntp	10
Random Hexamers (Life Technologies 51709)	2.5uM	2.5
RNase Inhibitor (Life Technologies 100021540)	0.4U/L	1
Reverse Transcriptase (Life Technologies 4308228)	1.25U/L	1.25
Total		30.75

Table 3-2. Components of the RT qPCR reaction

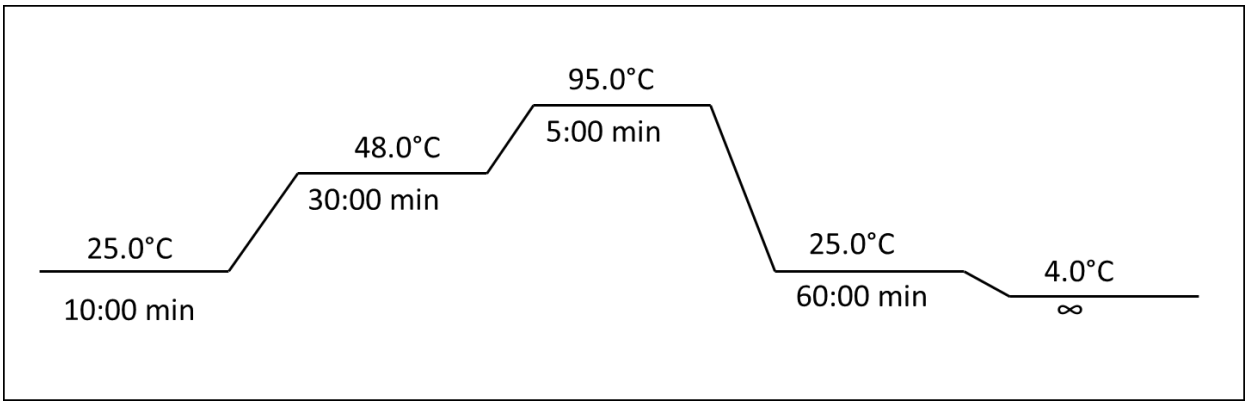


Figure 3-1. RT PCR Reaction Cycle

Flow Cytometry

Detection of surface Notch1 was achieved by culturing MCF-7 cells in the absence of estrogen for two days and then treating one group of cells with charcoal-stripped RPMI 1640 supplemented with 10µM 17-β estradiol in ethanol. After three days of growing cells in the presence or absence of 17-β estradiol, cells were rinsed with 1X PBS and incubated at 37°C with Cell Stripper to detach the cells from their culture dish. Cells collected and centrifuged and rinsed two times in FACS staining buffer. Cells were blocked with normal IgG for five minutes and then incubated for 45 minutes at 4°C with 5µl of 200µg/mL PE conjugated anti-Notch1 IgG (Biolegend 352106). Cells were suspended in 250µL FACS buffer and analyzed on the BD Canto II Flow Cytometer.

Mammosphere Forming Assays

Cells were grown in the presence or absence of estrogen for 48 hours prior to plating on six-well tissue culture treated plates. 300,000 cells per well were seeded in estrogen plus or minus conditions. 24 hours after seeding, cells were transfected with Notch1 siRNA or Scbi control. Six hours after transfection, cells were treated with 10µM U0126 or an equal volume of DMSO vehicle. 18 hours after U0126 administration, cells were trypsinized and counted. 30,000 cells were seeded to methylcellulose media and allowed to grow for seven days at 37°C 5% CO_2. Mammospheres were photographed at 20X and 40X magnification. To determine mammosphere forming efficiency (MFE), mammospheres were collected in 15mL conical tubes and centrifuged at 1200RPM for two minutes in 1X PBS. Mammospheres were resuspended in 2mL of 1X PBS and 50µL of this suspension was added to a well of a 96 well plate. Mammospheres in the 96 well plate were photographed at 4X magnification. Mammospheres were counted by only considering spheres at or above 50µm in diameter.

Antibodies

Notch1 was detected using the C-20 anti-Notch1 (Santa Cruz Biotechnologies sc-6014-R) at a primary antibody dilution of 1:1000 in 1X Roche Western Blocking Reagent (Roche Diagnostics 11921673001) and donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnologies sc-2313) at a dilution of 1:2000 in Roche Blocking Reagent. Total ERK1/2 was detected using anti-p44/42 (Cell Signaling Technologies 4695S) at 1:2000 in 1X Roche Western Blocking Reagent or 5% bovine serum albumin in 1X TBS-T and donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnologies sc-2313) at a dilution of 1:2000 in Roche Blocking Reagent or 5% BSA/TBS-T. Phospho-ERK1/2 was detected using anti-phospho-p44/42 (Cell Signaling Technologies 4370S) at 1:2000 in Roche Western Blocking Reagent or 5% BSA/TBS-T and donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnologies sc-2313) at a dilution of 1:2000 in Roche Blocking Reagent or 5% BSA/TBS-T. Cleaved Notch1 was detected using an antibody specific to the C-terminal valine of Notch1 produced as a result of γ-secretase cleavage (Cell Signaling Technologies 4147S) at 1:1000 in 5% BSA/TBS-T and donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase at a dilution of 1:1000 in Roche Blocking Reagent or 5% BSA/TBS-T. Anti-β-actin was used at a 1:5000 dilution in 5% milk (BioRad 170-6404) in TBS-T and donkey anti-mouse secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnologies sc-2314) at a dilution of 1:5000 in 5% milk.

Western Blotting

Before lysing the cells, they were rinsed briefly with 1X Phosphate Buffered Saline (Corning 21-040-CV). Cells were lysed by adding lysis buffer (50mM HEPES, 1% Triton X-100, 150mM Sodium chloride, 5mM EDTA, 1mM Sodium orthovanadate, 10mM Sodium

fluoride, 1mM PMSF, 1X protease inhibitor cocktail pill) directly to the adherent cells and incubating on ice for ten minutes. Cells were scraped from the culture dish and samples sonicated (Fisher Scientific FB120) for ten seconds at 20% amplitude. Protein concentration was determined by performing a bicinchoninic acid (BCA) assay (Fisher Scientific 23225). Lysates were brought to the same protein concentrations by adding lysis buffer. 2X Lammelli buffer (Biorad 161-037) with 0.5% β-mercaptoethanol (BP176-100) was added at a 1:1 ratio. Samples were heated to 95°C for 10 minutes.

Polyacrylamide gel electrophoresis was performed using the Invitrogen….. Lysates were loaded onto precast 10-well 7% Tris-Acetate Polyacrylamide gels (Thermo Fisher EA0358BOX) in 1X Tris-Acetate-SDS running buffer (Thermo Fisher LA0041). SeeBlue protein ladder was used as a molecular weight marker. Gels were electrophoresed at 150V for 60-90 minutes. After gel electrophoresis, the contents of the gel were transferred to a 0.45µm pore polyvinylidene fluoride (PVDF) membrane (Millipore IPVH00010) using the Invitrogen Novex system. The contents of the gel were electrophoresed to the PVDF membrane at 40V for 2 hours in 1x Transfer buffer. The PVDF membranes were then blocked with either 1X Roche Western Blocking Reagent in 1X Tris-Buffered Saline or 5% milk in Tris-Buffered Saline-Tween20 for 30-60 minutes. Blots were incubated on primary antibody overnight at 4°C on a rocker. The blots were washed rocking three times with TBS-T for ten minutes. The blots were incubated on secondary antibody for 60 minutes at room temperature on a rocker. Horseradish peroxidase (HRP) chemiluminescence was detected using the Pierce ECL substrate exposed on autoradiography film.

Luciferase Assays

MCF-7 ws8 cells were grown in estrogen-containing or estrogen-free RPMI 48 hours prior to being seeded onto a 12 well multi-dish. 24 hours after being seeded, the cells were transfected with the two plasmid constructs containing the luciferase components: the TRL plasmid bearing the CMV-Renilla Luciferase transfection control and AP-1 Firefly Luciferase. Plasmids were transfected using Lipofectamine 3000 reagent diluted in Opti-Mem. The luciferase assay was carried out using the Dual-Glo Luciferase Assay System (Promega E2920). The cells were lysed in 250µL of 1X passive lysis buffer. 20µL of lysate were aliquoted to three wells of a white 96-well microplate. The luciferase reactions were carried out in a microplate luminometer capable of automatically injecting the luciferase reagents.

DNA Cloning

The goal of performing subcloning was to move the inserts containing ERK1 WT and ERK1 R84S from the pET32 bacterial expression vector into the pcDNA3.1+ mammalian expression vector. The subcloning was designed to introduce a BamHI restriction site and a Kozak sequence 5' to the ERK1 open reading frame. The forward primer for the PCR cloning step was designed to bind to the 5' end of the ERK1 ORF on the first round of PCR. Since the forward primer contains a BamHI cleavage site upstream from the transcription start site, subsequent rounds of PCR yielded a cleavable product using the first round product as a template. The reverse PCR reaction used a primer complementary to the 3' multiple cloning site of the pET32 vector which contains a NotI cleavage site. Thus, PCR cloning will yielded a PCR product containing the insert with a 5' BamHI restriction site and a 3' NotI restriction site. PCR amplification was carried out using the high fidelity *Pfu* polymerase (Transgen AP221). Purified DNA was sent to ACGT for sequence analysis. ERK2-wt, ERK2-R65S, and ERK2-I84A were

supplied in the same vector background as ERK1. Therefore, the same cloning strategy for ERK1 was applied to ERK2. However, the ERK2 open reading frame contains a BamHI cleavage site so for ERK2, a 5' HindIII site had to be introduced for cloning into pcDNA3.1.

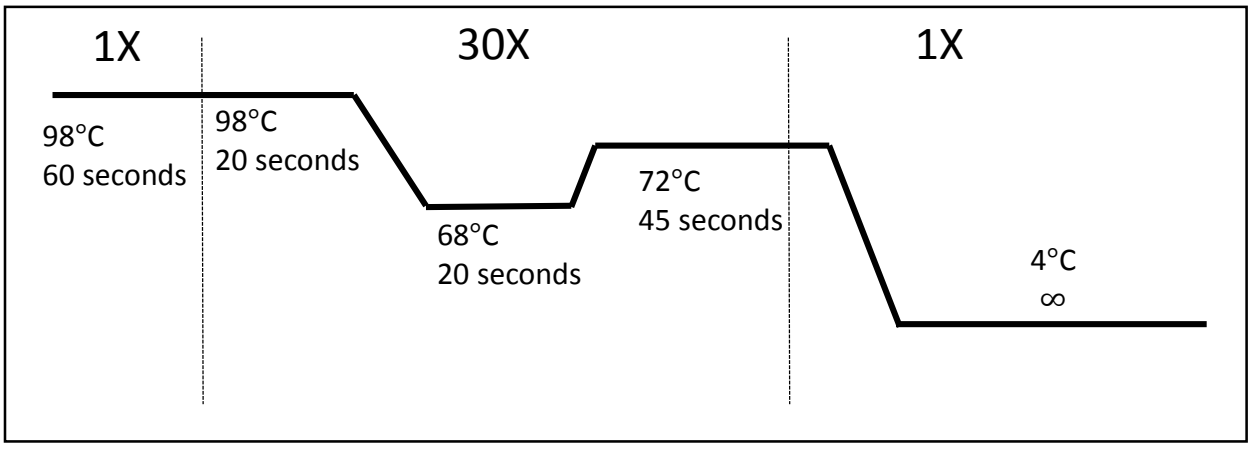


Figure 3-2. *Pfu* polymerase PCR reaction.

ERK1 FORWARD	5' TATCGGATCCAGAACCATGGCGGCGGCGGCGGCTCAGGGGGG 3'
ERK1 REVERSE	5' GGTGCTCGAGTGCGGCCGCAAGCTTGTCGACGGAGC 3'

Table 3-3. PCR primers used in the cloning of human ERK1.

CHAPTER 4

RESULTS

SPECIFIC AIM 1A: Determine whether the membrane-bound Notch1 receptor is required for ERK1/2 activation.

Work conducted previously by in our lab by Allsion Rogowski demonstrated that Notch1 is required for ERK1/2 phosphorylation in various cell lines including MCF-7. ERK1/2 is known to promote proliferation and survival characteristics. Furthermore, work published previously by members of our lab demonstrates that estrogen inhibits Notch1 activity possibly by maintaining its presence at the cell membrane. If estrogen prevents Notch1 cleavage, then any Notch1-specific effects elicited by a γ-secretase inhibitor would not be expected. Our data suggest that in the presence of estrogen, even though Notch1 expression is required for proliferation of MCF-7 cells, γ-secretase activity is not necessary for the maintenance of cell proliferation. One possible explanation for this γ-secretase independent effect of Notch1 is that Notch1 is able to elicit a signaling effect from its membrane-bound state. In order to test this hypothesis, a Western blotting approach was taken in order to compare the effects of Notch1 knockdown and GSI administration on ERK1/2 phosphorylation. If Notch1 activates ERK1/2 phosphorylation from its membrane-bound state, then the GSI will have no effect on ERK1/2 phosphorylation compared to vehicle (DMSO)-treated control. If overall Notch1 expression is depleted via knockdown, and a decrease in ERK1/2 phosphorylation is observed, then it can be concluded that the membrane-bound Notch1 receptor is required for ERK1/2 phosphorylation when compared to the GSI treated sample.

Estrogen depletion or administration of anti-estrogen targeted therapies such as tamoxifen is known to induce the expression of Notch target genes. In this case, if Notch1 is activated by estrogen depletion, then Notch1-specific effects elicited by a γ-secretase inhibition are to be expected. In the absence of estrogen in our model, γ-secretase activity is required for cell survival, which is shown by the decrease in cell number upon GSI administration. Furthermore, Notch1 knockdown in the absence of estrogen leads to an even more pronounced effect on cell viability than GSI administration alone. This suggests that even though γ-secretase dependent Notch1 activity is required for cell survival in the absence of estrogen, the effects that could be elicited by a membrane-bound Notch1 are still present.

Given the suggestions put forth by Rizzo, et al., by Rogowski, and by the preliminary data put forth here, we embarked to determine whether the uncleaved, membrane-bound form of Notch1 is required for ERK1/2 phosphorylation in an estrogen-dependent manner. In order to provide supporting evidence for our rationale, we first sought to demonstrate that estrogen causes an increase in the membrane-bound form of Notch1. Our approach employed a flow cytometry technique, which allows the detection of the extracellular domain of Notch1. If estrogen causes a membrane accumulation of Notch1, then the presence of the extracellular domain of Notch1 should be higher in the presence of estrogen than in the absence of estrogen. The results shown in Figure 4-1 demonstrate that compared to the unstained control, uncleaved Notch1 is detectable on MCF-7 cells which have been deprived of estrogen. However, when the cells were stimulated with 17-β Estradiol the mean fluorescence intensity doubles indicating that on average, each cell expresses more Notch1 on its surface when estrogen is present. This data corresponds with the immunofluorescence data reported by Rizzo, et al. Having provided further evidence for the

increased presence of surface Notch1 in the presence of estrogen, we next sought to determine the mechanism by which estrogen and Notch1 cooperate to influence ERK1/2 phosphorylation.

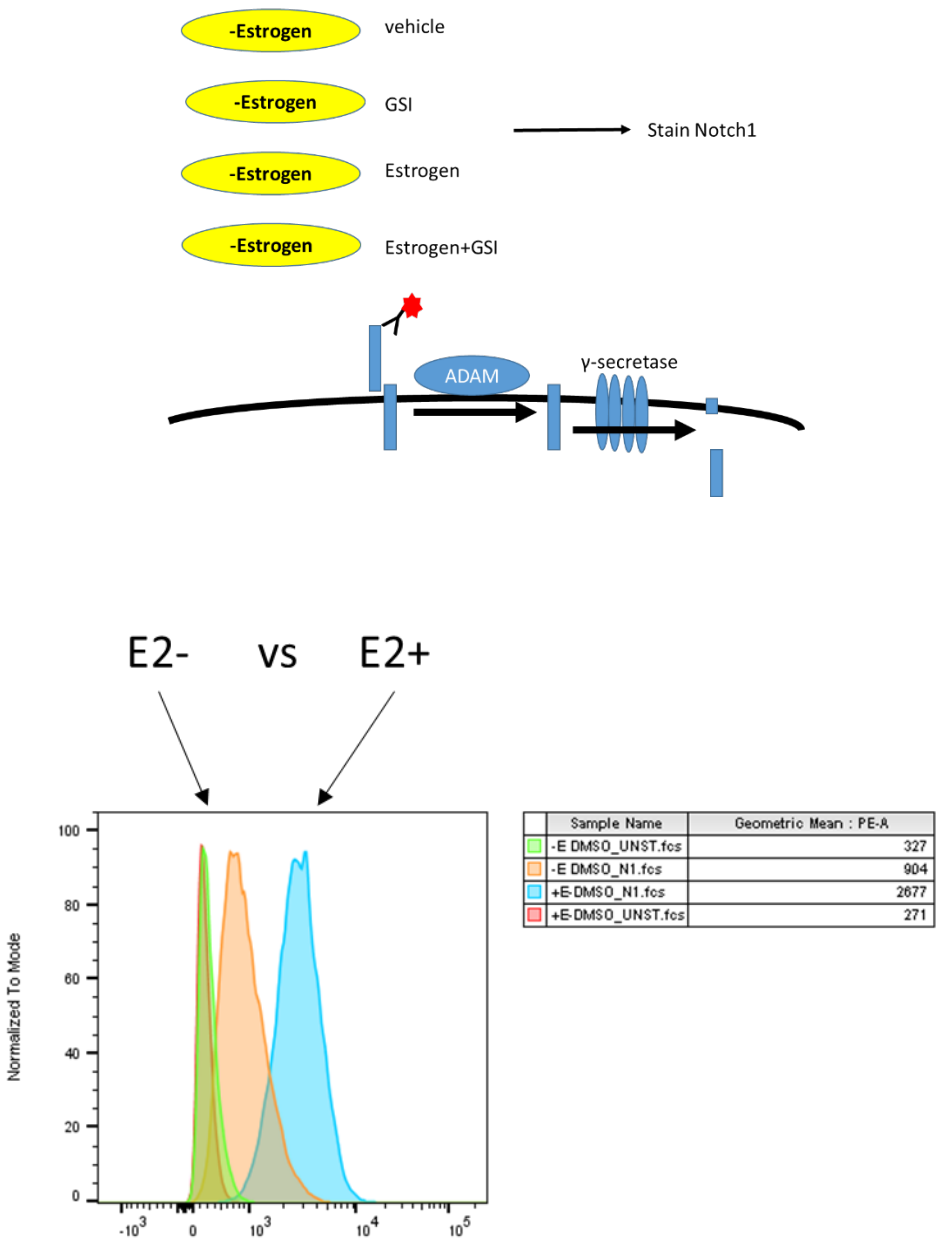


Figure 4-1. Strategy and results for detection of full length Notch1 via flow cytometry. The Notch1 antibody used for flow cytometry analysis binds to the extreme N-terminus of the Notch1 extracellular domain. Data are presented as Mean Fluorescence Intensity indicating the average amount of Notch1 located on the membrane of each cell. Unstained controls are presented as a control against autofluorescence.

Membrane-bound Notch1 is required for ERK1/2 phosphorylation.

A Western blotting approach was taken in order to determine whether the membrane-bound Notch1 receptor is required for ERK1/ 2 phosphorylation in an estrogen-dependent manner. If Notch1 is required for ERK1/2 phosphorylation, then Notch1 knockdown will cause a decrease in ERK1/2 phosphorylation. If the membrane-bound Notch1 receptor is required for ERK1/2 phosphorylation, then GSI administration will not decrease ERK1/2 phosphorylation. However, if the cleaved NICD1 is required for ERK1/2 phosphorylation then the GSI will cause a decrease in ERK1/2 phosphorylation. If estrogen controls the cleavage of Notch1, then a different set of expectations can be drawn for ERK1/2 phosphorylation under estrogen-deprived conditions. We hypothesize that Notch1 cleavage is increased in the absence of estrogen because of the decrease in Notch1 surface expression and concomitant activation of the Notch target genes HES1 and HEY1 under estrogen deprivation. If γ-secretase dependent Notch1 activation is required for ERK1/2 phosphorylation in the absence of estrogen, then ERK1/2 phosphorylation will decrease upon GSI administration. If membrane-bound Notch1 is required for ERK1/2 phosphorylation in the absence of estrogen, then Notch1 knockdown under these conditions will yield a decrease in ERK1/2 phosphorylation. However, if estrogen deprivation activates γ-secretase dependent Notch1 activity, the knockdown versus GSI approach will not necessarily determine whether ERK1/2 phosphorylation in estrogen deprived cells is due to membrane-bound Notch1 or cleaved NICD1 because Notch1 knockdown would inhibit the function of both.

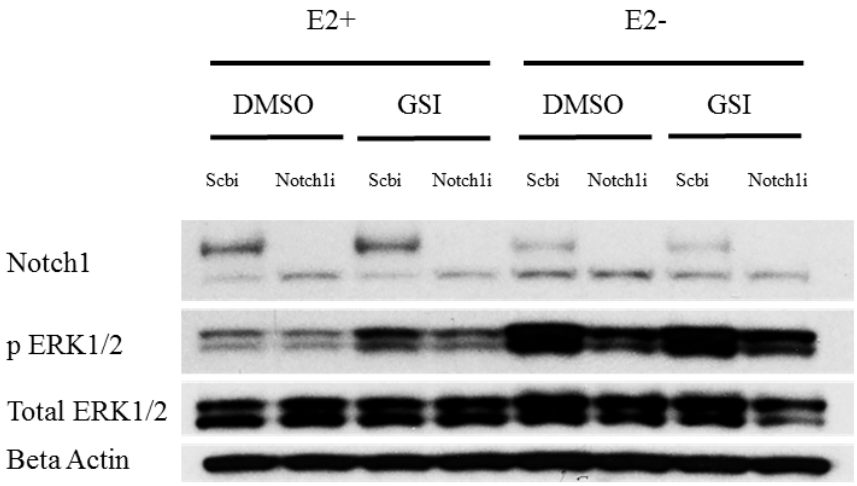
The data demonstrate that upon Notch1 knockdown, ERK1/2 phosphorylation is decreased. When the GSI is administered, an increase in ERK1/2 phosphorylation is observed. This agrees with our hypothesis because if Notch1 is stabilized at the membrane, then its ability to activate ERK1/2 could be augmented. However, since the GSI may have off-target effects,

administration of GSI alone does not necessarily mean that Notch1 is solely responsible for this increase in ERK1/2 phosphorylation. When the GSI is administered alongside Notch1 knockdown, ERK1/2 phosphorylation is decreased. This finding supports the notion that the increase in ERK1/2 phosphorylation seen upon GSI administration under Scbi control knockdown conditions is due to the effect of the GSI on Notch1 and not one of the other GSI targets.

The Western blots consistently demonstrate that Notch1 expression is decreased in estrogen-deprived cells compared to cells grown in the presence of estrogen. However, the data obtained for ERK1/2 phosphorylation under estrogen deprived are unable to be interpreted. The large standard deviations observed are due to a high variability in ERK1/2 phosphorylation levels observed whenever the cells are deprived of estrogen. In some experiments, ERK1/2 phosphorylation increase, as seen in the Western blot depicted. However, in others, ERK1/2 phosphorylation decreases upon estrogen deprivation. The purpose of depriving cells of estrogen is to mimic anti-estrogen therapy. Our hope was to be able to demonstrate a correlation between estrogen, Notch1, and ERK1/2 signaling pathways. However, estrogen is a hormone that is known to have multiple modes of action upon the ERK1/2 signaling pathway.

Given the high degree of consistency observed upon Notch1 expression between estrogen treatments and the low degree of consistency in ERK1/2 phosphorylation between estrogen treatments, it could be postulated that Notch1 and estrogen do not cooperate to influence ERK1/2 phosphorylation. An important future direction for this work will be to carefully control the estrogen-deprivation in order to obtain reproducible results. One possible variable that could have bearing on the observed results is the time of estrogen deprivation. In these experiments, the cells were grown in estrogen-free media for five days prior to harvest. If time of estrogen

deprivation is the confounding variable, then a time course experiment would be required in order to determine the optimal duration of estrogen deprivation. Alternatively, our lab has an MCF-7 cell line termed long-term estrogen deprived (LTED) which is optimized for growth and survival in the absence of estrogen. Since these cells are optimized for growth the absence estrogen-free media, a time-course would not be necessary to determine a consensus level of ERK1/2 phosphorylation.



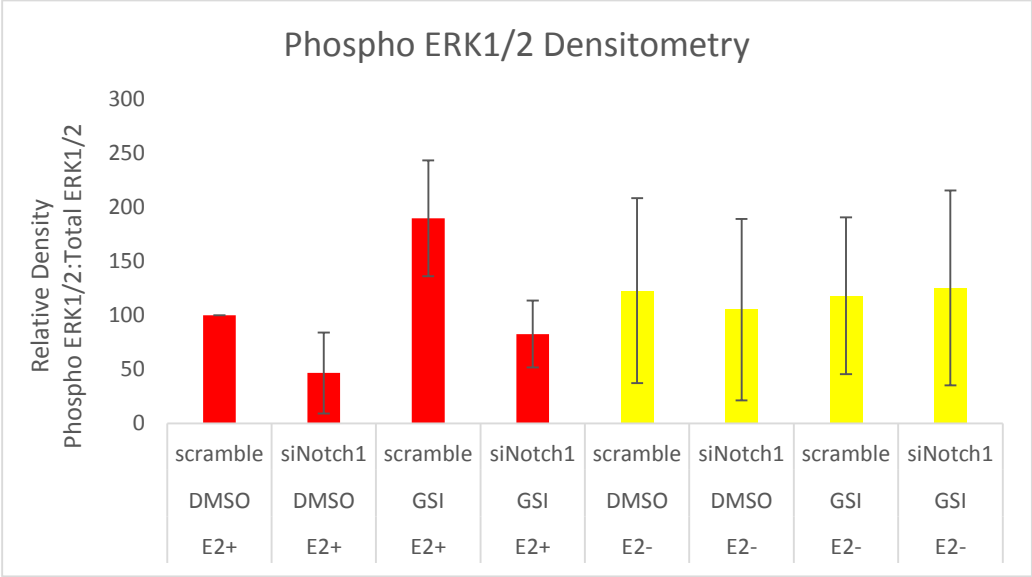


Figure 4-2. Effects of Notch1 knockdown, GSI administration, and estrogen deprivation on ERK1/2 phosphorylation. ERK1/2 phosphorylation is decreased upon Notch1 knockdown. ERK1/2 is elevated upon GSI administration unless Notch1 is concomitantly knocked down where is decreases to below negative control levels. Notch1 expression is decreased upon estrogen deprivation. The phospho ERK1/2: Total ERK1/2 ratio densitometry was calculated with ImageJ and expressed as mean ±standard deviation of three biological replicates.

Estrogen deprivation decreases overall expression of Notch1

We next sought to address the apparent decrease in Notch1 expression observed in our Western blots. Our data as well as data provided by others has shown that elevated levels of Notch1 are present on at the membrane when cells are grown in the presence of estrogen compared to estrogen-deprived growth conditions. At the same time, expression levels of Notch target genes increases upon estrogen deprivation. Taken together, these data suggest that γ-secretase activity is increased, which yields higher rates of Notch1 cleavage and subsequently higher levels of Notch target gene activation. However, an alternate hypothesis can be drawn to explain these findings. If estrogen deprivation causes an overall decrease in Notch1 expression while simultaneously causing an increase in the expression of another of the four Notch paralogs, then the results could be identical to those expected if estrogen deprivation causes an increase in Notch1 activity. If estrogen deprivation causes an increase in γ-secretase mediated Notch1 cleavage, then an increase in NICD1 protein levels can be expected under estrogen-deprived growth conditions.

The Western blotting technique employed allows the specific detection of NICD1. This is achieved because the epitope target of the Cleaved Notch1 primary antibody only binds to the N-terminal valine of NICD1, which is produced by γ-secretase cleavage. Our previous Western blots show a decrease in Notch1 when total protein is detected. However, this decrease may not account for NICD1 because once it translocates to the nucleus NICD1 is ubiquitinated and rapidly degraded by the 20S proteasome. Therefore, in order to observe the effects of estrogen deprivation on NICD1 levels, it is necessary to treat the cells with the 20S proteasome inhibitor, MG132. If estrogen deprivation causes an increase in the rate of Notch1 cleavage, then higher levels of NICD1 will be expected under estrogen-deprived growth conditions. Our results

demonstrate that contrary to this hypothesis, NICD1 levels decrease upon estrogen deprivation when stabilized by MG132. The loss of NICD1 expression upon GSI administration provides evidence that the band observed is indeed NICD1. Therefore, these results point to the alternate hypothesis that estrogen deprivation decreases the overall expression of Notch1.

The results from these experiments also provide support for our hypotheses with regard to the membrane-bound Notch1 requirement for ERK1/2 phosphorylation. If NICD1 is sufficient to induce ERK1/2 activation, then stabilizing NICD1 via MG132 administration may cause an increase in ERK1/2 phosphorylation. In the Western blot depicted, there is an increase in ERK1/2 phosphorylation when MG132 is administered. However, when NICD1 is inhibited via GSI administration, the increase in ERK1/2 phosphorylation is still observed indicating that the MG132 effect is not specific to NICD1. Whenever Notch1 is knocked down, ERK1/2 phosphorylation decreases in both the presence and absence of GSI and in the presence and absence of MG132. This suggests that the MG132-dependent effect on ERK1/2 phosphorylation is dependent on Notch1 but not in a way that requires Notch1 cleavage.

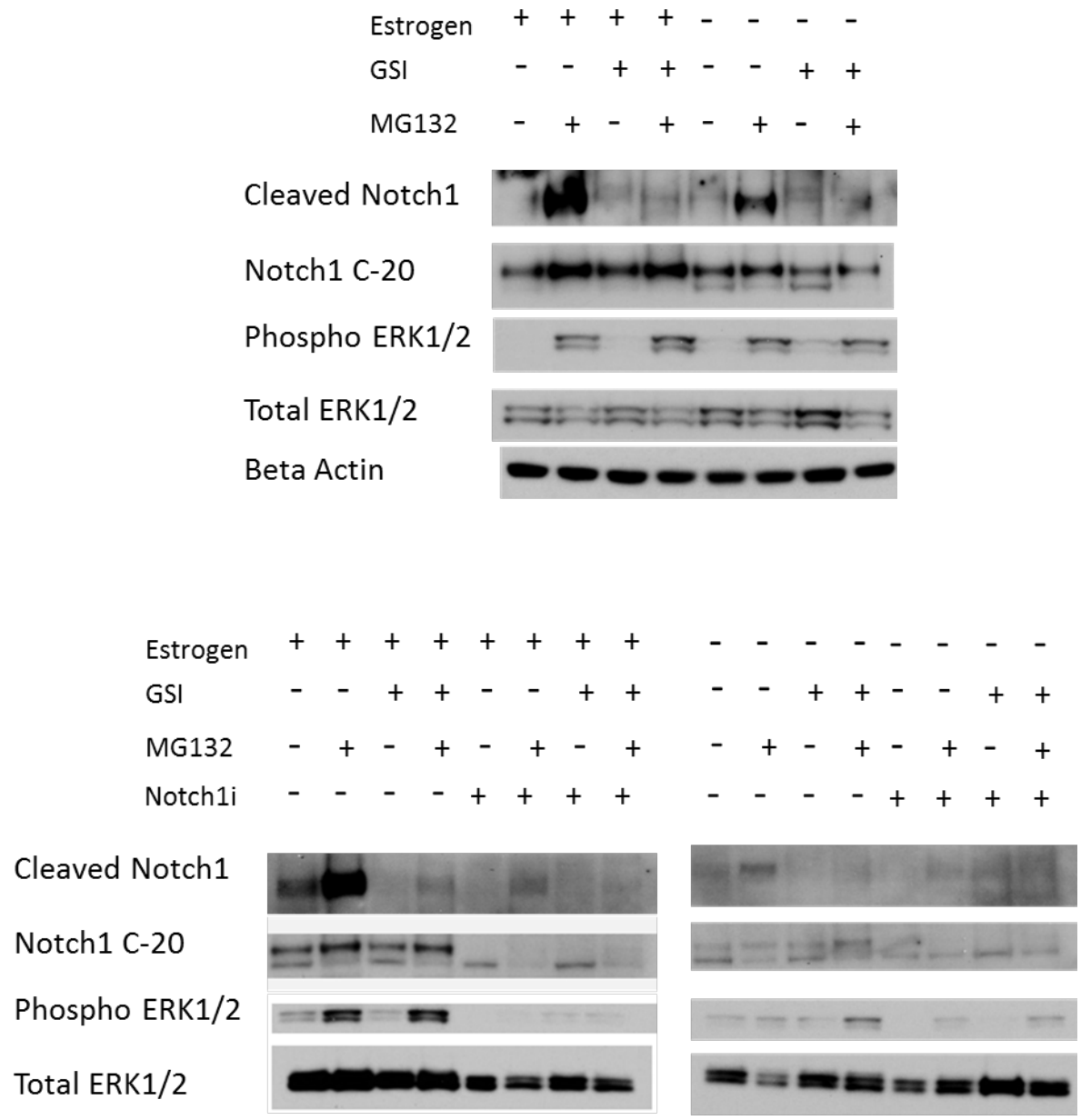


Figure 4-3. Effects of endogenous NICD1 on ERK1/2 phosphorylation. NICD1 can be stabilized by MG132 inhibition of the 20S proteasome and detected with the NICD1-specific antibody. GSI effectively inhibits NICD1 production. The Notch1 C-20 antibody detects membrane-bound Notch1. A decrease in membrane bound Notch1 and NICD1 is observed under estrogen deprivation. ERK1/2 phosphorylation increases upon MG132 administration. This increase is independent of estrogen deprivation and GSI administration, but the ERK1/2 increase is dependent upon Notch1 expression.

Notch4 expression increases upon estrogen deprivation.

Since putative Notch target genes are upregulated upon estrogen deprivation, this data suggests that other Notch receptors may be upregulated under these conditions. One receptor that others have shown that could be upregulated upon estrogen deprivation is Notch4. Data represented here demonstrate that Notch4 expression significantly increases upon estrogen deprivation. However, Notch target gene expression does not significantly decrease under estrogen deprived/Notch4 knockdown. This suggests that the increase in Notch4 alone cannot account for the increase in HES1 or HEY1 expression.

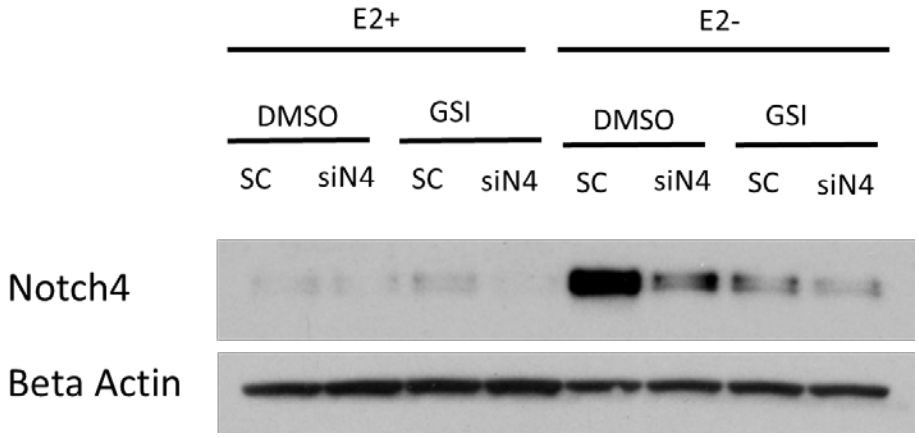


Figure 4-4. Notch4 expression is induced by estrogen deprivation. Notch4 expression is low when MCF-7 cells are grown in the presence of estrogen. Notch4 expression is induced by estrogen deprivation. This induction is decreased by Notch4 knockdown as well as GSI administration.

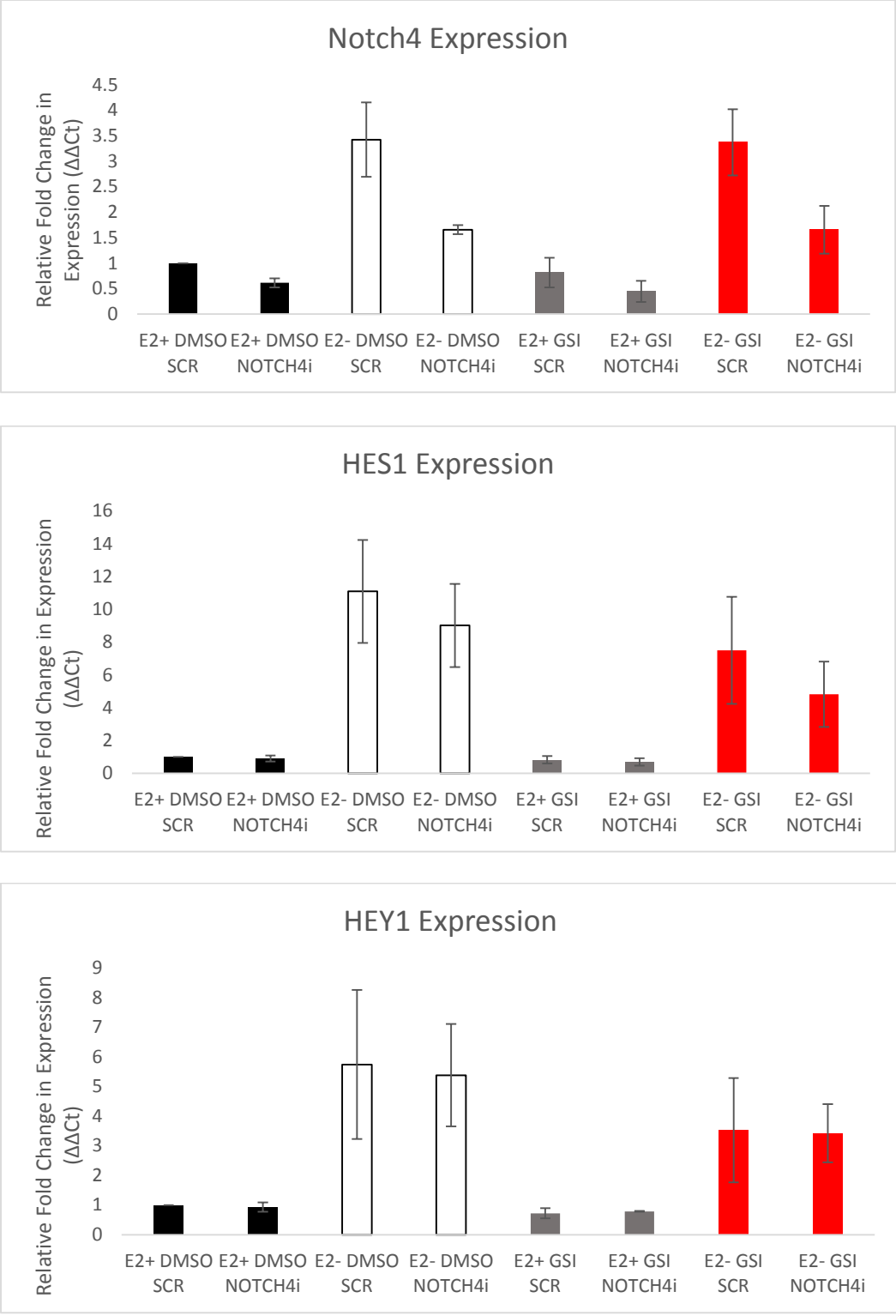


Figure 4-5. Effects of Notch4 knockdown, GSI administration, and estrogen deprivation upon Notch4 and Notch target gene expression. Notch4 expression as well as Notch target gene expression increase upon estrogen deprivation. However, Notch4 knockdown has no effect on target gene expression.

Aim 1B) Determine whether membrane-bound Notch1 is sufficient to activate ERK1/2 in an estrogen-dependent manner.

The results from Aim 1A suggest that the membrane-bound Notch1 receptor is necessary for ERK1/2 phosphorylation. These data also suggest that Notch1 is sufficient to induce ERK1/2 phosphorylation because when the GSI is administered, ERK1/2 phosphorylation is increased. However, since the GSI can inhibit the cleavage of multiple different membrane proteins, we set out to directly determine whether membrane-bound Notch1 is responsible for this increase in phosphorylated ERK1/2. If ERK1/2 phosphorylation can be induced by the ectopic expression of a membrane-bound Notch1 receptor, then it can be supposed that Notch1 is sufficient for ERK1/2 phosphorylation. The Notch1 construct that was expressed in these experiments mimics the ADAM cleaved NEXT domain, which is the substrate for γ-secretase. Thus, if membrane-bound Notch1 is necessary for ERK1/2 phosphorylation, then this construct will only induce phosphorylation if a GSI is present.

The results from the Western blot shows that in the presence of estrogen, the GSI induces ERK1/2 phosphorylation as seen in previous figures. Notch1ΔE is sufficient to induce ERK1/2 phosphorylation only when the GSI is coadministered. Overexpression of NICD1 is not sufficient to induce ERK1/2 phosphorylation. In the absence of estrogen, GSI administration is not sufficient to induce ERK1/2 phosphorylation. Interestingly, Notch1ΔE expression is sufficient to induce ERK1/2 phosphorylation regardless of whether the GSI is administered. However, NICD1 expression is not sufficient to induce ERK1/2.

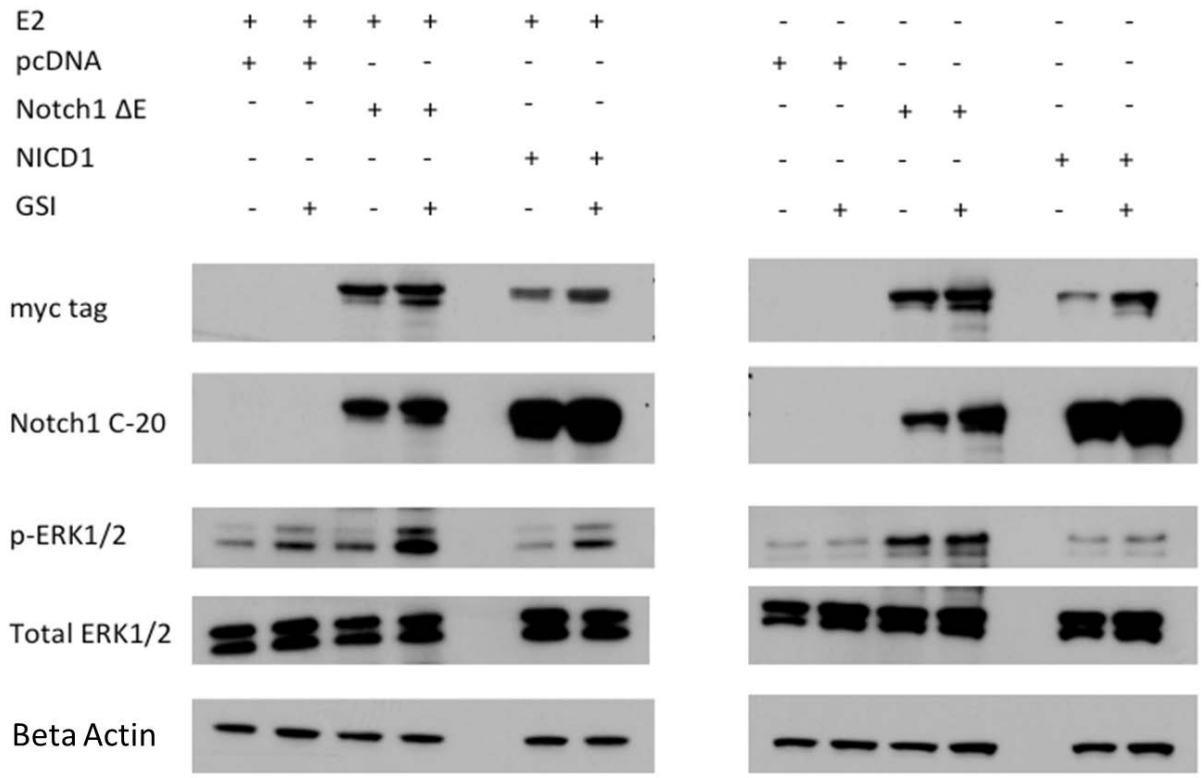


Figure 4-6. Membrane-bound Notch1 overexpression is sufficient to induce ERK1/2 phosphorylation when the GSI is co-administered. Both Notch1 ΔE and NICD1 are tagged with the myc epitope. Overexpression can also be detected via the Notch1 C-20 antibody. ERK1/2 phosphorylation can be induced by GSI alone as shown previously. Notch1 ΔE expression alone is not sufficient to induce ERK1/2 phosphorylation. GSI administration in combination with Notch1 ΔE overexpression is sufficient to induce ERK1/2 phosphorylation. NICD1 expression is unable to induce ERK1/2 above pcDNA transfected control conditions. In the absence of estrogen, GSI administration is not required for Notch1 ΔE induction of ERK1/2. However, NICD1 overexpression is not sufficient to induce ERK1/2 phosphorylation.

Specific Aim 2: Determine whether the Notch1-dependent effects on MCF-7 cell proliferation and survival are due to ERK1/2 activation.

The results from Aim 1 demonstrate that the membrane-bound Notch1 receptor is required for activation of ERK1/2. ERK1/2 is known to positively regulate cell growth and proliferation. Thus, our preliminary data and the data from Aim 1 circumstantially suggest that Notch1 and ERK1/2 interact in a noncanonical manner in order to promote MCF-7 growth and survival in an estrogen-dependent manner. Following this logic, the hypothesis can be drawn that if Notch1 knockdown causes a decrease in proliferation/survival in an ERK1/2 activation-dependent manner, then constitutive ERK1/2 activation will rescue proliferation/survival even in the absence of Notch1.

ERK1 R84S activates AP-1 luciferase even in the presence of MEK inhibitor.

The human ERK1 wild type and ERK1 R84S mutant were supplied to our lab in a bacterial expression vector. In order to express these proteins in our MCF-7 cells the inserts had to be cloned, restriction digested, ligated into a mammalian expression vector, pcDNA3.1+. In order to verify the expression, the ERK1 WT and ERK1 R84S plasmids were transfected into MCF-7 cells, and expression was analyzed via Western blot. In order to determine whether the ERK1 R84S mutant is constitutively active, the cells were treated with U0126, an inhibitor of the upstream MAP kinase kinase that activates ERK1/2. Previous publications demonstrate that the ERK1 R84S mutant is able to autophosphorylate itself independently of MEK activity. Our data demonstrate that U0126 can inhibit the phosphorylation of the ectopically expressed ERK1 WT, but is unable to inhibit the phosphorylation of ERK1 R84S. These data agree with previous reports wherein ERK1 R84S is constitutively phosphorylated suggesting that it is constitutively activated.

In order to demonstrate that constitutive ERK1 R84S phosphorylation confers constitutive activity, an AP1 luciferase assay was employed. Whenever ERK1 is phosphorylated, it in turn phosphorylates and stabilizes subunits of the AP1 family of transcription factors, namely c-Fos. In this experimental system, the AP1 consensus sequence (5'-TGA G/C TCA-3') is located upstream of the firefly luciferase gene in an ectopically expressed mammalian expression vector. If ERK1 R84S is indeed constitutively active, then it will activate AP1-mediated luciferase transcription even in the presence of U0126. Since ERα is known to activate the AP1 consensus sequence and recruit AP1, we conducted these experiments in the presence and absence of Estrogen. Estrogen depletion is known to increase the expression of ERα so in the absence Estrogen, AP1-driven luciferase transcription should increase compared to cells grown in the presence of Estrogen. Our data demonstrate that expression of ERK1 WT is insufficient to activate AP1 driven luciferase activity in the presence of Estrogen. This occurs in spite of the Western blot data, which demonstrates that phosphorylated ERK1 levels were higher in cells transfected with ERK1 WT. AP1 luciferase activity is elevated whenever the ERK1 R84S mutant is expressed even in the presence of U0126 confirming this mutant as a bona fide constitutively active ERK1. When the cells are deprived of Estrogen, the trend is consistent with that observed in cells grown in the presence of Estrogen. AP1 luciferase activity is elevated across all samples whenever cells are deprived of estrogen.

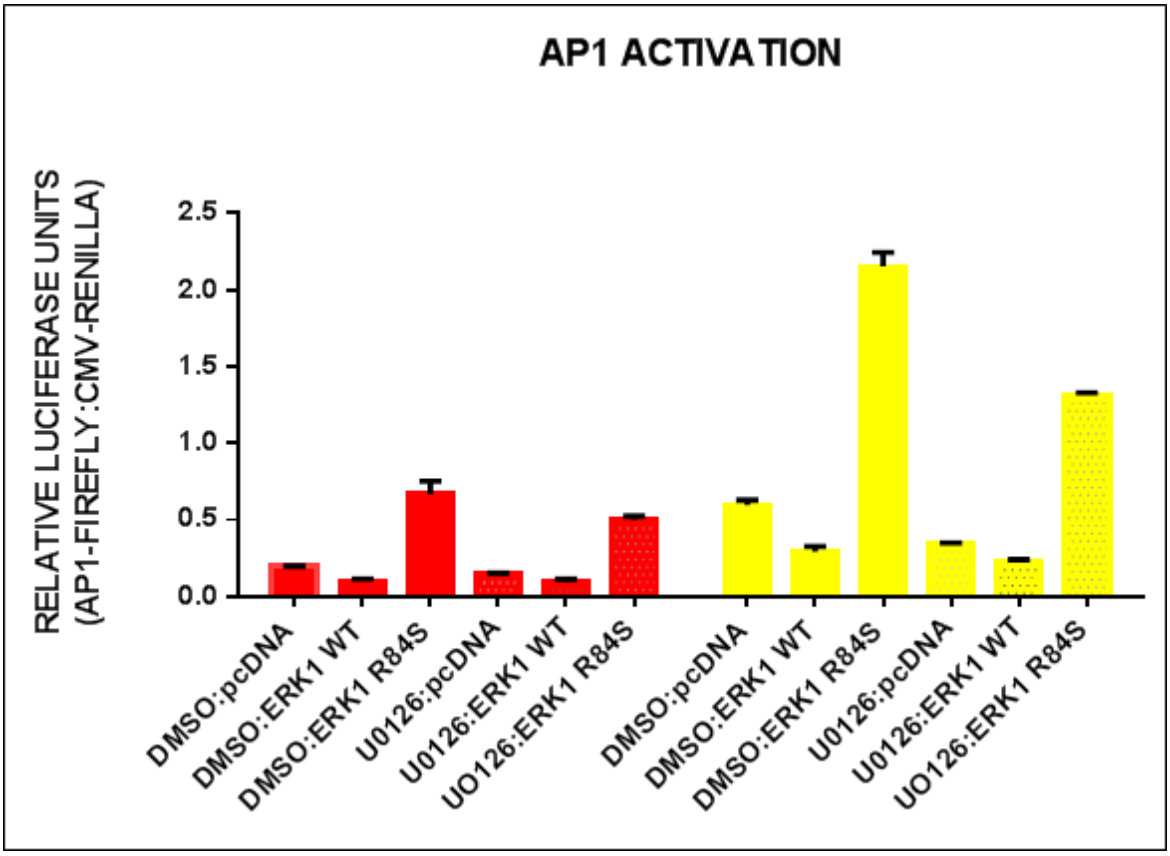


Figure 4-7. AP-1 luciferase activity upon ERK1 WT and ERK1 R84S transfection under E2+ and E2- MCF-7 cell culture conditions. ERK1 R84S is sufficient to induce AP-1 Luciferase activity even in the presence of the MEK inhibitor U0126. Estrogen deprivation induces AP-1 luciferase activity, and the ratio of AP-1 induction is similar to that seen when cells are grown in the presence of estrogen.

ERK1 R84S is not sufficient to constitutively phosphorylate p90 RSK.

In order to gather further supporting evidence that ERK1 R84S is a constitutively active mutant, we sought to determine whether the putative ERK1/2 substrate p90RSK is constitutively phosphorylated upon ERK1 R84S transfection. MCF-7 cells were transfected with ERK1 WT and ERK1 R84S in the presence or absence of the MEK inhibitor U0126. If ERK1 R84S is constitutively activate, then it will phosphorylate its substrate p90RSK even in the presence of U0126. The results demonstrate that though U0126 attenuates ERK1 WT phosphorylation, ERK1 R84S remains phosphorylated upon U0126 treatment. In the absence of U0126, p90RSK phosphorylation is decreased whenever ERK1 WT is expressed. ERK1 R84S has no effect on p90RSK phosphorylation compared to negative control transfection under DMSO treatment. U0126 inhibits p90RSK phosphorylation, and neither ERK1 WT nor ERK1 R84S are able to restore phosphorylation to levels seen under control conditions. This suggests that though ERK1 R84S may be sufficient to activate AP-1 mediated transcription, it may not be deemed fully constitutively active due to its inability to hyperactivate p90RSK or to activate p90RSK in the presence of the MEK inhibitory U0126.

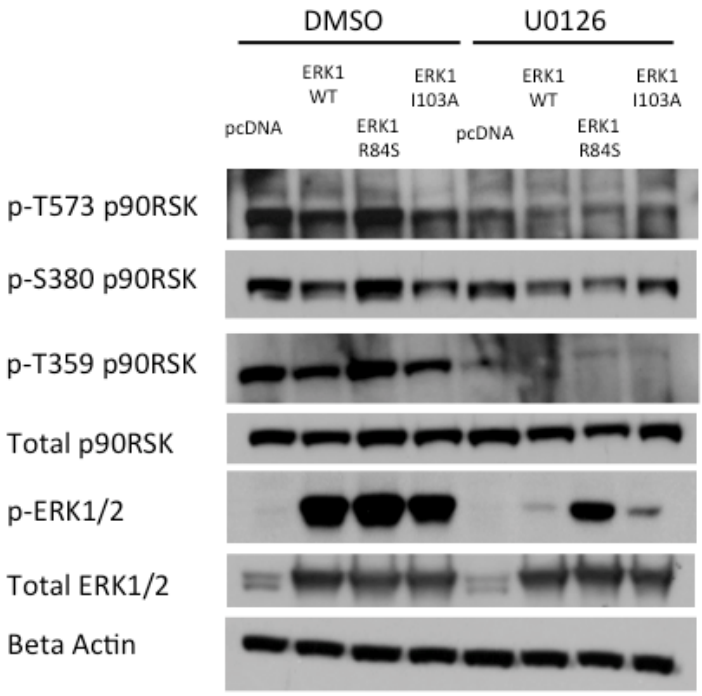


Figure 4-8. ERK1 WT, ERK1 R84S, and ERK1 I103A are not sufficient to constitutively activate p90 RSK. P90 RSK is phosphorylated at numerous serine and threonine residues. Antibodies against three of its most commonly phosphorylated residues were used to determine whether the ERK1 R84S or ERK1 I103A mutant can constitutively phosphorylate these putative sites. p90 RSK phosphorylation is not induced above negative control conditions upon ERK1 expression, and U0126 abolishes the ability of T573 and T359 to be phosphorylated altogether. However, this Western blot demonstrates that the ERK1 mutants are able to be expressed. Also, This blot shows that ERK1 R84S is able to be constitutively phosphorylated even in the presence of the MEK inhibitor, U0126.

ERK1 R84S is unable to rescue proliferation upon Notch1 knockdown.

We hypothesized that the loss of proliferation observed upon Notch1 knockdown was due to the subsequent loss of ERK1/2 activity. If the expression of a constitutively active ERK1 in Notch1 knockdown cells is able to rescue proliferation, then the loss of ERK1 upon Notch1 knockdown is responsible for the proliferation phenotype. We first conducted an experiment similar to our proliferation assays used for our preliminary data. MCF-7 cells were transfected with Notch1 siRNA in combination with ERK1 WT or ERK1 R84S and cell numbers were determined after seven days of continuous growth in culture. The results show that though Notch1 knockdown increases proliferation (as in the preliminary data), no benefit was conferred to cells that were co-transfected with ERK1 WT or ERK1 R84S.

We next sought to determine whether ERK1 could have an effect on proliferation at earlier time points. The cell growth assays described previously take place over a seven-day time span. The effects of transient plasmid transfection are known to diminish over time. Since the cells are being transiently transfected with the ERK1 constructs, effects rendered on proliferation after seven days may not be apparent. In order to observe effects at a 48 hour time point, the same transfection conditions were conducted but instead of cell counts, cells were monitored for perturbations in the cell cycle. DNA content was detected by fixing and permeabilizing the cells and staining with propidium iodide. Flow cytometry was used to determine DNA content and results are reported as percentages of cells in G1 phase, S phase, and G2 phase. Though not necessarily considered part of the cell cycle, cells in sub G0 were considered in these percentages as they represent cells with death-associated DNA fragmentation.

As expected, of the MCF-7 cells grown in the presence of estrogen and negative control transfection conditions, approximately 35% are in S phase, 50% are in G1 phase, 13% are in G2

phase, and 1% are in sub G0. When cells are deprived of estrogen, the percentage of cells in S phase significantly decreases to 15%, and cells in G1 phase significantly increase to 65% indicating that these cells undergo growth arrest upon estrogen deprivation. In the presence of estrogen, Notch1 knockdown causes a 5% decrease in the number of cells in S phase and approximately a 3% increase in cells in sub G0. In the absence of estrogen, there is also a 5% decrease upon Notch1 knockdown, but this difference is accounted for by a 5% increase in the number of cells in G1. Significant effects are observed upon estrogen deprivation and Notch1 knockdown. However, expression of ERK1 WT or ERK1 R84S did not effectively alter the cell cycle status of these cells.

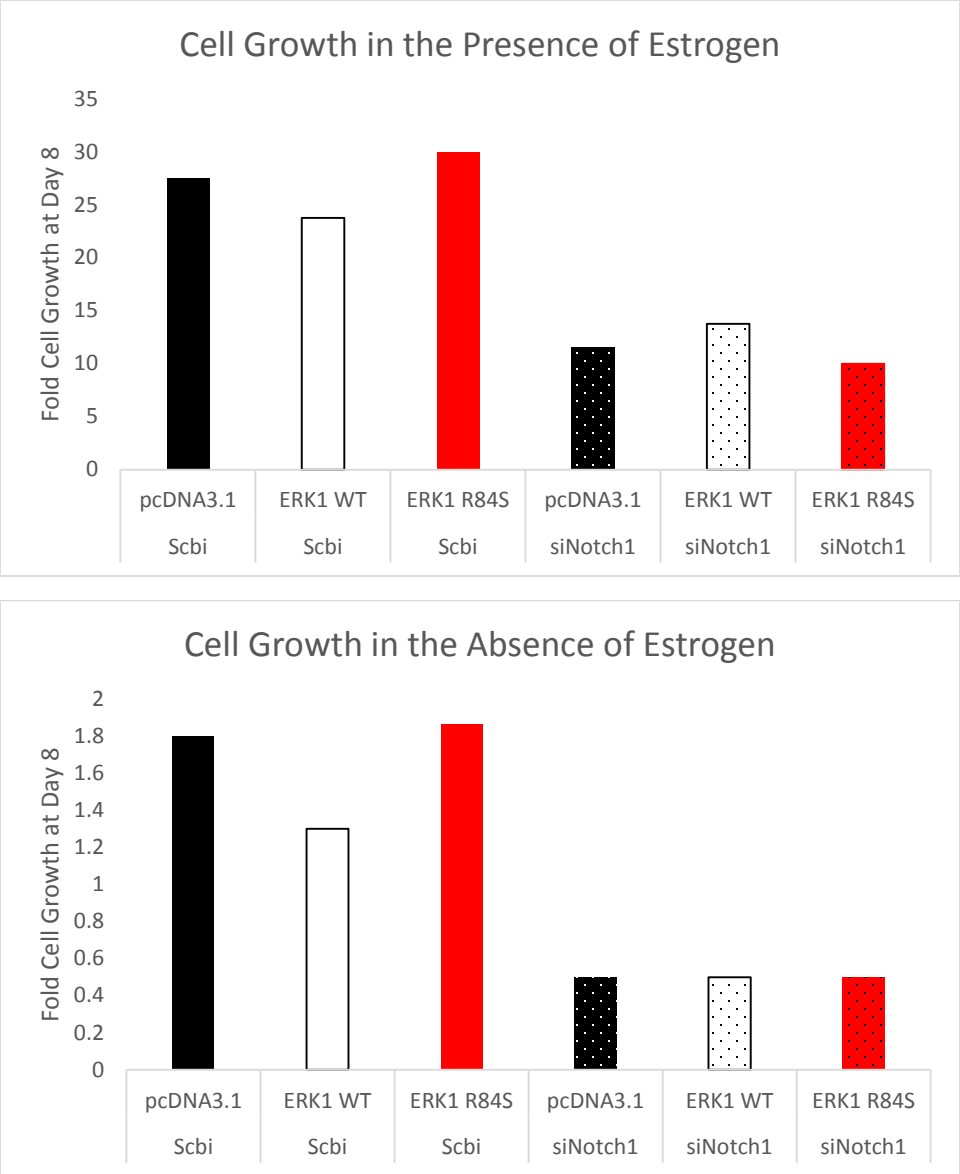


Figure 4-9. ERK1 WT and ERK1 R84S are unable to rescue MCF-7 growth on an 8-day time scale. At day 8 after transfection of Notch1 siRNA a decrease in cell number in cells transfected with Notch1 siRNA is observed. Expression of ERK1 WT or ERK1 R84S are unable to rescue this loss in cell survival.

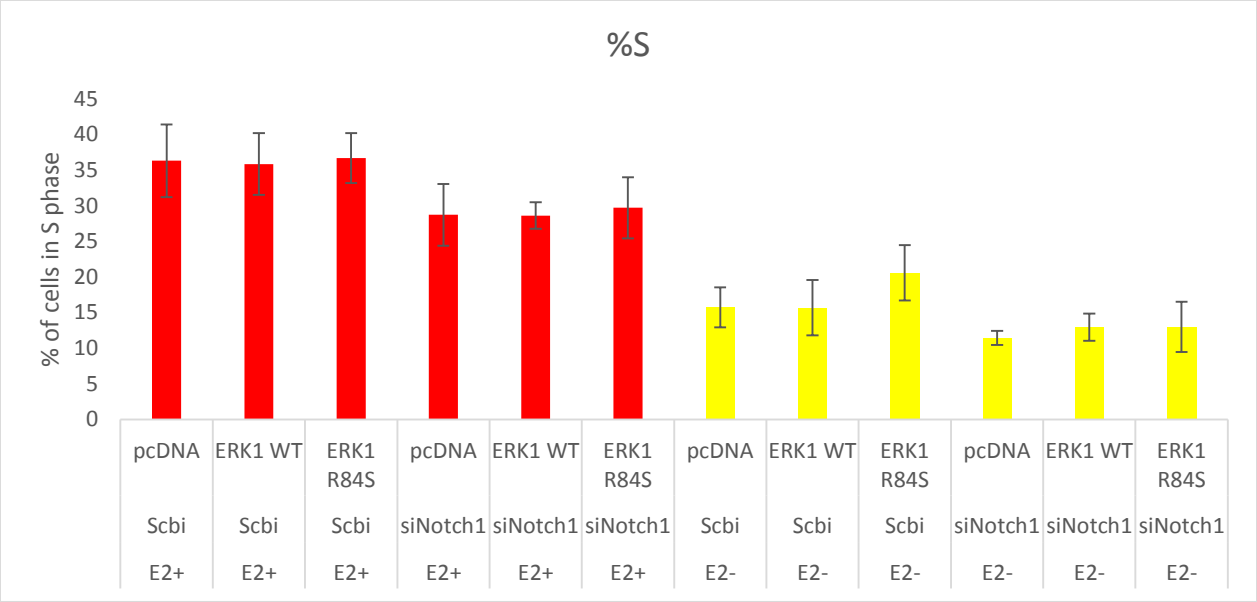


Figure 4-10. ERK1 WT and ERK1 R84S are unable to rescue the decrease in % MCF-7 cells in S-phase. Upon Notch1 knockdown, there is a decrease in the percentage of cells in S-phase of the cell cycle. However, no rescue is observed upon ERK1 WT or ERK1 R84S expression.

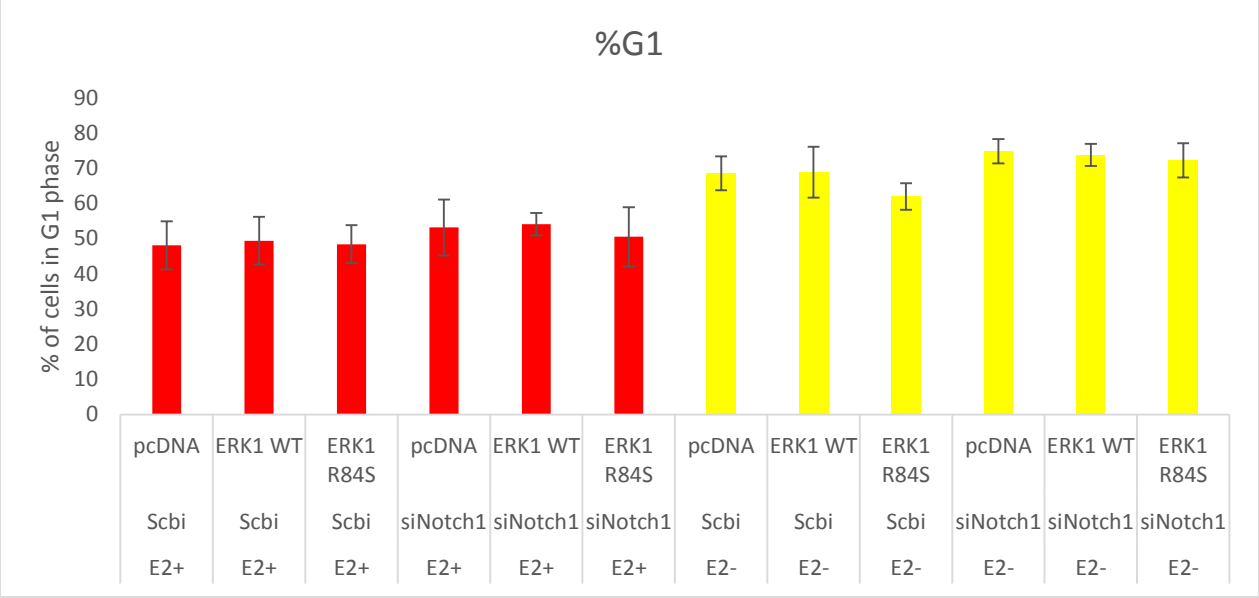


Figure 4-11. Neither Notch1 knockdown, nor ERK1 WT or ERK1 R84S expression has an effect on % cells in G1 phase of the cell cycle.

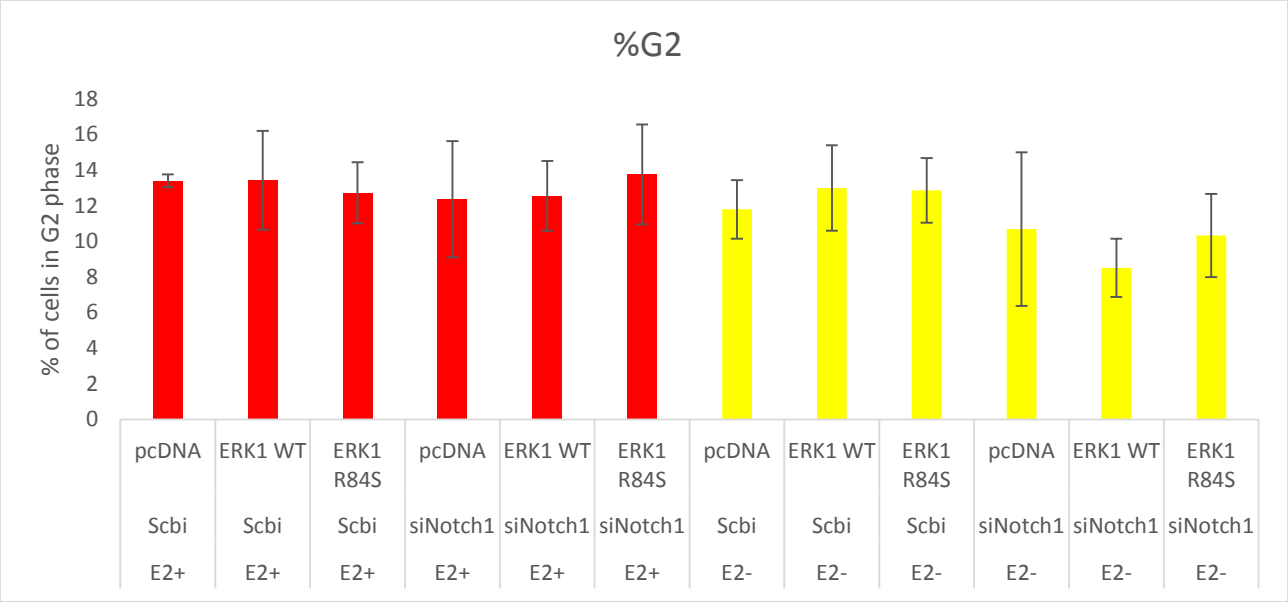


Figure 4-12. Neither Notch1 knockdown, nor ERK1 WT or ERK1 R84S expression has an effect on % cells in G2 phase of the cell cycle.

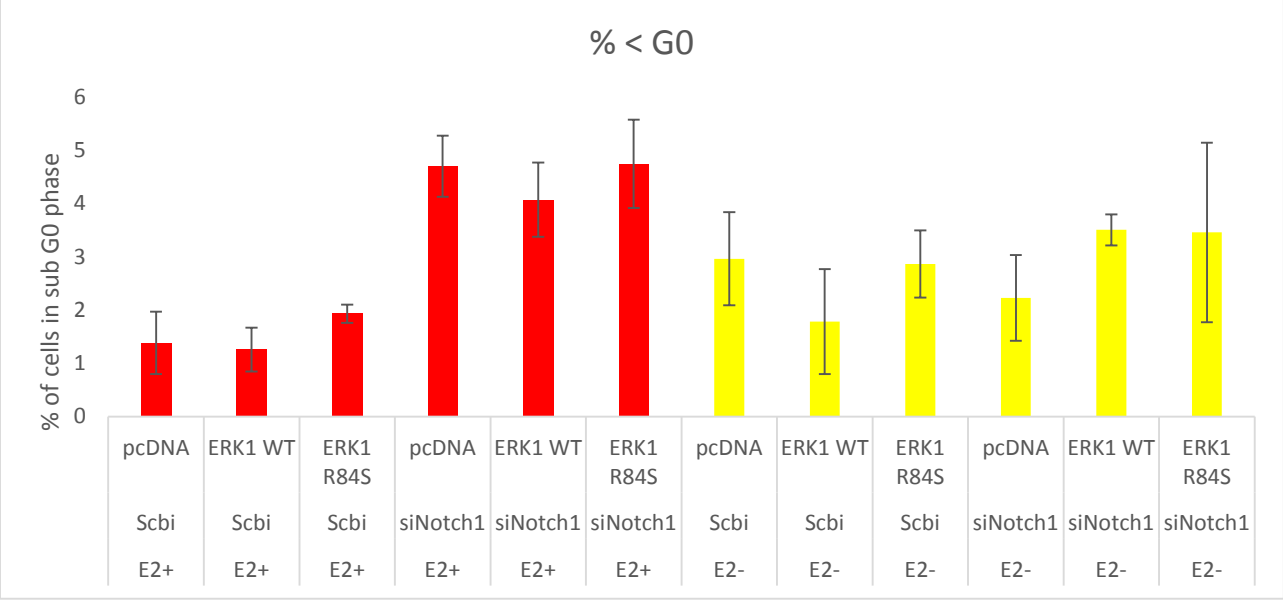


Figure 4-13. ERK1 WT and ERK1 R84S are unable to rescue the increase in % MCF-7 cells in sub-G0. Upon Notch1 knockdown, there is an increase in the percentage of cells in sub-G0 indicating cells undergoing death-related DNA fragmentation. However, no rescue is observed upon ERK1 WT or ERK1 R84S expression.

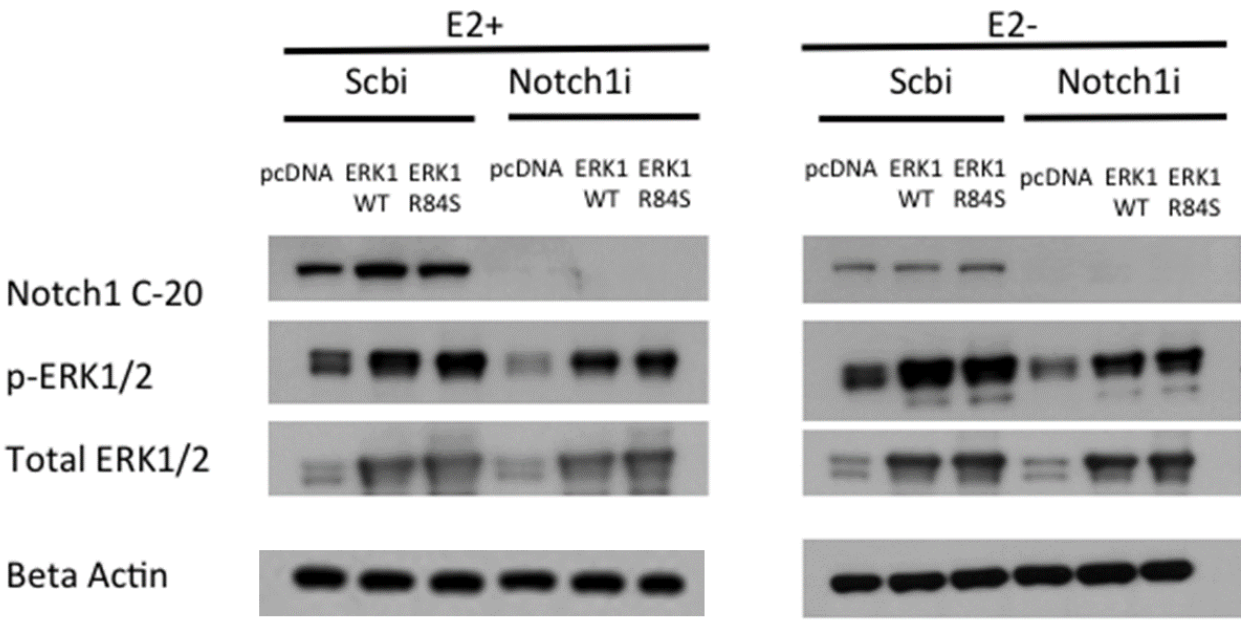


Figure 4-14. Validation of concomitant Notch1 knockdown and ERK1 overexpression.

ERK1/2 activity may inhibit mammosphere formation in a Notch1-dependent manner.

Our results suggest that ERK1/2 may not have an effect on MCF-7 cell survival and proliferation. However, the proliferation assays and cell cycle assays performed are suited to observing effects on the bulk population of cancer cells. Current research in breast cancer seeks to understand the dynamics of a small sub-population of cells termed the breast cancer stem cell (BCSC). The BCSC hypothesis states that a small subpopulation of cells bearing stem-like characteristics exists within a breast tumor. BCSCs are hypothesized to have low proliferation potential but high resistance to cell stress, characteristics that could circumstantially make BCSCs prone to drug resistance. Within a breast tumor population, it is possible to isolate cells which bear stem like characteristics. Stem-like cells bear molecular features such as high aldehyde dehydrogenase activity and CD44+/CD24- cell surface expression (Ricardo). Recent publications suggest that the Notch signaling pathway activity positively correlates with these molecular features. One of the key features of the BCSC is its ability to survive in an attachment-free environment. When suspended in a methylcellulose pseudomatrix, the majority of cells undergo anoikis. The cells that survive typically bear stem-like molecular features, and these cells differentiate and proliferate to give rise to spheroid structures termed mammospheres. The Notch signaling pathway is known to facilitate mammosphere formation in MCF-7 cells, and GSI administration inhibits mammosphere formation. Estrogen deprivation also facilitates mammosphere formation in a Notch-dependent manner.

Since ERK1/2 activity does not seem to have a significant effect on bulk cell proliferation, we hypothesize that ERK1/2 activation is required for BCSC survival. If ERK1/2 activity is required for BCSC survival, then a decrease in mammosphere forming efficiency (MFE) can be expected upon administration of U0126. If Notch1-dependent ERK1/2 activation

is required for this anticipated effect, then Notch1 knockdown will mimic U0126 administration. Mammosphere formation is typically low in MCF-7 cells when grown in the presence of estrogen but increases significantly upon estrogen deprivation. These experiments will be conducted both in the presence and absence of estrogen because an effect may not be observed if only conducted in the presence of estrogen.

Mammosphere formation increases upon U0126 administration when grown in both the presence and absence of estrogen. Notch1 knockdown has no effect on MFE when considered alone. However, in the presence of U0126, Notch1 knockdown rescues the increased MFE effect seen under negative control knockdown conditions in both the presence and absence of estrogen. These findings suggest that Notch1 inhibits mammosphere formation in an ERK1/2-dependent manner.

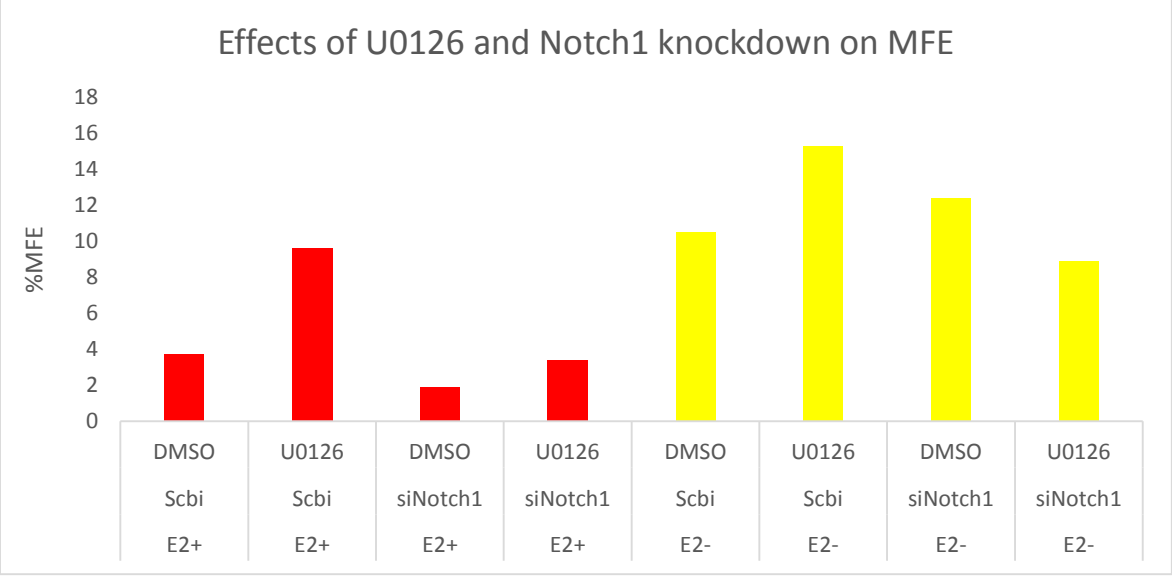


Figure 4-15. Effects of ERK1/2 inhibition and Notch1 knockdown on mammosphere formation. An increase in mammosphere forming efficiency (MFE) is observed upon MEK inhibition. This U0126 effect is rescued by Notch1 knockdown. This effect is observed when cells are grown under both the presence and absence of estrogen. Though, overall MFE increases under estrogen-deprived conditions.

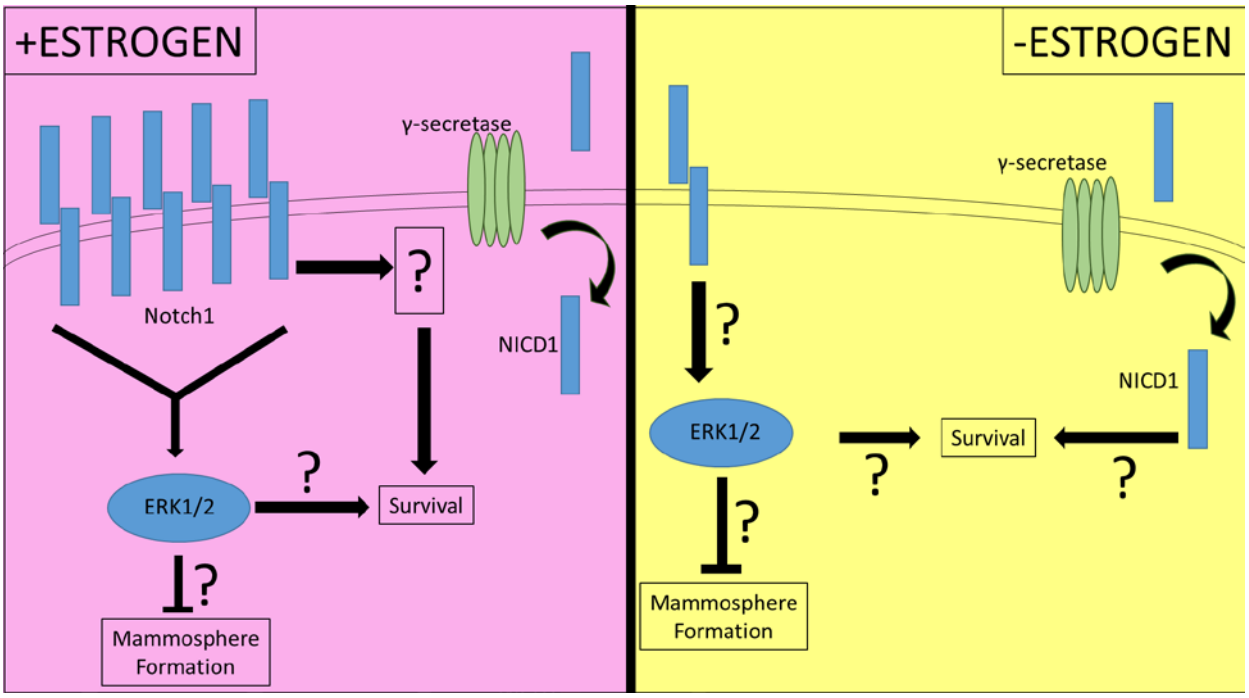


Figure 4-16. A model for Notch-1-mediated activation of ERK1/2 and the effects on cell survival. Our data suggest that in the presence of estrogen, membrane-bound Notch1 is necessary and sufficient for ERK1/2 activation. Our data also suggest that in the presence of estrogen, membrane-bound Notch1 is required for MCF-7 cell survival. However, our data do not demonstrate whether the membrane-bound Notch1 effect ERK1/2 phosphorylation is responsible for the membrane-bound Notch1 effect on survival. The constitutively active ERK1 mutant may be insufficient to fully address the hypothesis put forth. However, it is possible that membrane-bound Notch1 acts on cell survival through other pathways that were not explored. In the presence of estrogen, Notch1 is cleaved by γ-secretase, but NICD does not seem to affect cell survival or ERK1/2 phosphorylation. In the absence of estrogen, Notch1 expression is decreased, and Notch1 is required for cell survival, and γ-secretase activity is required for survival. However, it is not known whether the γ-secretase requirement is specifically due to NICD1. Due to inconsistency, our data neither suggest nor deny the ability of Notch1 to activate ERK1/2 in the absence of estrogen. However, our data suggest that ERK1/2 suppresses mammosphere formation in both the presence and absence of estrogen in a Notch1-dependent manner.

CHAPTER 5

DISCUSSION

Crosstalk between Notch1 and Estrogen signaling pathways

The current clinical standard of care for the treatment of estrogen receptor positive breast cancer is anti-estrogen therapy. The reason that we are interested in Notch1 signaling in breast cancer is because members of the Notch signaling pathway are dysregulated in breast cancer, especially when the disease recurs or acquires resistance. These correlations have led scientists to investigate the signaling mechanisms that could explain the relationship between estrogen signaling and Notch signaling. The key finding that is consequential to this work is that inhibition of estrogen signaling activates canonical Notch signaling. This finding is disconcerting because although anti-estrogen therapies are typically successful at debulking ER+ breast tumors, these drugs may be selecting for or even creating a subpopulation of cancer cells that are dependent on Notch signaling and independent of estrogen signaling. In light of this hypothesis, combination therapies have been proposed which include the administration of GSIs along wtith anti-estrogen therapy.

The results that were obtained from the preliminary proliferation assays are surprising. These results suggest that Notch1 may have an important role, which does not require activity along its canonical pathway. Some have suggested non-canonical roles for Notch proteins. However, suggested non-canonical roles for Notch typically involve γ-secretase cleavage. Our hypothesis suggests that interactions between Notch1 and other signaling factors are consequential to cell survival and proliferation even while Notch1 is still localized to the membrane. This thesis provides supporting evidence for this hypothesis. However, a key future

direction for this work is to gather more evidence to underpin an exact signaling mechanism to explain the Notch1 membrane-localization requirement.

Is Notch1 required for proliferation or survival?

A key question in explaining the effects seen upon proliferation is whether the effects seen are due to a lower proliferation rate or a higher death rate upon Notch1 knockdown or GSI treatment. This question is valid because either phenomenon could explain the results: on day 8, cells with a . For instance, with the cells grown in estrogen, the lower cell count observed eight days after Notch1 knockdown could be due to a lower rate of cell cycle progression, which here we are terming proliferation. However, if along the eight-day time course cells undergo death and detach from the culture plate, they would be lost because the media was replenished daily. Thus, either could explain these results.

Whenever the cells are deprived of estrogen, a different observation is made. Instead of the 70-fold increase in cell number seen in the presence of estrogen, cells cultured in estrogen-deprived media undergo growth arrest as evidenced by neither an increase or decrease in cell number. Whenever the cells are given a GSI or Notch1 knockdown, the number of cells drops below one after eight days of culture. Therefore, it can be assumed that cells are undergoing death when the GSI is administered or Notch1 is knocked down. Furthermore, we take this to mean that γ-secretase activity and Notch1 are required for survival of estrogen-deprived cells, not for their proliferation.

To summarize this section so far, we have assumed that Notch1 is required for proliferation when cells are grown in the presence of estrogen and that Notch1 is required for survival in the absence of estrogen. However, newer data suggests that these assumptions may not be true. The cell cycle analysis was performed in order to observe cell proliferation potential

on a shorter time scale. The purpose of the experiments was to observe potentially short-term effects elicited by our ERK1 mutant. Though we were unable to conclude that the ERK1 mutant had an effect on the cell cycle, the results from our controls revealed some interesting clues as to the effect of Notch1 on the cell cycle. In the presence of estrogen, the only statistically significant difference between control knockdown and Notch1 knockdown conditions is the percentage of cells in <G0. As mentioned previously, <G0 is not a phase of the cell cycle. The cells in this population are characterized by their fragmented DNA, an indicator that these cells have undergone some form of cell death. This data suggests that the decrease in cell number seen in our preliminary proliferation assays is due to high cell mortality and not low cell proliferation when Notch1 is knocked down. In order to confirm this finding, more detailed analysis will need to be performed. An AnnexinV staining was performed in order to determine whether cells undergo apoptosis under Notch1 knockdown. However, no differences were observed between control and Notch1 knockdown conditions (data not shown). Several forms of cell death exist, and a variety of studies would need to be performed to determine whether the observed DNA fragmentation is due to apoptosis, necrosis, senescense, anoikis, etc.

Does estrogen deprivation activate canonical Notch1?

Two observations combine to suggest that when MCF-7 cells are deprived of estrogen, γ-secretase-mediated cleavage of Notch1 increases and induces its canonical activity. First, when MCF-7 cells are grown in the presence of estrogen, membrane staining of Notch1 is high, and Notch1 staining decreases upon estrogen deprivation. Second, canonical Notch target gene activation is observed upon estrogen deprivation. This suggests that in the absence of estrogen, Notch1 is cleaved from the membrane whereby it translocates to the nucleus and activates its target genes. However, the finding that both total Notch1 protein levels as well as NICD1 levels

decrease upon estrogen deprivation confounds this explanation. As an alternative, we have data suggesting that the observed decrease in Notch1 expression coincides with an increase in Notch4 expression. Therefore, an alternate hypothesis would be that if estrogen deprivation decreases Notch1 expression and increases Notch4 expression, then Notch4 activates canonical Notch target gene expression in absence of estrogen. This would provide an interesting avenue of future research because this could refine our understanding of the dynamics between estrogen signaling and Notch signaling. Furthermore, it could enhance our ability to effectively administer a combination of anti-estrogen and GSI therapy to ER+ breast cancer patients, which could in turn yield improved outcomes for cancer patients.

Membrane-bound Notch1 is necessary and sufficient for ERK1/2 phosphorylation in the presence of estrogen.

Taken together, the proliferation assays and cell cycle assays suggest that membrane-bound Notch1 is required for cell survival whenever cells are grown in the presence of estrogen. In order to provide further evidence for this, we performed a series of Western blots that take advantage of the ability of the GSI to stabilize Notch1 at the membrane. The Western blots depicted in Figure 4-2 demonstrate that in the presence of estrogen, ERK1/2 phosphorylation is decreased upon Notch1 knockdown. However, his effect could be due to either membrane-bound Notch1 or γ-secretase cleavage-dependent NICD1 activity. When the GSI is administered, an increase in ERK1/2 phosphorylation is observed. This increase could be explained by an increase in membrane-bound Notch1. However, due to the multiple off-target effects that can be attributed to GSI administration, GSI administration alone does not specifically address whether this effect is due to Notch1. When the GSI is administered under Notch1 knockdown conditions, a decrease in ERK1/2 phosphorylation is observed compared to DMSO/Scbi control conditions.

Since Notch1 knockdown specifically targets Notch1, it can be reasoned that the increased ERK1/2 phosphorylation observed under GSI/Scbi conditions are attributed to the membrane stabilization of Notch1.

The observations made whenever the MCF-7 cells are grown in the presence of estrogen are consistently reproducible. One consistent observation made is that Notch1 expression decreases whenever cells are grown in the absence of estrogen. However, results obtained for ERK1/2 phosphorylation in the absence of estrogen are inconclusive due to high variability of ERK1/2 phosphorylation observed when estrogen is deprived.

This finding has a number of implications and possible explanations. Since Notch1 knockdown specifically targets Notch1 mRNA for degradation, it can be alleged that Notch1 is required for MCF-7 survival. However, these data do not confirm the role of γ-secretase inhibition under estrogen-deprived conditions. It is possible that the GSI inhibits proliferation by inhibiting the classical function of Notch1. However, these proliferation data do not rule out the possibility that Notch1 knockdown and γ-secretase inhibition act independently on proliferation. Thus, it is possible that Notch1 knockdown and GSI have a greater effect on cell proliferation because Notch1 knockdown specifically targets Notch1 and the GSI inhibits other pro-proliferative targets besides Notch1.

The ERK1 R84S mutant is not fully constitutively active.

In the paper describing the ERK1 R84S mutant, the authors used AP-1 luciferase activity and phosphorylated ERK Western blots as biochemical means to demonstrate that this mutant is constitutively active. Those findings were recapitulated in this work. Furthermore, our data demonstrates that ERK1 R84S activates AP-1 luciferase and maintains phosphorylation of ite TEY activation motif even when cells are treated with the MEK inhibitor U0126. An extra step

was taken in order to determine whether this mutant is able to act on other of its various substrates. AP-1 luciferase is activated when members of the AP-1 transcription factor dimerize and bind to their cognate DNA consensus sequences. Phosphorylated ERK1/2 facilitates this process by phosphorylating and stabilizing AP-1 members such as c-fos, which assume transcriptional activity upon dimerizing with other AP-1 members. ERK1/2 has numerous other substrates besides c-fos and in turn has the ability to influence other cellular processes. One well-described substrate that operates independently of c-fos is p90 RSK. In spite of the observed AP-1 luciferase activity, ERK1 R84S is unable to induce phosphorylation of the three p90 RSK residues that are putatively targeted by ERK1/2. These experiments tested a second ERK1 mutant, ERK1 I103A. This mutant was shown by others to bear enzymatic activity even in the absence of ERK1 TEY motif phosphorylation. Nonetheless, expression of this mutant in MCF-7 cells did not induce p90 RSK phosphorylation. Though ERK1 R84S and ERK1 I103A may be able to constitutively stabilize the AP-1 dimer, this mutation does not confer full constitutive activity to ERK1/2.

The ERK1 R84S mutant is unable to rescue the effects that Notch1 has on MCF-7 survival and cell cycle.

If the Notch1 requirement for cell survival is dependent upon its ability to activate ERK1 or ERK2, then expressing a constitutively active ERK1 or ERK2 under Notch1 knockdown will rescue the loss of survival. Whenever we conducted these experiments, we were unable to rescue cell survival with either the ERK1 WT or ERK1 R84S mutant. Though it is possible that Notch1-mediated ERK phosphorylation is not required for cell survival, other explanations for these observations exist. For instance, though ERK1 R84S is sufficient to induce AP-1 mediated gene expression, the effect of Notch1 on survival may not be through AP-1. P90 RSK activation is

known to promote cell survival, and we show that ERK1 R84S does not induce p90 RSK phosphorylation above negative control transfection conditions. Therefore, if p90 RSK lies downstream of the Notch1→ERK1/2 pathway, then expression of this mutant would not be expected to rescue survival under Notch1 knockdown.

It is possible that the techniques and materials used are not sufficient to address the role of Notch1/ERK1/2 in cell survival. However, if we assume that the Notch1→ERK1/2 signal is not required for cell survival, then a question remains to be answered: what is the biological role of the Notch1→ERK1/2 signal if not for cell survival? In order to address this question, we must first refine the definition of survival that we have used thus far. Two techniques are used to quantify survival of these cells, cell counting and flow cytometric cell cycle assay. These techniques account for the entire population of cells grown in culture, which we now distinguish as bulk proliferation.

When quantifying the proliferation of a bulk population of cells, one must not be misled by the assumption that all of the cells are equal. Notably, mounting evidence from experiments performed in breast cancer cell lines such as MCF-7 suggests that a BCSC subpopulation exists as described in the Results section. Since we were able to demonstrate the connection between Notch1, ERK1/2, and bulk cell survival, the mammosphere experiment was conducted to determine whether Notch1-mediated ERK1/2 activation has a role in BCSC survival. We hypothesized that if ERK1/2 activation is required for BCSC survival, then ERK1/2 inhibition via U0126 administration or Notch1 knockdown will decrease mammosphere forming efficiency (MFE). Our results demonstrate that MFE increases upon U0126 administration, which is contrary to our expectation. Since Notch1 is required for ERK1/2 phosphorylation, then Notch1 knockdown is expected to mimic the effects of U0126. However, whenever Notch1 is knocked

down, no effect on MFE is observed. Furthermore, Notch1 knockdown rescues the increased MFE observed upon U0126 administration. This suggests that Notch1 and ERK1/2 do interact to affect MFE. Our original hypothesis proposes that ERK1/2 is downstream of Notch1 and that Notch1 and ERK1/2 should interact to promote BCSC survival. However, this data suggests that Notch1 inhibits mammosphere formation and that ERK1/2 lies upstream of Notch1.

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VITA

Jeffrey Clay Bloodworth was born on June 19, 1987 to Clay and Starr Bloodworth in Griffin, GA. At the age of three, he moved to Big Point, MS where he spent his childhood. He is the younger brother of Selena Eppinger and the older brother of Trent Bloodworth. He graduated from East Central High School in Hurley, MS in 2005.

Jeffrey attended college at the University of Mississippi where he majored in Biology and Biochemistry. Jeffrey conducted research in two labs while at Ole Miss. Under the direction of Brad Jones, Ph.D. he performed immunohistochemistry on Drosophila embryos for the purpose of understanding neuronal versus glial cell fate decisions. Under the direction of Susan Pedigo, Ph.D. he cloned the human epidermal cadherin gene and the secreted aspartic protease gene from *Candida albicans* and purified the respective proteins in order to investigate host-microbe interactions. He graduated in 2011 with a B.S. in Biology and a B.A. in Biochemistry. In October 2011, Jeffrey accepted a position as a Research Assistant at The Ohio State University Division of Pharmacology. Under the direction of Nam Lee, Ph.D., Jeffrey was involved in a number of projects which sought to understand the roles of TGF-β signaling in tumor vascular endothelium.

In August 2014, Jeffrey joined the Molecular Biology and Biochemistry Program at Loyola University Chicago. He joined the lab of Clodia Osipo, Ph.D. in November of 2014.