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LOYOLA UNIVERSITY CHICAGO

ROLE OF DAXX AS A PREDICTIVE BIOMARKER IN BREAST CANCER

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

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ABSTRACT

Breast cancer is the most commonly diagnosed form of cancer in women, besides skin cancer, and second only to lung cancer for cause of mortality. It is divided into three subtypes, depending on different molecular or pathologic characteristics, including estrogen or progesterone receptor-positive (ER/PR+), human epidermal growth factor receptor-2-positive (HER2+) and triple negative breast cancer (TNBC). There are several therapies used to target these subtypes, but there is still a chance that the cancer will recur into a more aggressive, resistant form even if the therapies were successful before. This recurrence is believed to be due to the Cancer Stem Cell (CSC) hypothesis which states that within the heterogeneous breast cancer tumor, there is a population of CSCs that are responsible for resistance to therapy and tumor recurrence. Evidence shows that Notch signaling, a pathway regulating several cellular processes, could be the reason these CSCs survive, so new therapies are being developed to target Notch signaling. The problem is, that Notch gene targets are not enough to provide a predictive response in breast cancer, so new potential biomarkers have been identified to predict a response to therapy—such as DAXX protein. This protein, which has been associated in pro-apoptotic pathways and gene expression repression, has been shown to be inhibiting bulk cell proliferation in cell lines representative of all three breast cancer subtypes. In a HER2+ and TNBC cell line this has been shown even in the presence of a Notch inhibitor. Because of this, it was believed that DAXX expression may be inhibiting Notch signaling.

Results show that DAXX expression is required to limit proliferation of TNBC and other subtypes such as the HER2+ (BT474) and not ER+ (MCF-7). Interestingly, DAXX depletion using a DAXX siRNA decreases sensitivity of breast cancer cells to standard of care therapy in the TNBC subtype, MDA-MB-231 cells, only. In other TNBC cell lines, this is not the case—showing just how heterogenous this subtype is. Further, DAXX appears to be potentially regulating PARP activity in MDA-MB-231 cells, which is key to stabilize cells that have some type of DNA damage, since depletion of DAXX results in increased PARP-1 and PAR chains expression.

These results indicate that DAXX expression plays a role in limiting proliferation. The mechanism by which this occurs is largely unknown. However, preliminary findings demonstrate that a DNA damaging agent such as Carboplatin may induce JNK phosphorylation and activation. This activation of JNK seems to be dependent on DAXX expression in MDA-MB-232 cells. We assessed whether targeting JNK would be a novel therapeutic strategy in combination with carboplatin. Results showed that the JNK inhibitor (SP600125) partially prevents the increase in cell proliferation upon DAXX depletion. Results together suggest: 1. DAXX expression limits proliferation of most subtypes of breast cancer; 2. DAXX expression is required for drug sensitivity to standard of care but this differs in TNBC subtypes; 3. DAXX activates JNK to limit proliferation in the mesenchymal stem-like subtype of TNBC; and 4. DAXX may regulate poly(ADP) ribosylation (PAR) of PARP1 to possibly regulate the DNA damage response.

CHAPTER I

INTRODUCTION

Breast Cancer

Breast cancer is the most commonly diagnosed form of cancer in women, followed by skin cancer, and second only to lung cancer for cause of mortality (Akram, Iqbal, Daniyal, & Khan, 2017; Desantis, Ma, Bryan, & Jemal, 2014). It is a disease that is characterized, like all other cancers, as uncontrolled growth of cells due to mutations or abnormal expression changes of genes that promote proliferation (Majeed et al., 2014). These hyper-proliferative cells are localized to the luminal or basal cells within the breast organ. The disease is primarily caused by inherited mutations in genes, such as BRCA1 or BRCA2, or by other factors such as obesity, alcohol, hormone therapies or increased breast tissue density (Emens & Jaffee, 2005; Majeed et al., 2014). However, thanks to new screening tools, the mortality rate of breast cancer for women in developed countries has significantly declined since cancers are being detected earlier (Akram et al., 2017). Screening for the disease includes several diagnostic exams such as mammograms, ultrasounds, magnetic resonance imaging (MRI), computerized axial tomography (CAT) scans, and position emission tomography (PET) scans. If there is an abnormal mass found, then a biopsy is used to confirm cancer, subsequently leading to its pathological and molecular characterization. Upon characterization, breast cancer can be divided into various subtypes, dependent on certain pathologic or molecular characteristics of cancerous cells (Akram et al., 2017). There are five known subtypes of breast cancer: normal-like, luminal A, luminal B, HER2-positive (HER2/

ERBB2+), and triple negative (Anderson, Rosenberg, Prat, Perou, & Sherman, 2014). Nearly 70% of breast cancer cases are Luminal A and B. These cancers express estrogen and or progesterone receptors (ER/PR) and express wildtype levels of HER2. The proliferation of Luminal A and B breast cancers are mainly driven by the female steroid hormone, 17β-estradiol (Anderson et al., 2014; Dai et al., 2015). The HER2+ subtype represents 15-25% of breast cancers. In these cancers, there is an overexpression of the human epidermal growth factor 2 (HER2) protein which is due to amplification of the *ERBB2* proto-oncogene (Anderson et al., 2014; Fragomeni & Sciallis, 2018). The triple negative or also referred to as basal-like breast cancers lack expression of ER/PR and overexpression of HER2 and represent nearly 10% of diagnosed breast cancers (Anderson et al., 2014; Dai et al., 2015). The final subtype, normal-like, has similar features to Luminal A but is considered a benign disease.

Estrogen Receptor Positive Breast Cancer and Signaling Pathways

In both females and males, hormones play a key role in developing and maintaining normal sexual and reproductive function. Estrogen, specifically, or 17β-estradiol (E_2) has biological effects as a key regulator in various systems and tissues of the body such as mammary glands, cardiovascular, musculoskeletal, immune, and central nervous systems (Hall, Couse, & Korach, 2001; Heldring et al., 2019). There are two nuclear receptors that mediate cellular signaling of E_2: ERα and ERβ. Both receptors exhibit a high degree of homology thus they interact with the same DNA response elements referred to as estrogen response elements (ERE) to mediate transcriptional regulation. ERα is primarily expressed in the breast, cervix, and vagina, while ERβ expression is much more limited to the ovary, prostate, testis, spleen, lung, hypothalamus, and thymus (Couse et al., 1997; Hall et al., 2001). There are four distinct pathways in which estrogens and the ERs regulate cellular processes [(summarized in **figure 1** (Heldring et al., 2019)]. The classical ligand

dependent pathway can be divided into two different yet similar pathways—direct or tethered. In the direct ligand dependent pathway, ER is sequestered in an inhibitory complex with heat shock proteins in the cytosol until the hormone binds (Hall et al., 2001). Once E_2 binds within the ligand binding domain, the monomeric receptor undergoes a conformation change releasing heat shock proteins. The ER forms a dimer as an ERα or ERβ homodimer or an ERα/β heterodimer. Once dimerization is facilitated, the receptor is in its active form and can enter the nucleus, bind its ERE, recruit coregulators including histone acetyltransferases to initiate transcription. The tethered ligand dependent pathway is similar to the classical pathway, except that the active ER binds to another transcription factor, such as AP-1, and helps direct AP-1-driven transcription (Hall et al., 2001). The non-genomic pathway also requires ligand binding to activate a receptor. However, in this pathway, the "ER" is a non-nuclear, membrane bound receptor referred to as G Protein-coupled estrogen receptor (GPER). In this pathway, the ligand, E_2 binds the GPER at the plasma membrane or at the mitochondrial membrane initiating a rapid kinase cascade including the mitogen-activated protein kinase (MAPK) to induce a physiological response without regulating gene expression (Heldring et al., 2019). Finally, the ligand independent pathway utilizes various growth factors, such as epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1), to activate the ER in the absence of E_2. Either growth factor receptors can activate a cascade of intracellular kinases to initiate phosphorylation of the ER, resulting in dimerization, nuclear translocation, and binding to ERE to regulate transcription of genes in an E_2-independent manner (Hall et al., 2001; Heldring et al., 2019). Proliferation of ER+ breast cancer is thought to be promoted mainly by the classical pathway. However, the other pathways have been shown to promote drug resistance to targeted therapies.

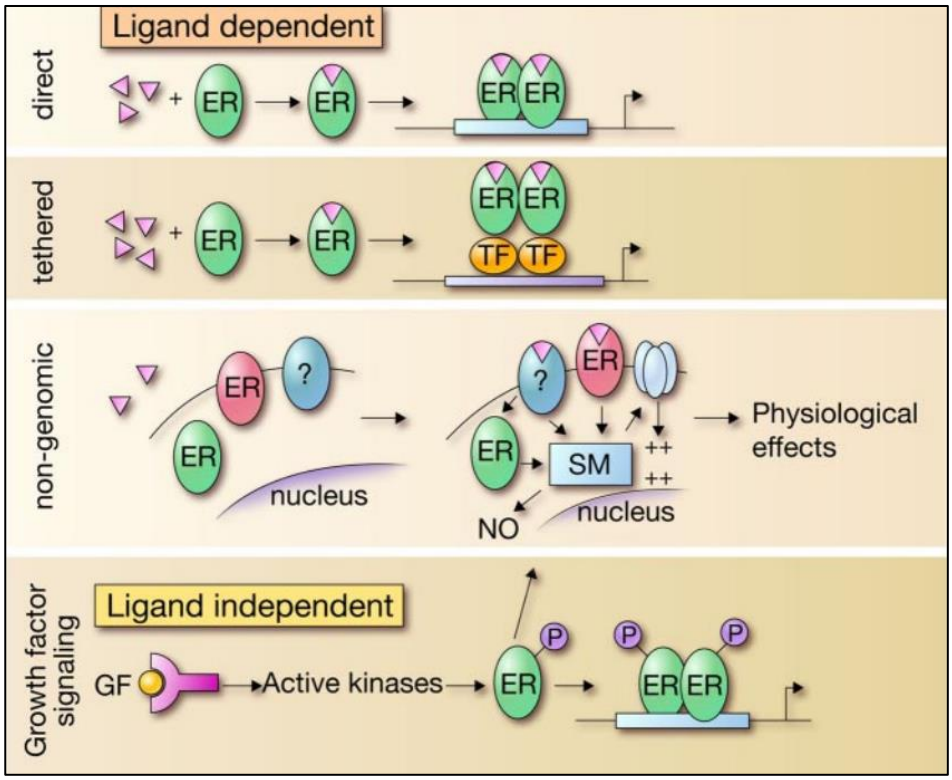

Figure 1: Model Representing Four Molecular Pathways Used by Estrogen Receptors to Regulate Genes or Physiological Processes.

Clinical Therapies Used to Treat ER+ Breast Cancer

Current clinical therapies to treat women with ER+ breast cancer include endocrine therapy (ET) that target the synthesis of the ligand, estrogen or antagonize the ER through direct competitive inhibition. As a first line therapy for women over the age of 50, the synthesis of estrogen is targeted using aromatase inhibitors (AI). The enzyme, aromatase catalyzes the conversion of androgens into estrogens therefore, inhibiting this process will deplete the amount of estrogen available to interact with ER and has shown to do so effectively (Chen et al., 2007). Examples of AIs include letrozole and anastrozole. However, long term exposure to this form of ET eventually results in endocrine resistance, thus other therapies must be explored as second line therapies to effectively treat the cancer. To directly target the ER, two types of antagonists are

used: selective estrogen receptor modulators (SERM) or selective estrogen receptor degraders (SERD). SERMs are cytostatic agents that modulate ER by competitively binding to the ligand binding domain, inhibiting tissue specific estrogenic effects (Kaklamani & Gradishar, 2017). Examples of SERMs include tamoxifen and raloxifene. Tamoxifen, a non-steroidal molecule, was the first SERM to be approved for ER+ breast cancer (ref: Jordan) and more recently is first line therapy for women under the age of 50 (ref: Rugo). SERDs are pure antagonists due to their structural similarity to estrogen and their ability to promote the degradation of ER and subsequently down regulate ER activity (Kaklamani & Gradishar, 2017). SERDs are categorized as steroidal (i.e. fulvestrant), acrylic acids (i.e. AZD9496) or bases (i.e. arzoxifene) (Mcdonnell, Wardell, Norris, & Biology, 2016). For a patient with ER+ breast cancer, the type of therapy they receive is heavily dependent on menopausal status and can be a combination of the therapies described.

Human Epidermal Growth Factor Receptor 2 Breast Cancer and Signaling Pathways

The human epidermal receptor tyrosine kinase-2 (HER2) is a type I, transmembrane, receptor tyrosine kinase (RTK) and the second member of the HER family of RTKs which includes HER1 (EGFR), HER3, and HER4. The HERs can homo- or hetero-dimerize upon growth factor binding. Dimerization initiates auto- and trans-phosphorylation of tyrosine residues within the intracellular tyrosine kinase domain (Hsu & Hung, 2017). These phosphorylated tyrosines recruit src-homology-2 domain (SH2)-containing adaptor proteins that initiate activation of several kinase cascades including PI3K/AKT, Ras/MEK/ERK, PKC, and JAK/STAT to promote cell survival, proliferation, differentiation, motility, apoptosis, invasion, migration, adhesion and angiogenesis, as shown in **figure 2** (Hsu & Hung, 2017; Nautiyal, Kanwar, & Majumdar, 2010). HER2 is considered an orphan receptor that does not need a growth factor to facilitate activation. As it is

overexpressed in breast cancer, it can homodimerize with itself or heterodimerize with other members (Veeraraghan et al., 2017). Although this family of proteins are critical for regulating normal cellular processes, a consequence of dysregulating these processes is the development of cancers. In HER2+ breast cancer, the *ErbB2* proto-oncogene is amplified, resulting in an overexpression of HER2 protein at the plasma membrane (Fragomeni & Sciallis, 2018). This overexpression is sufficient to initiate breast cancer development. HER2+ breast cancer is an aggressive form of breast cancer that frequently presents as metastatic with poorer survival rates than ER+ breast cancer (Baselga, Coleman, Janni, & Kettering, 2017; Veeraraghan et al., 2017).

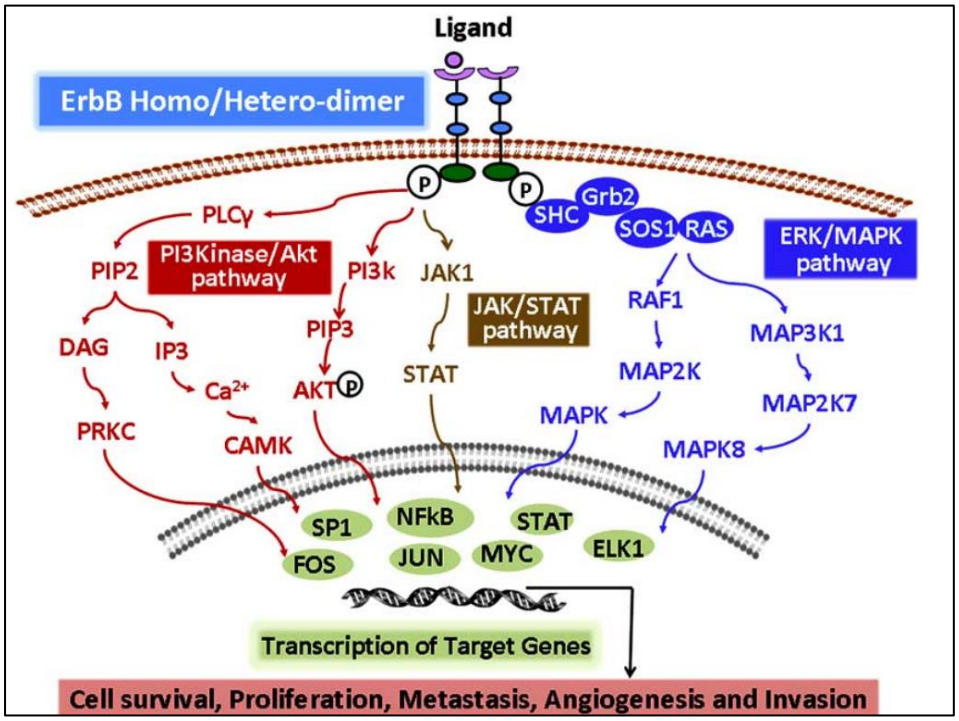

Figure 2: The Various Signaling Pathways Initiated Upon Activation of HER (ERBB) Receptor Proteins. The binding of the ligand leads to homo or heterodimerization of the receptors which will activate several pathways. These pathways then result in cellular processes that are related to maintaining homeostasis. Depending on which hetero or homodimer is formed will dictate which pathway is activated.

Clinical Therapies Used to Treat HER2+ Breast Cancer

To treat HER2+ breast cancer, current therapies include first line use of humanized, monoclonal antibodies: trastuzumab and pertuzumab. These antibodies bind to different ectodomains of HER2 thus inhibiting dimerization, promoting receptor-mediated endocytosis, downregulation of the receptor, decreased signaling, proliferation, and survival (Hsu & Hung, 2017). Second line therapies are used when resistance to antibodies occurs. These include tyrosine kinase inhibitors (TKIs) such as lapatinib (Hurvitz, Gelmon, & Tolaney, 2017; Segovia-mendoza, González-gonzález, Barrera, & Díaz, 2015) or neratinib (Baselga et al., 2017; Hsu & Hung, 2017; Hurvitz et al., 2017) TKIs are small molecules that mimic ATP. They competitively bind within

the kinase domain of HER2 preventing ATP hydrolysis and thus tyrosine phosphorylation (Segovia-mendoza et al., 2015). The goal of anti-HER2 targeted therapy is inhibit signal transduction pathways downstream of HER2 (Baselga et al., 2017). These compounds are most effective when combined with chemotherapy agents such as capecitabine or paclitaxel. Unlike lapatinib, neratinib is a pan-inhibitor because it interacts with the domain of several members of the epidermal growth factor receptor (EGFR) family (Segovia-mendoza et al., 2015). Neratinib is more effective than lapatinib.

Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is molecularly characterized as lacking expression of ER, PR, and overexpression of HER2. (Pal, Childs, & Pegram, 2011). When compared to the other breast cancer subtypes, TNBC is more aggressive, presents as an advanced stage disease, and has an unfavorable or low prognosis (Fragomeni & Sciallis, 2018; Pal et al., 2011). TNBC tumors are poorly differentiated making them more difficult to routinely diagnose due to heterogeneity and have a high frequency to metastasize (Bianchini, Balko, Mayer, Sanders, & Gianni, 2016; Jitariu, Cîmpean, & Ribatti, 2017). They develop primarily in pre-menopausal women and progress rapidly. Histologically, most of TNBC's are invasive mammary carcinomas of no specific type, meaning they do not have any specific differentiating features and iare molecularly characterized into several subtypes (Lehmann et al. 2011, Bianchini et al., 2016). It is very difficult to characterize TNBC as there are no known targets that predict outcome and response. This subtype is subdivided into at least 6 different categories, increasing the complexity of this subtype in general (Lehmann et al., 2011). There have been some characterization of TNBC suggesting that critical pathways are hyperactive. The PI3K/AKT/mTOR pathway is one of the main signaling pathways that is responsible for a number of cellular events including proliferation

and survival. Approximately 10% of TNBC have mutations genes within in the PI3K pathway (Massihnia et al., 2016; Oualla et al., 2017). There is also evidence that the mTOR pathway is more frequently activated in TNBC compared to other subtypes and that this activation correlates to poorer prognosis (Massihnia et al., 2016).

Clinical Therapies Used to Treat Triple Negative Breast Cancer

Since TNBC doesn't have many defining targets, such as ER or HER2, the primary form of targeted therapy are DNA-damaging, tubulin-disrupting, cytotoxic agents in combination with radiation therapy. Chemotherapies include platinum agents, such as cisplatin and carboplatin, anthracyclines, such as doxorubicin, daunorubicin, epirubicin and idarubicin and taxanes, such as paclitaxel and docetaxel (Mcgowan et al., 2017). Platinum agents take advantage of the fact that cancer cells have poor DNA damage repair pathways. This provides the opportunity to promote more DNA damage with the goal of accumulating enough DNA damage to induce cell death (Bianchini et al., 2016). Cells that cannot properly repair this damage undergo programmed cell death or apoptosis. Anthracyclines are a class of cardiotoxic drugs derived from the bacterium, *Streptomyces peucetius*. This drug intercalates DNA by binding with topoisomerase enzymes to form a topoisomerase-doxorubicin-DNA complex that will cause double stranded breaks in the DNA, again taking advantage of the fact that these cancerous cells have poor DNA damage repair pathways (Mcgowan et al., 2017). Taxanes are a class of diterpenes (large organic molecules) derived from plants of the genus Taxus (Laurentiis & Press, 2015). Taxanes are mitotic inhibitors; they function by disrupting microtubule function therefore inhibiting the process of cell division, ultimately leading to cell death (Sibaud et al., 2017). As previously mentioned, the PI3K/AKT/mTOR pathway is hyper-activated in some TNBC, so now there are also therapies being generated to inhibit this pathway. DNA repair mechanisms are also being targeted because

reducing this activity would increase and maintain DNA damage, inducing apoptosis. PARP enzymes are key in these repair mechanisms. TNBC that contain mutations in key DNA repair genes, BRCA1 and BRCA2, are sensitive to PARP inhibition. Therefore PARP inhibitors are being developed to target TNBC with these DNA-repair deficiencies (Oualla et al., 2017).

DNA Damage Repair Mechanisms

Although there are some potential new therapies being generated, a primary form to target cancers, mainly TNBC, is DNA damaging agents. Cancerous cells have defective DNA damage repair mechanisms, making these therapies a very effective way to combat the disease. In a healthy cell, there are five main repair DNA repair pathways which resolve single strand or double strand breaks in the DNA (Chatterjee & Walker, 2018). Base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) are pathways involved in single stranded breaks. BER is the primary mechanism used to remove base alterations in the DNA (Chatterjee & Walker, 2018; Gomes, Menck, & Leandro, 2017). Once the damage or alteration is identified, a DNA glycosylase excises the base and apurinic/apyrimidinic endonuclease 1 (AP1) cleaves the phosphodiester chain at an AP site. DNA polymerase fills in the newly formed gap and a ligase, such as DNA ligase 3 and XRCC1 (X-ray cross complementing) (Gomes et al., 2017; Shafirovich & Geacintov, 2018; Whitaker, Schaich, Smith, Flynn, & City, 2017). NER is similar to BER, but this repair mechanism is used when there is a bigger portion of the DNA to removed and repaired, such as when there is a lesion that distort the DNA double helix (Shafirovich & Geacintov, 2018). Platinums used in chemotherapy take advantage of the fact that cancerous cells do not have effective NER repair mechanisms, since the mechanism of action of the drug is to form adducts or cross links in the DNA with the drugs themselves

(Bianchini et al., 2016). Two different NER repair systems exist: global genomic NER (GG-NER) and transcription coupled NER (TC-NER)—which occurs when either the double helix distortions made by lesions are sensed or when RNA polymerase is stalled because of lesions, respectively (Shafirovich & Geacintov, 2018). Although there are two forms of NER initiated, the overall basic process is still conserved. Upon damage recognition, signaling within the cell will recruit NER factors (TFIIH, XPA, XPF, and XPG) that will excise almost 30 nucleotides on the damaged strand, before DNA polymerase fills in what was removed (Abbotts & Wilson III, 2017; Shafirovich & Geacintov, 2018). In MMR, mismatched bases that arise after replication are repaired. Mismatch repair proteins, MutS homolog (MSH), recognize the site in which the incorrect base is in place; in humans this protein is the heterodimer MSH2/MSH6 (MSHα) which recognizes the mismatch and 1-2 addition bases or the heterodimer MSH1/MSH2 (MSHβ) which recognizes the mismatch and more than 2 bases (Chatterjee & Walker, 2018). The mismatched base, and surrounding bases, on the nascent strand are incised by MutL homologs (MLH) and DNA polymerase δ or ε synthesizes the new strand, followed by nick sealing (Carolina, Hill, & Carolina, 2017; Chatterjee & Walker, 2018).

For double stranded breaks, the DNA repair mechanisms include homologous recombination (HR) and canonical nonhomologous end joining (c-NHEJ). HR, the more complex pathway to repair a DSB, uses an undamaged sister chromatid to repair the DNA and does so to refrain from losing any of the sequence and several motor proteins to complete the repair process. First, 3’ overhangs are created by resecting parts of the DNA with several nucleases such as MRE11-RAD50-NBS complex (an endo/exonuclease) that resects 3’-5’ towards the break and exonuclease 1 (EXO1) which resects 5’-3’ away from the break (Shibata, Jeggo, & Löbrich, 2018). Utilizing these overhangs, Replication Protein A (RPA) binds to the

DNA in order to prevent spontaneous reannealing. Helicase protein BLM unwinds the DNA and WRN promotes branch migration to allow for the recombinase RAD51 to mediate strand invasion and resolve the DSB (Kaniecki, Tullio, Greene, Universitaria, & Biophysics, 2018; Shibata et al., 2018). c-NHEJ, on the other hand, is mediated by Ku heterodimer proteins, that dimerize at the DSB (Shibata et al., 2018). This dimerization recruits DNA-dependent protein kinase catalytic subunits (DNA-PKcs) that create a complex with recruit other proteins such as XRCC, which stabilize the ends of the break. XRCC then forms a complex with DNA ligase IV to ligate the ends and finish the repair (Shibata et al., 2018). ***Figure 3*** depicts pictorially these pathways discussed.

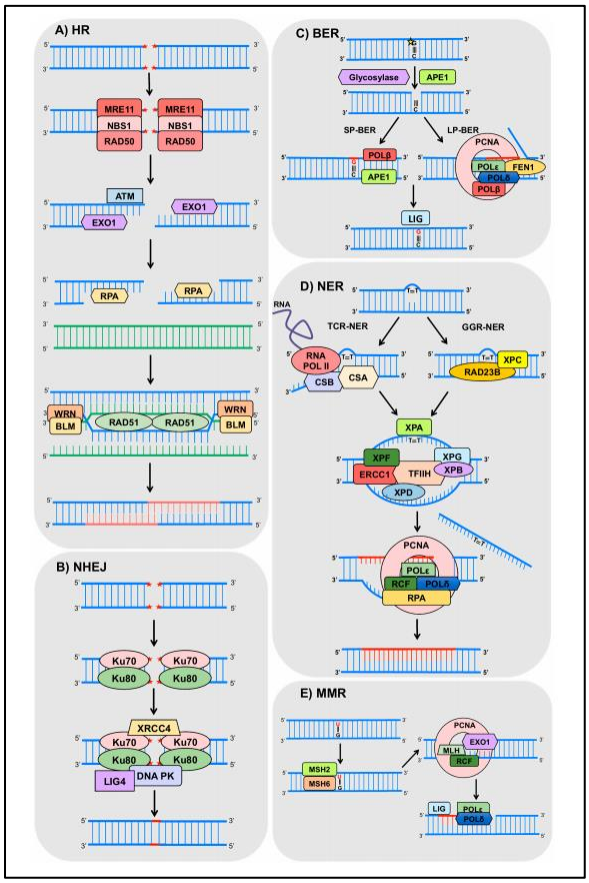

Figure 3: Schematic Summarizing Five Main DNA Repair Mechanisms.

Cancer Stem Cell Hypothesis

Drug resistance is a major cause of tumor relapse, metastatic spread, and mortality for women with breast cancer. There is a hypothesis that recurring cancers arise from a subpopulation of cells that have undergone oncogenic transformations that have acquired resistance to the initial treatment (Celi, 2018). The subpopulation of cells are able to persist after treatment, and eventually self-renew, giving rise to differentiated or stem-like daughter cells. A key pathway that promotes survival of these persistant cells is Notch signaling (Dontu et al., Celi, 2018; Reya, Morrison, Clarke, & Weissman, 2001; Shima, Yamada, Ishikawa, & Endo, 2017). These cells are referred to as cancer stem cells (CSCs) and are thought to be a small population of cells within a heterogeneous tumor. These CSCs are capable of driving tumor growth, progression and metastasis because of the stem-like properties they possess (Celi, 2018; Charafe-Jauffret et al., 2008; Kakarala & Wicha, 2008; Morrison, Schmidt, Lakhani, Reynolds, & Lopez, 2008; Shima et al., 2017). Because breast cancers are molecularly complex, the cancer stem cell hypothesis, depicted below in **figure 3**, has gained attention in the field as a means to explain why breast cancer models and treatments are becoming more and more limited (Kakarala & Wicha, 2008). Classically, it was believed that all cells in a cancerous tissue arise from mutations, making the cells present equally malignant. However, current stem-cell biology studies are now sufficient to directly test and attempt to support this hypothesis: a heterogeneous tumor contains several cell types, including those that have stem-like properties (Charafe-Jauffret et al., 2008; Kakarala & Wicha, 2008). As more information about CSCs come to light, so does the clinical implications these cells in regards to treatment resistance. New targeted therapies are being developed to inhibit survival of CSCs in combination with current standard of care with the goal of eliminating the entire tumor and preventing recurrence (Reya et al., 2001). However, there are severe side effects

associated with targeting pathways such as Notch due to both normal stem-cells and CSCs use the Notch pathway.

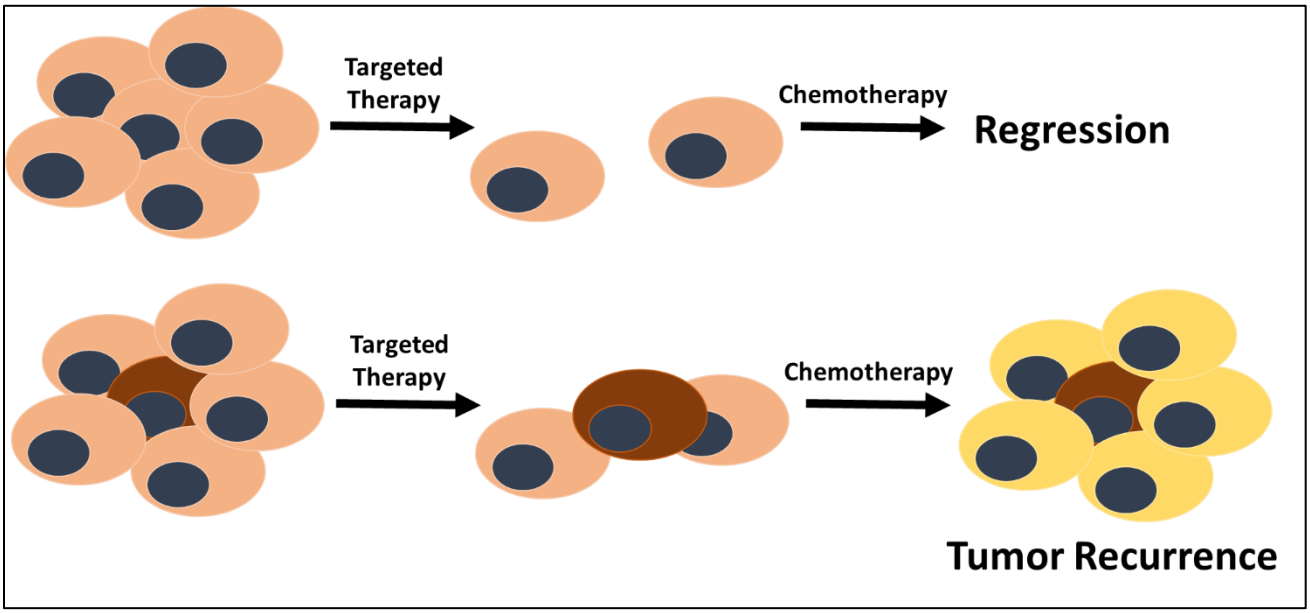

Figure 4: Summary of the Cancer Stem Cell Hypothesis. In certain bulk tumors, there are cancer cells that are sensitive to a specific therapy, depending on which type of Breast Cancer that tumor is. After targeted therapy, the tumor shrinks, and the remaining cancer cells are eliminated through chemotherapy, leading to regression of the tumor (top). However, this is not always the case in breast cancer treatment, and it is now believed that within the heterogenous tumor, there are stem-like cells that are not affected by targeted therapy or chemotherapy, thus persist after treatment and can give rise to a new, resistant bulk tumor (bottom).

Notch Signaling

There are several lines of evidence that show CSCs survive by upregulating the Notch signaling pathway. Notch receptors are highly conserved, single pass Type I transmembrane proteins comprised of four homologs (Notch1, Notch2, Notch3, and Notch4) (Kopan & Ilagan, 2009; Previs, Coleman, Harris, & Sood, 2015). Notch signaling is a form of cell-cell communication that results in regulating transcription of target genes (Kopan & Ilagan, 2009; Previs et al., 2015). Other than maintaining the survival of stem cells, this pathway also regulates other cellular processes such as self-renewal, cell fate determination, differentiation, proliferation,

apoptosis, cell polarity, adhesion, and migration (Lamy, Ferreira, Sales, & Silva, 2017). Notch is synthesized as a single polypeptide that undergoes a series of glycosylation events in the endoplasmic reticulum and Golgi apparatus. Once in the Golgi, the single protein is cleaved into two domains: extracellular (NEC) and transmembrane (NTM). The Notch receptor on the plasma membrane is a heterodimeric protein comprised of the extracellular domain bound to the transmembrane portion by a cation. To activate the canonical signaling pathway, one of the four receptors on the signal receiving cell encounters its ligand on the signal sending cell. There are five ligands that are known to induce Notch signaling and these are a family of DSL ligands, which are characterized by the presence of a Delta, Serrate and Lag2 (DSL) domain (D'Souza, Meloty-Kapelle, & Weinmaster, 2010). There are five of these ligands in total that interact with the receptors and activate the pathway: Delta-like1, 2, 3 and Jagged1 and 2 (D'Souza et al., 2010; Kopan & Ilagan, 2009; Lamy et al., 2017). Once there is contact, the extracellular portion of the Notch receptor is pulled away from the transmembrane portion by the ligand on the adjacent cell. This contact also activates a series of proteolytic cleavages that will first remove a portion of the transmembrane domain forming a product, NEXT. This first step is mediated by a metalloproteinase, ADAM 10/17. This event by ADAM10/17 exposes the cleavage site for the γ-secretase complex, that will cleave and release the Notch Intracellular Domain (NICD) (D'Souza et al., 2010; Kopan & Ilagan, 2009; Kovall, Gebelein, Sprinzak, & Kopan, 2017). Within the cell, the NICD translocates into the nucleus where it will interact with a transcriptional repressor, CSL, leading to recruitment of other coactivators (i.e. Mastermind-Like family of proteins) that will de-repress CSL and lead to activation of transcription (D'Souza et al., 2010; Talora et al., 2008). Classic Notch target genes include those in the HES and HEY family of genes, CCND1/3, NF-κB genes, p21KIP1, BCL2 and MYC (D'Souza et al., 2010; Lamy et al., 2017; Previs et al., 2015).

A visual representation of Notch signaling is shown below in **figure 4** (Takebe, Nguyen, & Yang, 2014). Dysregulation of this pathway has been implicated in several malignancies as it can be oncogenic or tumor suppressive. Notch receptors and ligands are overexpressed in breast cancer (Reedijk et al.). NICD1, 3, or 4 expression in the mammary gland leads to spontaneous mammary tumors in mice (Callaghan et al.). Hyperactive Notch signaling has been shown to promote drug resistance (Rizzo et al. 2008, Osipo et al. 2008), metastatic disease progression and tumor recurrence (Pandya et al. 2011), which is why new therapies are being developed to target this pathway (Previs et al., 2015).

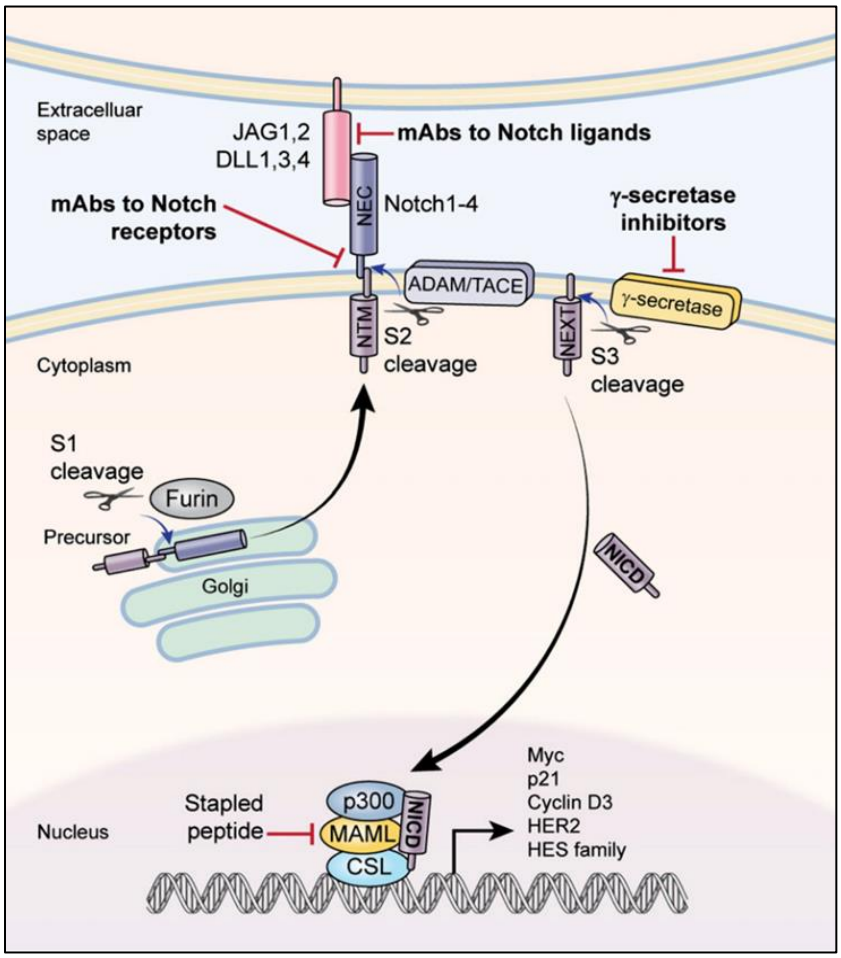

Figure 5: The Canonical Notch Signaling Pathway. The Notch receptor is synthesized in the ER as a precursor before being cleaved and moving to the plasma membrane. At the membrane, the receptor interacts with its ligand, initiating a series of cleavages that will eventually release the NICD. This domain translocates to the nucleus, where it binds to CSL, recruit additional co-activators, and activate the transcription of target genes.

Inhibition of Notch Signaling

Since Notch signaling has been associated with maintaining the population of stem-like cancer cells, new therapies are being developed in order to target this signaling pathway. There are several ways to inhibit the Notch pathway, one of which being through the use of a drug that will inhibit a key component in the pathway: the γ-secretase complex using a γ-secretase inhibitor

(GSI) (Kovall et al., 2017; Lamy et al., 2017; Previs et al., 2015; Shih & Wang, 2007; Takebe et al., 2014; Yuan et al., 2015). As previously mentioned, the γ-secretase complex cleaves NEXT allowing for the release of the NICD to activate transcription of Notch target genes. However, by using a GSI, this cleavage step is inhibited thus preventing release of NICD and de-repression of CSL genes (D'Souza et al., 2010; Talora et al., 2008). Several GSIs have been synthesized and proven to be quite effective. However, the side effects of inhibiting Notch signaling is a challenge since the pathway is required for normal cellular functions (D'Souza et al., 2010; Previs et al., 2015; Shih & Wang, 2007; Takebe et al., 2014). Also, there is a large amount of cytotoxicity associated with the use of GSIs, specifically in the gastrointestinal tract, which is only worsened by other cancer therapies such as chemotherapy. Thus, the use of GSIs pose a great challenge in the clinic (Lamy et al., 2017; Previs et al., 2015; Shih & Wang, 2007; Takebe et al., 2014). Other potential inhibitors of the Notch signaling pathway include Notch antibodies, antibody drug conjugates (ADCs), blocking peptides or combination therapies (Lamy et al., 2017; Previs et al., 2015; Takebe et al., 2014). Monoclonal antibodies can be used to inhibit either the receptors or the ligands themselves or by blocking the cleavage of Notch by γ-secretases—a less cytotoxic solution to GSI (Lamy et al., 2017; Takebe et al., 2014). ADCs are antibodies that are linked to a biologically active cytotoxic drug to have the same targeting abilities as a Notch monoclonal antibody with the ability of anticancer drugs (Takebe et al., 2014). Blocking peptides are permeable peptides that block the interaction between NICD, CSL, and co-activators, such as MAML, in the nucleus to prevent transcription (Takebe et al., 2014). All of which are still under investigation.

Clinical Biomarkers

Clinical biomarkers are detectable biological properties or molecules that can be used to monitor disease progression, further characterize a disease, or assess the prognosis of the disease (Günther, 2015). Because of this, they are used as prognostic markers for survival, critical for management of the disease, type of therapy used, and possibly predictive of tumor response to the therapy (Duffy, Harbeck, Nap, Molina, & Nicolini, 2017). Current biomarkers that are used for prediction of response and survival outcomes remain ER, PR or HER2. Looking at these biomarkers are limiting because they only predict if a certain tumor can benefit from receiving endocrine therapy or anti-HER2 therapy (Duffy et al., 2017). Due to the interest in Notch signaling as a promoter of CSCs, Notch gene targets (i.e. HES1 and HEY1) are considered possible biomarkers for the activation status of Notch and presence of CSCs. There is work suggesting that exosome-encapsulating miRNAs, serum levels, metabolites or multi-gene sequencing can provide more precise biomarkers (Duffy et al., 2017; Günther, 2015; Joyce, Kerin, & Dwyer, 2016). However, these methods have yet to provide predictive biomarkers of response to anti-Notch therapy. Thus, there is a critical need to identify new predictive biomarkers for Notch activation, presence of CSCs, and importantly response to anti-Notch therapy and overall survival outcomes (Duffy et al., 2017; Harris et al., 2016).

Death Domain-Associated Protein 6 (DAXX)

Death Domain-Associated Protein 6 (DAXX) is a multifunctional nuclear and cytoplasmic protein that is critical for several cellular processes. It has been shown to be able to shuttle between both locations in response to various cellular stresses (Khelifi, Alcontres, & Salomoni, 2005). At first, it was identified as a cytoplasmic protein that interacts with the Fas death domain to aid in the activation of Fas-mediated cell death through JNK signaling, independently of FADD, hence

the naming of the protein (Khelifi et al., 2005; Pluta, Earnshaw, & Goldberg, 1998; Salomoni & Khelifi, 2006). DAXX also plays a role in other cell death pathways, such as TGFβ-induced apoptosis, necrosis and autophagy (Salomoni & Khelifi, 2006). Conversely, DAXX may have an anti-apoptotic role. To study the function of DAXX, siRNA was used to deplete the protein, but interestingly, it was found that DAXX depletion led to an increase apoptosis and sensitized cells to apoptosis-inducing factors (Michaelson & Leder, 2003; Salomoni & Khelifi, 2006). DAXX has also been shown to be critical for embryonic development, since knockout studies in mice showed embryonic lethality (Michaelson, Bader, Kuo, Kozak, & Leder, 1999; Salomoni & Khelifi, 2006). Upon further characterization, it was determined that DAXX plays several roles in various cellular processes not only in the cytoplasm but in the nucleus of cells. In the nucleus, it has been found to associate with promyelocytic leukemia protein nuclear bodies (PML-NB), where it recruits DNA methyltransferase1 (DMNT1) to methylate DNA at differentially methylated regions (DMR), to repress gene expression (Salomoni & Khelifi, 2006). This methylation event, however, is not only to repress gene expression but is also associated with differential histone modification (Voon & Gibbons, 2016). These DMRs are important to maintain imprinted gene expression, and must remain methylated (Voon & Gibbons, 2016). This methylation status has been shown to be dependent of histone 3.3 (H3.3), a histone variant that is deposited at various repetitive sites that are required to maintain heterochromatin stability. DAXX acts as an H3.3 chaperone and associates with the chromatin remodeler, ATRX (α-thalassemia/mental retardation syndrome; SWI/SNF) protein, to form a complex that is essential to deposit H3.3 at telomeres in chromatin dense regions of the nucleus that can be modified with methylation of lysine 9 (H3K9me3) to enable heterochromatin maintenance (Fan et al., 2019; Salomoni & Khelifi, 2006; Voon & Gibbons, 2016; Voon & Wong, 2016). Mutations in this complex have been identified in various

cancers and implicated in aiding cancerous cells in escaping apoptosis (promoting oncogenesis). Thus, maintaining the proper function of this complex could be key in destroying cancerous tumor cells (Dyer, Qadeer, Valle-garcia, & Bernstein, 2017; Fan et al., 2019).

C-Jun-N-Terminal Kinases

C-Jun-N-Terminal Kinases (JNK), also known as Stress-Activated Protein Kinases (SAPK), are part of the Mitogen-Activated Protein Kinase (MAPK) family (Zeke, et al., 2016; Derijard, et al., 1994; Hibi, et al., 1993). There are three genes that encode for JNK proteins, *JNK1*, *JNK2*, and *JNK3*, and while *JNK1* and *JNK2* are present throughout the organism, *JNK3* has tissue-specific expression patterns (Davis, 2000). These genes can be spliced into ten different splicing variants, which correlate to different cellular functions. Within the protein structure of JNK, there is an activating loop that contains either a threonine or tyrosine residue that can be phosphorylated to activate JNK (Bogoyevitch & Kobe, 2006). Being that they are kinases, JNK proteins catalyze the transfer of a phosphoryl group on ATP to a specific substrate in order to carry out a certain function (Bogoyevitch & Kobe, 2006). JNK related substrates are either nuclear or cytoplasmic and phosphorylation of these substrates can lead to functions that are either activating or inhibitory. These substrates include the Jun family, activating transcription factor (ATF) family, Jun dimerization Protein 2 (JDP2), B-cell lymphoma 2 (Bcl2) regulatory protein, Bcl2-associtated death protein (BAD), Bcl2 associated X protein (BAX), protein kinase B (PKB or Akt), Elk-1, c-Myc, Nuclear factor of activated T cells (NFAT) family, signal transducer and activator of transcription (STAT) family, Pax family, tumor suppressor protein p53, as well as several nuclear hormone receptors and proteins (Bogoyevitch & Kobe, 2006; Yazgan & Pfarr, 2002; McElhinny, Li, & Wu, 2008; Leppa & Bohmann, 1999; Katz, Heinrich, & Aronheim, 2001; Gupta et al., 1996; Yang, Whitmarsh, Davis, & Sharrocks, 1998; Yamana et al., 2002; Adler et al., 1997). There are

external stimuli, such as environmental cellular stresses, that results in phosphorylation of substrates, initiating a cascade of regulatory events leading to a multitude of cellular processes. These process are primarily dealing with the activity of transcription factors that can then regulate oncogenic transformation, growth, differentiation, metabolism, cell division, movement, cell survival and/or cell death (Zeke et al., 2016; Bogoyevitch & Kobe, 2006). Shown in **figure 5**, is a summary of various JNK signaling pathways and the complexity of intracellular signal transduction (Bogoyevitch & Kobe, 2006).

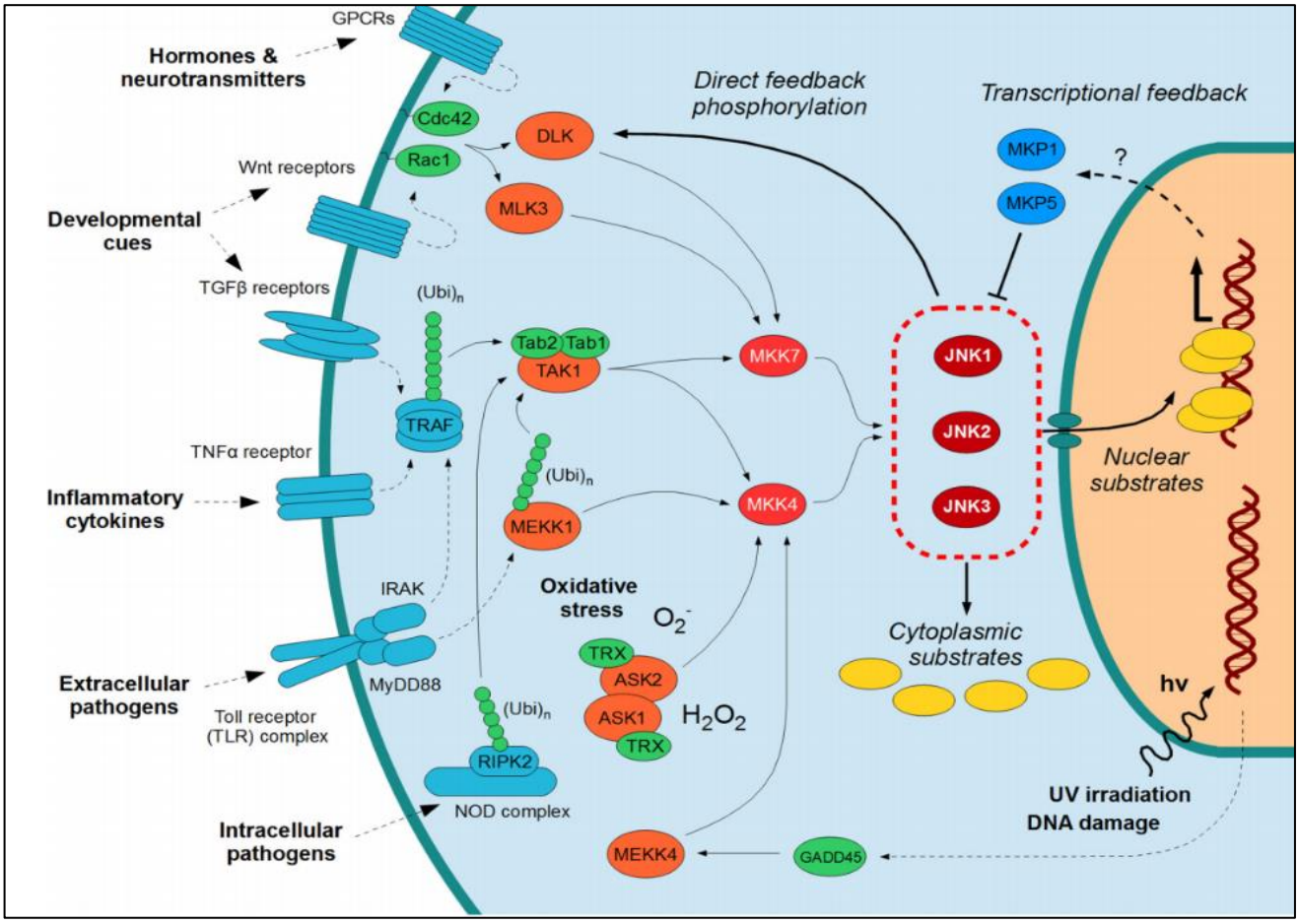

Figure 6: Overall JNK Signaling Pathways. Pathways are initiated by several external stimuli that eventually will activate JNK, resulting in phosphorylation of several substrates within a cell. This phosphorylation can then lead to further activation or inhibition of target substrate.

JNK Signaling and Apoptosis

JNK signaling, as mentioned before, results in various cellular events via intracellular signaling. The JNK signaling pathway has been shown to play a key role in both cell proliferation and programmed cell death depending on the type and duration of extracellular signal (Dhanasekaran & Reddy, 2017). In terms of cell death, it has been shown that JNK signaling-mediated regulation of programmed cell death has been implicated in resistance to targeted therapy, chemotherapy and cancer growth (Dhanasekaran & Reddy, 2017). All three JNKs have been shown to play roles in apoptotic signaling both in the cytoplasm to regulate mitochondrial-mediated apoptosis and in the nucleus. There are several apoptotic signals, such as oxidative stress or DNA damage, that lead to a release of apoptotic proteins from the inner membrane space of the mitochondria (Dhanasekaran & Reddy, 2011). However, it has been determined that JNK proteins play a role in the modulation of these pro-apoptotic proteins in the mitochondria (Aoki et al., 2002; Schroeter et al., 2003). JNK translocates to the mitochondria, and it has been shown to be required for the release of cytochrome C, a protein required to initiate the caspase 9 cascade inducing apoptosis (Dhanasekaran, 2011). On the other hand, extracellular signals can activate MAPK which in turn phosphorylates JNK. Because of this event, JNK can translocate into the nucleus where it can phosphorylate and subsequently activate c-Jun (Dhanasekaran, 2011; (Raman, Chen, & Cobb, 2007). Upon activation, c-Jun can form, along with other nuclear proteins, activator protein (AP-1) complexes which are responsible for regulating gene expression and in this case, the expression of pro-apoptotic genes (Gutkind, Turjanski, & Vaque, 2007). Not only does an activated JNK phosphorylate c-Jun, it can also phosphorylate other transcription factors that are responsible for transcribing pro-apoptotic genes or decrease the expression of pro-survival genes (Dhanasekaran & Reddy, 2011, 2017; Larhammar et al., 2017). **Figure 6** summarizes both nuclear

and mitochondrial pro-apoptotic events that are initiated by JNK signaling, as previously described (Dhanasekaran & Reddy, 2017).

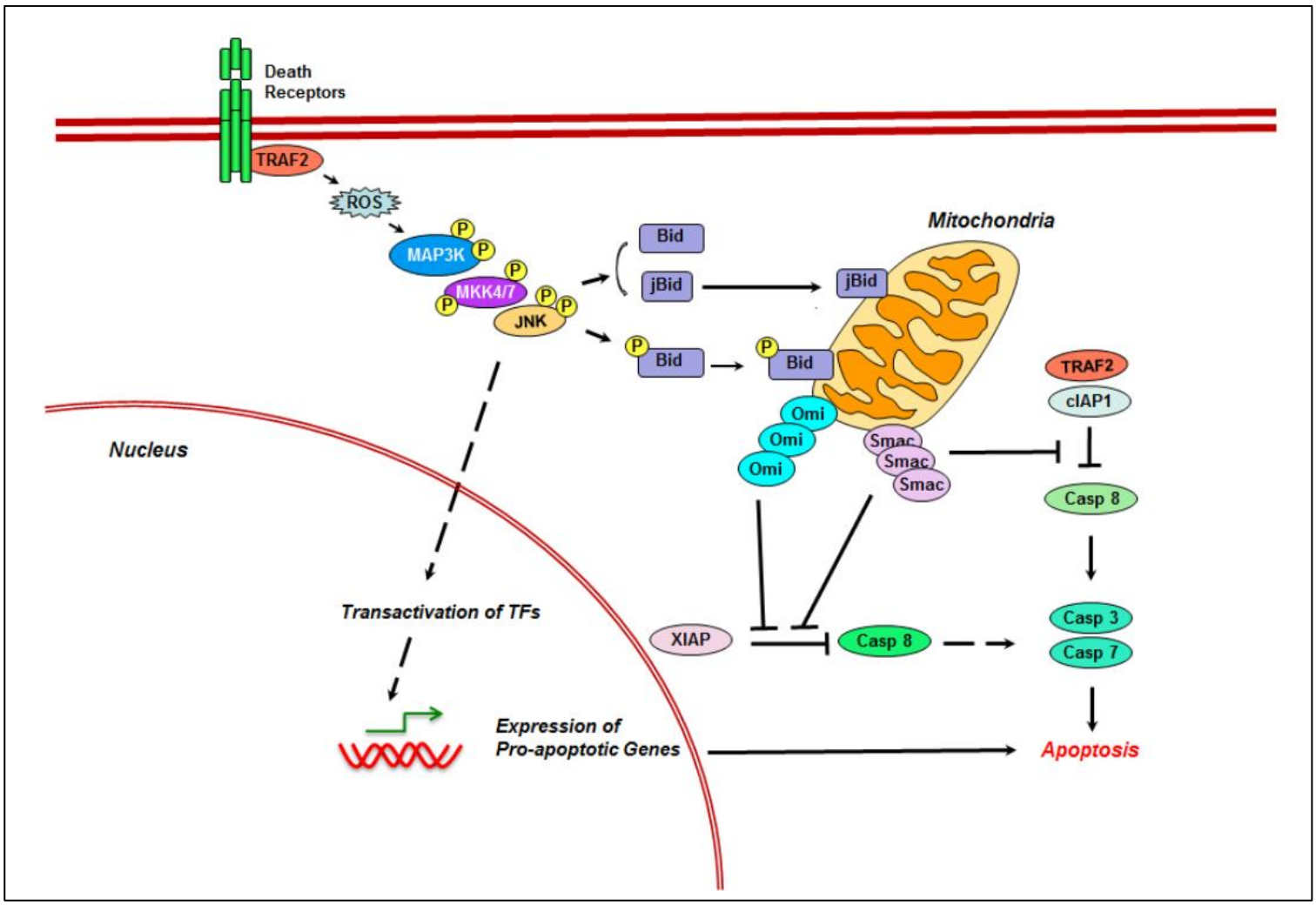

Figure 7: The Role of JNK Signaling in Both Nuclear and Mitochondrial Related Apoptosis. Transactivation of transcriptions factors (TFs) are those that have been previous described.

CHAPTER II

PRELIMINARY DATA, HYPOTHESIS AND SPECIFIC AIMS

Preliminary Data

Since breast cancer recurrence continues to be a problem for many patients that undergo remission, specific gene targets are used to predict how well a patient will respond to a therapy. Currently, Notch gene targets HES1 and HEY1 are being used since this signaling pathway has been implicated in maintaining the survival of CSCs. However, these gene targets offer no predictive value, which is why a biomarker trial was initiated to elucidate potential new Notch targets, that predict therapeutic response. Twenty-two women with ER+ breast cancer were enrolled in the trial. An initial biopsy was taken prior to beginning 2 weeks of endocrine therapy (tamoxifen or an aromatase inhibitor). A second biopsy was taken at this point and for 10 more days these women were treated with endocrine therapy in combination with a GSI, MRK-0752. After treatment, these women underwent their final surgery, and the RNA from the samples collected on day 0, 14 and 25 were analyzed by real-time PCR and Affymetrics microarray. Several gene transcripts were found to be significantly upregulated or downregulated **(figure 7)**. To test whether these genes were required for anti-Notch efficacy by the GSI, overexpression or depletion studies were conducted for each gene under no treatment or GSI treatment, using the ER+ MCF-7 cell line, followed by assessment of CSC survival as measured by mammosphere forming efficiency (MFE) **(figure 8)**. Only one gene was identified to be necessary for anti-CSC efficacy by the GSI, Death-associated protein 6 (DAXX).

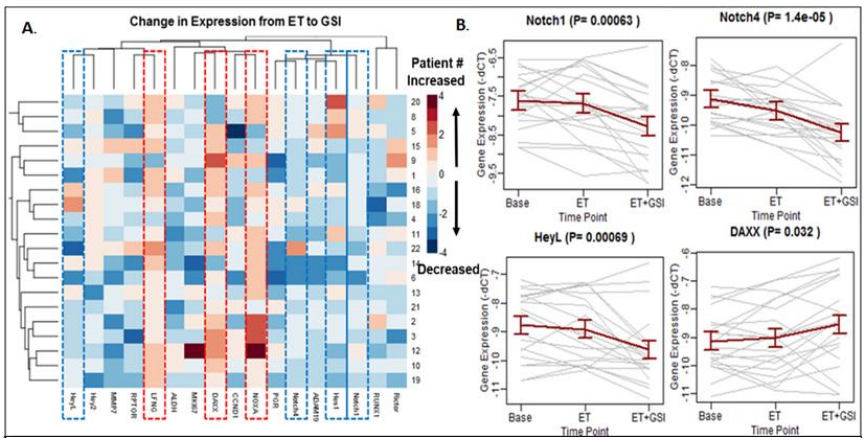

Figure 8: Upregulation of DAXX shown in Clinical Trial Patients Treated with ET and GSI. Data from clinical trial that assessed the expression of several genes from 22 patient tumors that first underwent endocrine therapy and treatment with GSI. **(A)** Heat map of the gene transcripts whose varying gene expression was due to the GSI treatment. **(B)** The expression of 4 transcripts, highlighted in the microarray, to determine the efficacy of the GSI used from the tissue samples, showing that out of these four transcripts, DAXX is the only one upregulated.

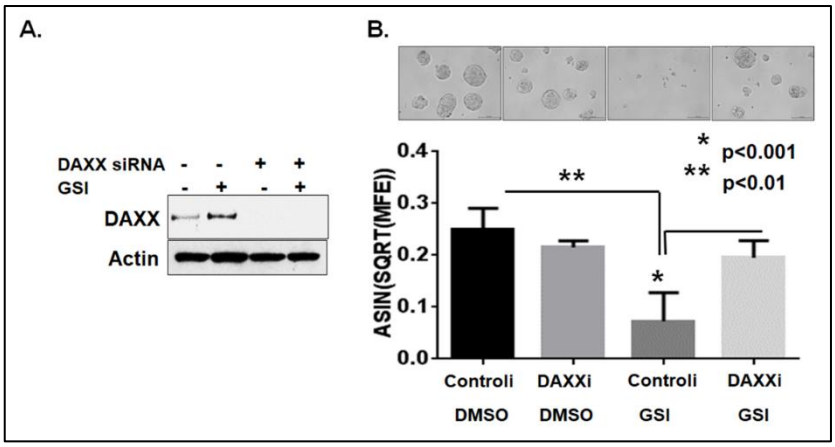

Figure 9: Effects of Depleting DAXX in CSC Population of an ER+ Cell Line. (A) Western blot showing that not only can DAXX be depleted by siRNA, but treatment of GSI also upregulate expression of the protein. **(B)** CSC assay showing effects of depleting DAXX under conditions with or without GSI (top). Quantification of the assay seen on the bottom under each condition tested.

Since DAXX was shown to be the gene of interest from the clinical trial and in vitro results using ER+ cells, more in vitro studies were performed to assess the role of DAXX on bulk cell proliferation using other breast cancer cell lines (HER2+ and TNBC). The cells were transfected with a control or DAXX siRNA to knockdown the DAXX protein. Cells were then treated, daily, with the vehicle (DMSO), 5μM MRK-003 GSI or Pfizer GSI for up to 7 days followed by the measurement of cell proliferation using the Countess Cell Counter. As **figure 9** shows, DAXX depletion by siRNA increases proliferation of HER2+, BT474 cells in the absence of GSI treatment. In contrast, the Pfizer GSI has little effect on proliferation, while the MRK-003 GSI significantly inhibits proliferation of BT474 cells . Under any GSI treatment, DAXX depletion has little effect on proliferation. These results suggest two possible conclusions: 1) DAXX limits proliferation of HER2+, BT474 cells through a Notch dependent mechanism and/or 2) DAXX inhibits Notch to promote resistance to a GSI.

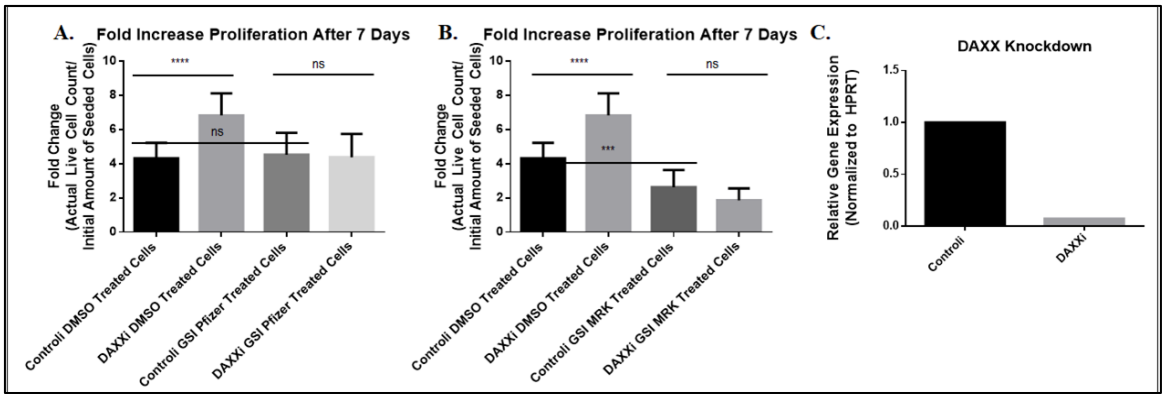

Figure 10: Bulk Cell Proliferation Data in BT474 Trastuzumab Resistant Cells: BT474 TR cells were transfected with control siRNA (SCBi) and DAXX siRNA for 48 hours. After transfection, the cells were grown in 10% FBS DMEM in three 6-well plates and treated daily for seven day utilizing two different GSIs: Pfizer (**A**) and MRK-003 (**B**). the fold increase proliferation over the course of the experiment was calculated and statistical significance was calculated using an ANOVA and multiple comparison test. (**C**) shows the confirmation of the DAXX knockdown using the relative gene expression via RT-qPCR.

Similar results were observed in TNBC cells (MDA-MB-231). These cells have a high proliferation rate and surprisingly, DAXX depletion further increased proliferation from 20 to 80-fold in the absence of GSI (**figure 10**). Like the results from BT474 cells, the Pfizer GSI was ineffective at inhibiting proliferation of MDA-MB-231 cells, while the MRK-003 GSI significantly blocked growth. DAXX depletion increased sensitivity to a GSI, suggesting that DAXX limits the proliferation of TNBC by possibly inhibiting Notch and thus promotes resistance to a GSI.

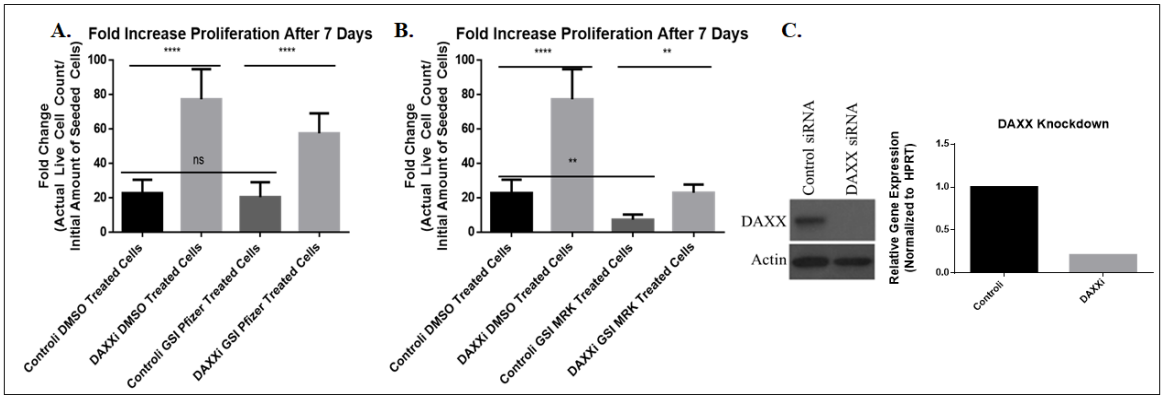

Figure 11: Bulk Cell Proliferation Data in MDA-MB-231 Cells: MDA-MB-231 cells were transfected with control siRNA (SCBi) and DAXX siRNA for 48 hours. After transfection, the cells were grown in 5% FBS IMEM in three 6-well plates and treated daily for seven day utilizing two different GSIs: Pfizer (**A**) and MRK-003 (**B**). the fold increase proliferation over the course of the experiment was calculated and statistical significance was calculated using an ANOVA and multiple comparison test. (**C**) shows the confirmation of the DAXX knockdown in a Western blot (left) and the relative gene expression via RT-qPCR (right).

Preliminary data suggest that DAXX is required to limit the proliferative capacity of HER2+ and TNBC cells, possibly by inhibiting Notch signaling. These results indicate that there could be a cross-regulation of DAXX and Notch that is necessary to limit proliferation of breast

cancer cells and possibly sensitivity to growth inhibiting agents, such as carboplatin, the current standard of care **(figure 11)**.

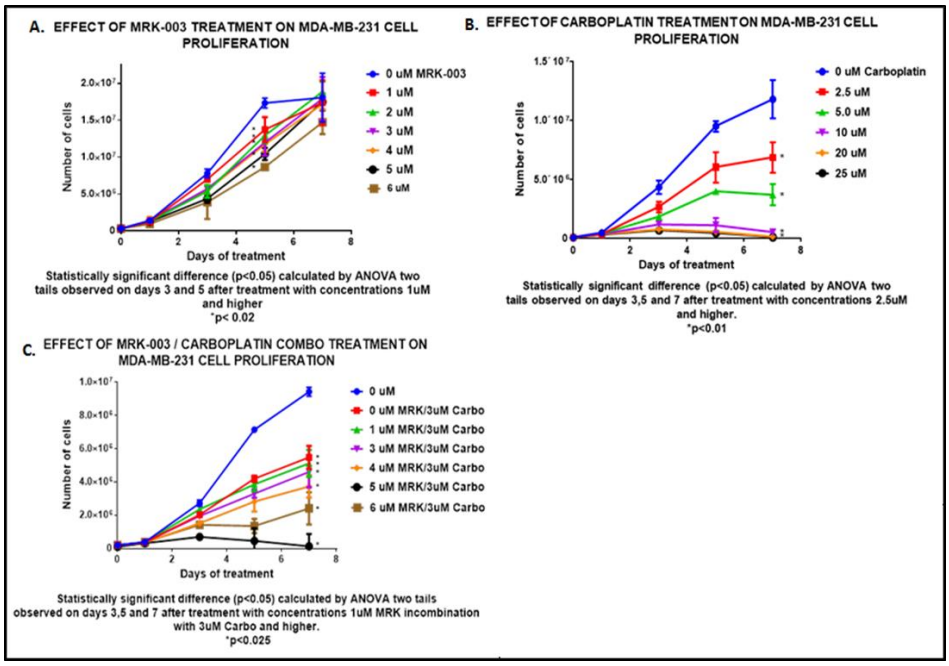

Figure 12: Growth Progression Studies of MDA-MB-231 Cells. Proliferation of MDA-MB-231 cells treated with GSI MRK-003 (**A**), carboplatin (**B**), or in combinations of the two treatments (**C**), were measured over the course of 7 days. Varying concentrations of each drug were compared against one another, until the combination treatments, where the IC50 of carboplatin was used and kept constant throughout.

Hypothesis

Based on previous reports and new preliminary data, the following hypothesis is proposed**: DAXX limits proliferation of distinct breast cancer subtypes by attenuating Notch signaling. In addition, DAXX expression could be a potential predictive biomarker of response to current targeted therapy.** The following aims will determine if DAXX is a key regulator of breast cancer growth and establish if its expression is to modulate sensitivity to targeted and/or cytotoxic therapies.

Specific Aims

Aim 1. Determine if DAXX is a Suppressor of Breast Cancer Cells of Distinct Subtypes by Regulating the Notch Pathway.

Preliminary data show that DAXX expression is required to limit bulk cell proliferation of two out of the three breast cancer subtypes: HER2+ and TNBC. By siRNA, the expression of DAXX is depleted, and the proliferation over the course of 7 days increases by 2-fold in BT474 cells (HER2+) and almost 4-fold in MDA-MB-231 cells (TNBC). The requirement of DAXX to limit proliferation of these breast cancer cells seems to be dependent on Notch signaling. However, what is not known, is whether DAXX expression modulates Notch signaling to limit the proliferation and survival of breast CSCs from distinct subtypes of breast cancer.

Aim 1a: To assess the role of Notch signaling, real-time PCR will be performed under control and Dominant Negative Mastermind-like (DN-MAML1) conditions to detect changes in cellular phenotypes when Notch signaling is disrupted via the detection of Notch target gene transcripts (i.e. HES1, HEY1, HES4, HEYL, CMYC, and CCND1). Under these conditions, bulk cell proliferation studies and stem cell assays, assessed by mammosphere forming efficiency, will be conducted using ER+ MCF-7, HER2+ BT474, and TNBC MDA-MB-231 cells to see if the results compare to the preliminary data that utilizes GSI to inhibit Notch signaling.

Aim 1b: To assess the role of DAXX on Notch signaling, real-time PCR will be performed under control and DAXX siRNA-transfected conditions to detect Notch target gene transcripts (i.e. HES1, HEY1, HES4, HEYL, CMYC, and CCND1). Additional bulk cell proliferation studies and stem cell assays, as assessed by mammosphere forming efficiency, will be conducted under control conditions (scrambled siRNA) and DAXX depletion (DAXX siRNA) using ER+ MCF-7, HER2+ BT474, and TNBC MDA-MB-231 cells.

Aim 2. Determine if DAXX Expression is Regulated by Current Therapy and Modulates Sensitivity.

Preliminary data from the TNBC MDA-MB-231 cells show that DAXX expression is required to limit proliferation through a Notch dependent mechanism. This indicates that the expression of DAXX may attenuate the efficacy of the GSI.

Aim 2a: To assess whether DAXX expression is modulated in response to other types of therapies, TNBC cells will be treated with carboplatin. HER2+ cells will be treated with anti-HER2 therapy such as lapatinib or trastuzumab. ER+ cells will be treated with anti-estrogens, including estrogen deprivation, tamoxifen, or fulvestrant. DAXX expression will be measured by real-time PCR and Western blotting after 24- and 72-hours post-treatment. Further, based on preliminary data generated by former members of the Osipo laboratory, it was shown that carboplatin increased expression of Notch receptors and sensitivity to the MRK-003 GSI in TNBC. Based on these results, carboplatin could decrease DAXX, thus enhancing Notch signaling and sensitivity to Notch blockade. Thus, if RNA and protein expression results demonstrate that therapy, such as carboplatin, decreases DAXX expression, then DAXX will be ectopically overexpressed to assess proliferation and expression of Notch genes and targets in response to a GSI, carboplatin, and the combination. Similar studies will be conducted in ER+ and HER2+ breast cancer cells using the appropriate standard of care therapy.

Aim 2b: Alternatively, DAXX may limit proliferation of cells, thus decreasing efficacy to carboplatin in TNBC cell, to anti-HER2 therapy in HER2+ cells, and/or to anti-estrogens in ER+ cells. To assess the role of DAXX as a promoter of resistance to current therapy, DAXX will be depleted using siRNA and different breast cancer subtypes will be treated with carboplatin

(TNBC), lapatinib (HER2+) or fulvestrant (ER+), then proliferation and CSC survival will be measured.

CHAPTER III

MATERIALS AND METHODS

Materials

Cell Culture

Each Breast Cancer cell line was maintained and cultured in the appropriate medium and incubated at 37°C with 5% CO_2. Listed below are the cell lines used and the medium in which they were cultured for maintenance or experiments.

MDA-MB-231: MDA-MB-231 Breast Cancer cells were acquired from Dr. Ruth Lupu (Mayo Clinic, Rochester, MN). These cells were cultured in Improved Minimal Essential Medium (IMEM) enhanced with 5% Fetal Bovine Serum (FBS; Corning Cellgro, Corning Inc., Corning, NY), 1% Nonessential Amino Acids (NEAA; Corning Cellgro), and 1% L-Glutamine (Corning Cellgro).

BT474 TR and TS: BT474 Breast Cancer cells that are sensitive to Trastuzumab (TS) were received from American Type Culture Collection depository (ATCC, Rockland, MD) and cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Corning Cellgro) enhanced with 10% FBS (Corning Cellgro), 1% NEAA (Corning Cellgro), and 1% L-Glutamine (Corning Cellgro). BT474 cells were treated over 6 months with increasing concentrations of Trastuzumab until they acquired resistance to the treatment by Dr. Clodia Osipo (Osipo et al., 2008. Oncogene) (TR). These cells are cultured in 10µg/mL of Trastuzumab to maintain resistance.

MCF-7: MCF-7 Breast Cancer cells were provided by Dr. V. Craig Jordan (University of Texas, M.D. Anderson Cancer Center, Houston, TX). These cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640; Corning Cellgro) enhanced with 10% FBS (Corning Cellgro), 1% NEAA (Corning Cellgro), and 1% L-Glutamine (Corning Cellgro). When these cells were deprived of Estrogen, they were cultured in Roswell Park Memorial Institute medium without phenol red (RPMI 1640 w/o phenol red) and enhanced with 10% charcoal stripped FBS (Corning Cellgro), 1% NEAA (Corning Cellgro), and 1% L-Glutamine (Corning Cellgro).

MDA-MB-468: MDA-MB-468 Breast Cancer cells were cultured in Dulbecco's Modification of Eagles Medium (DMEM, Corning Cellgro) and enhanced in the same manner as described above.

Plasmid DNA

DAXX: Human death domain associated protein (DAXX) expression plasmid consists of the entire ORF with a Myc-DDK expression tag cloned into a pCMV6-entry vector. (OriGene, Rockville, MD, #RC226603)

HES1: Human hairy and enhancer of split 1 (HES1) expression plasmid consists of the entire ORF with a Myc-DDK expression tag at the end of the sequence cloned into a pCMV6-entry vector. (OriGene, #RC211709)

Small Interfering RNA (siRNA)

DAXXi: DAXX siRNA (GE Healthcare Dharmacon, Inc., Lafayette, CO) was used to knockdown DAXX protein. The siRNA came in a set of 4 (J-004420-05, J-004420-06, J-004420-07, J-004420-08) to knockdown the entire protein. DAXX siRNA was transfected using Lipofectamine RNAi iMax (Invitrogen, Carlsbad, CA, #13778150) and Opti-MEM (Gibco; Thermo Fisher Scientific, Waltham, MA, #31985070).

HES1i: Hes-1 siRNA (Santa Cruz, sc-37938) was used to knock down Hes1 protein. Hes1 siRNA was transfected using Lipofectamine RNAi iMax (Invitrogen) and Opti-MEM (Gibco; Thermo Fisher Scientific).

As a control, Scrambled siRNA purchased from Integrated DNA Technologies, IDT, was used (Integrated DNA Technologies, Coralville, IA, custom-made; no reference number).

Antibodies

The DAXX antibody is monoclonal and purchased from Cell Signaling Technologies (Cell Signaling Technology, Danvers, MA, #4533). Total JNK and Phosphorylated-JNK (T183/Y185) antibodies are monoclonal rabbitand purchased from Cell Signaling (Cell Signaling, #9258 and #4668). The Hes-1 antibody is polyclonal rabbit purcchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-25392). Monoclonal mouse anti-β-Actin antibody was used as the loading control for all Western Blot analysis and cytoplasmic localization studies (Sigma-Aldrich, St. Louis, MO, A-5441). Polyclonal rabbit anti-Lamin B (M-20) antibody was used as the loading control for nuclear localization studies via Western Blot analysis (Santa Cruz, sc-6217).

Primers

All primers used in Reverse Transcription, quantitative real-time Polymerase Chain Reaction (RT-qPCR) were designed using the NCBI Primer Blast and purchased from Invitrogen. They were resuspended in Nuclease-Free water to create working stocks with a concentration of 50µM. Listed in the table below are the sequences for each primer used.

Table 1. Forward and Reverse Sequences of the Primers Used to Target Notch Signaling Gene Targets and Notch Receptors.

Primer	Forward Sequence	Reverse Sequence
HPRT	5' ATG AAC CAG GTT ATG ACC TTG AT 3'	5' CCT GTT GAC TGG TCA TTA CAA TA 3'
DAXX	5' GAT GAT GAA GAG GAG GAC GAA G 3'	5' TGG AGA TCT GTA GTG AGG ACA 3'
HES 1	5' CGG ACA TTC TGG AAA TGA CA 3'	5' CAT TGA TCT GGG TCA TGC AG 3'
HEY 1	5' TCA TTT GGA GTG TTG GTG GA 3'	5' CTC GCA CAC CAT GAT CAC TT 3'
HES 4	5' GGA CGC CCT CAG AAA AGA G 3'	5' GGT ACT TGC CCA GAA CGG 3'
HEY 2	5' GAC AGT GGA TCA TTT GAA GAT GC 3'	5' AAA AGC AGT TGG CAC AAG TC 3'
c-Myc	5' CGA CTC TGA GGA GGA ACA AG 3'	5' TGC GTA GTT GTG CTG ATG T 3'
Notch 1	5' ATC AAC GCC GTA GAT GAC C 3'	5' TTG TTA GCC CCG TTC TTC AG 3'
Notch 3	5' TCC TGG CTA CAA TGG TGA TAA C 3'	5' ATT AAT CTC GCA GAG CAC CC 3'
Notch 4	5' TGC AGG CAT ATG GGA TGT AA 3'	5' CAT CCC CAC AGT GGA GTT CT 3'

Drug Treatments

MRK-003 GSI: A γ-secretase inhibitor, MRK-003, was provided from Merck Oncology and Co. (Whitehouse Station, NJ) and resuspended in Dimethyl sulfoxide (DMSO) from Thermo Fisher Scientific (Thermo Fisher Scientific, 67-68-5) to create a 10mM stock solution. The working concentrations for cell treatments were 5µM or 10µM.

Pfizer GSI: A γ-secretase inhibitor was purchased from Pfizer (Pfizer, New York, NY, PF-03084014) and resuspended in DMSO to create a 10mM stock solution. The working concentration for cell treatments were 5µM or 10µM.

Carboplatin: Carboplatin, a DNA damaging agent, was purchased from Sigma Aldrich (Sigma, C2538) and resuspended in DMSO (Thermo Fisher) to make a stock solution of 10mM. The stock was diluted to make various concentrations—1.25mM, 2.5mM, and 5.0mM. The working concentrations for cell treatments were 1.25µM, 2.5µM, 5.0µM and 10µM.

Estradiol: 17β-Estradiol was purchased from Sigma-Aldrich (Sigma-Aldrich, #50-28-2) and resuspended in 200 proof absolute molecular grade ethanol from Thermo Fisher Scientific (Thermo Fisher Scientific, BP2818100) to make a 5mM solution. A 5µM stock solution was then created and the working concentration for cell treatments was 5nM.

4-Hyrdroxytamoxifen**:** 4-Hydroxytamoxifen (4OHT) was purchased from Selleck Chemicals (Selleck Chemicals, Houston, TX, S7827) and resuspended in 200 proof molecular grade ethanol (Thermo Fisher) to make a solution of 40mM. A working stock solution of 100µM was created by diluting the 40mM stock solution and from this various concentrations of the drug were created, ranging from 0.0001µM to 10µM, in logarithmic increases. The concentration for cell treatments were 0.0001nM, 0.001nM, 0.01nM 0.1nM, 1.0nM, 10nM and 100nM.

Fulvestrant: Fulvestrant was purchased from Selleck Chemicals (Selleck Chemicals, S1191) and resuspended in 200 proof molecular grade ethanol (Thermo Fisher) to make a solution of 100mM. A working stock solution of 100µM was created by diluting the 100mM stock solution and from this various concentrations of the drug were created, ranging from 0.0001µM to 10µM, in logarithmic increases. The concentration for cell treatments were 0.0001nM, 0.001nM, 0.01nM 0.1nM, 1.0nM, 10nM and 100nM

Trastuzumab: Trastuzumab (Herceptin™) was received from Genentech (Genentech Inc., South San Francisco, CA, 50242-134-68) as a 22mg lyophilized pellet. A working concentration of 22µg/mL was created by adding sterile PBS. The stock was diluted with Phosphate-Buffered Saline (PBS, Corning Cellgro) to create various concentrations for cell treatments—2.5µg/mL, 5.0µg/mL, 10µg/mL and 20µg/mL.

SP600125: JNK inhibitor (SP600125) was purchased from Selleck Chemicals (Selleck Chemicals, S1460) and resuspended in DMSO (Thermo Fisher) to create a 50mM stock

concentration. The stock was diluted to make various concentrations, 10mM, 5mM and 1mM and the working concentrations for cell treatments were 10µM, 5µM and 1µM.

Experimental Methods

Transfection

Protein Knockdown. In 10cm sterile tissue culture treated plates, cells were seeded the day before transfection at nearly 80% confluency. On the day of the transfection, 2mL of Opti-MEM media were mixed with 120µL of Lipofectamine RNAi iMAX. This solution was left to incubate at room temperature for 5 minutes, to form the lipofectamine mixture in the media. In separate tubes, 1mL of Opti-MEM media was mixed with 60µL of either control (scrambled) siRNA or the target protein siRNA. These solutions were also left to incubate for 5 minutes at room temperature. Then, 1mL of the Lipofectamine RNAi iMax+Opti-MEM solution were added to each tube with Opti-MEM+siRNA. These solutions were left to incubate for 20 minutes at room temperature. This was to allow the now formed, positively charged liposomes to interact with the negatively charged siRNA and encapsulate the siRNA for delivery. Fresh media was added to the plates while the siRNA incubated with the Lipofectamine RNAi iMax+Opti-MEM solution (9mL). After the solutions had incubated, each were added carefully to each plate of cells, in a dropwise manner and left to incubate for 48hrs at 37°C. This allowed for the liposomes to be endocytosed by the cells to release the siRNA once it had entered the cell. The siRNA is then able to travel to the nucleus where it can bind to the target mRNA, and degrade the transcript thus knocking down the protein of interest. When transfecting in a 6cm dish, the same process was followed but the amounts of reagent and siRNA used were decreased by a factor of 2.6, which was the ratio of the 10cm dish surface area to the 6cm dish surface area. This was to compensate for the change in

surface area of the smaller plate. When transfecting in a 6-well tissue culture plate, the amounts were decreased by a factor of 5.85.

Overexpression of Protein by DNA Plasmid. In 10cm sterile tissue culture treated plates, cells were plated the day before the transfection at about 80% confluency. On the day of the transfection, 1mL of Opti-MEM media were mixed with 24µL of polyethyleneimine (PEI). This solution was left to incubate at room temperature for 10 minutes. Then 6µg of plasmid DNA were added to the PEI+Opti-MEM solution and left to incubate at room temperature for 20 minutes. This allows for the PEI to interact with the plasmid DNA to form a complex of polymer and DNA, polyplexes, which are positively charged, condense the DNA and provide protection from degradation. Fresh media was added to the plates during incubation (9mL). After incubation, the PEI+Opti-MEM+plasmid DNA solutions were carefully added to the plates, in a dropwise manner and left to incubate for 24hrs at 37°C. Once the positively charge polyplexes encounter the negatively charged cell surface, the DNA is released into the cell, where it can travel to the nucleus and transiently express the plasmid—resulting in the protein of interest to be overexpressed. When transfecting in a 6cm dish, the same process was followed but the amounts of reagent and plasmid DNA were decreased by a factor of 2.6, which was the ration of the 10cm dish surface area to the 6cm dish surface area, compensating for the change in surface area of the smaller plate. When transfecting in a 6-well tissue culture plate, the amounts were decreased by a factor of 5.85.

Western Blotting

Bicinchoninic Acid (BCA) Assay. Lysis buffer was prepared as shown in the table below. Cells could either be trypsinized and pelleted prior to adding lysis buffer or directly lysed and scraped off with a rubber cell scraper. After the addition of the buffer, cells were incubated on ice for 20 minutes. After incubation, cells were sonicated twice for 10 seconds at 20% amplification.

Protein standards of bovine serum albumin (BSA) of varying concentrations, from 0 to 10µg/mL, were added in duplicate to a 96-well plate to create a standard curve. 10µL of each sample were added to the 96-well plate, as well, in duplicate. 200µL of BCA working reagent were added to each well (10mL of Reagent A mixed with 200µL of Reagent B). The 96-well plate was incubated at 37°C for 30 minutes. After incubation, the plate was read by a microplate reader. The total amount of protein was determined by comparing the absorbance values of the samples to that of the standard curve, with an R^2 value greater than 0.98, generated. Western blot samples were then prepared with the known amounts of protein from each sample collected, 2X sample buffer with 2-Mercaptoethanol and additional lysis buffer, if needed. The samples were then boiled on a hot plate at 95°C for 10 minutes and stored at 4°C.

Table 2. Reagents Needed and Amounts to Make 5mL of Lysis Buffer.

Reagent	Amount (µL)
1M HEPES	250
10% Triton X-100	500
5M NaCl	150
0.5M EDTA	50
200mM Na Vanadate	25
200mM PMSF	50
200x Protease Inhibitor Cocktail	25
Nuclease-Free Water	3700

Gel Preparation. For Western blot analysis, Sodium Dodecyl Sulfate (SDS) gels were prepared, to separate proteins based on molecular weight. Two portions of the gel were casted: the stacking portion, where the samples are loaded, and the resolving portion, where the samples run through and separate. Depending on the protein of interest and its molecular weight, an 8% or 10%

acrylamide gel was created, with the higher percentage used to visualize proteins of lower molecular weight. Listed in the table below are the reagents and amounts needed to make both portions of the gel. A clean, short glass plate was placed in front of a clean, 1.5mm tall glass spacer plate, and secured inside a casting frame, with the bottom of the plates completely flat. The casting frame was then secured into place in the casting stand. 8mL of the resolving solution were added in between the two plates. 1mL of isopropanol was added to the top of the solution, to flatten the top of the gel forming. The gel was left to solidify for 20 minutes. Once the resolving portion of the gel was casted, the isopropanol was rinsed off and the stacking portion was added. 2mL of the stacking solution were added to the top of the gel and a 1.5mm gel comb was place on top of the gel, in the stacking solution. (either 10 or 15 well). The gel was left to solidify again for 20 minutes and was ready to be used.

Table 3. List of Reagents and Amounts for Both an 8% and 10% SDS Resolving Gel.

Reagent (8% Gel)	Amount (mL)	Reagent (10% Gel)	Amount (mL)
Nuclease-Free Water	4.6	Nuclease-Free Water	4.0
30% Acrylamide	2.7	30% Acrylamide	3.3
1.5 M Tris Buffer (pH 8.8)	2.5	1.5 M Tris Buffer (pH 8.8)	2.5
10% SDS	0.1	10% SDS	0.1
10% Ammonium Persulfate (APS)	0.1	10% Ammonium Persulfate (APS)	0.1
TEMED*	0.006	TEMED*	0.004

Table 4. List of Reagents and Amounts for the Stacking Gel.

Reagent	Amount (mL)
Nuclease-Free Water	1.4
30% Acrylamide	0.33
1.5 M Tris Buffer (pH 8.8)	0.25
10% SDS	0.02
10% Ammonium Persulfate (APS)	0.02
TEMED*	0.002

Western Blot. Begin with preparation of separating and stacking gels first and then the apparatus set up. The Western blot separating apparatus was filled with 1X Running buffer (80mL 10X Running buffer + 720mL ddH_2O). The gels were inserted inside, with the short glass plate facing inward. All protein samples were boiled and sonicated prior to loading onto gels. Protein separation was conducted using 150V for 1 hour or until separation was completed. To transfer the proteins from the gel to the nitrocellulose membrane, the transfer apparatus was set up (with a buffer tank, electrode assembly and two gel holder cassettes). 2 sponges, 2 pieces of Whatman filter paper and 1 nitrocellulose membrane (per gel) were soaked with 1X Transfer Buffer (80mL 10X Transfer buffer + 560mL of ddH2O + 160mL of methanol) . The gels were then carefully taken out from the glass plates and placed on the nitrocellulose membrane- sandwiched between two pieces of filter paper, oriented in the correct direction that would transfer the ladder onto the left side of the membrane. The filter papers-nitrocellulose membrane-gel were then smoothened using a flat spatula to remove any potential bubbles that would have interfered with the transfer. The filter papers-nitrocellulose membrane-gel bundle was then placed in between two buffer-soaked sponges and placed inside a gel holder cassette, where the orientation of the gel coincided with the black portion of the cassette. The cassette was then placed inside the electrode assembly, with the black portion of electrode assembly facing the back and the black portion of the cassette facing the same way. The buffer tank was filled with the remaining 1X Transfer Buffer and two icepacks were placed in the remaining space of the buffer tank, to ovoid over-heating. The transfer of proteins from gel to nitrocellulose membrane was conducted at 100V for 1 hour. After transfer, membranes were cut at the appropriate weight marker, depending on the protein of interest. Pieces of membrane were then blocked and gently rocked for an hour at room temperature with 3mL of nonfat milk (5g of powdered nonfat milk in 100mL of TBST), to prevent nonspecific binding of

antibodies to the membrane. After blocking, 3mL of nonfat milk with 3µL of primary antibody, specific to the protein of interest, were added to the membranes and left to rock gently overnight, at 4°C. The following day, membranes were washed with 1X TBST and rocked for 10 minutes (3 times/each). After the final wash, 3mL of nonfat milk and 2µL of secondary antibody (either anti mouse or anti rabbit) were added to the membranes and rocked gently for 1 hour at room temperature. The membranes were washed again, as previously described, and then developed using Pierce ECL Western Blotting Substrate and pieces of film. If needed, then SuperSignal West PICO Plus Chemiluminescent Substrate was used to develop proteins that were more difficult to detect.

Growth Assay

After transfection, cells were seeded in sterile 12-well, tissue culture plates, in triplicate wells. The number of cells seeded varied among cell type and are listed in the table below. Cells were treated daily for a total of seven days with varying concentrations of the standard of care for each cell type: Triple negative breast cancer cell lines were treated with Carboplatin (MDA-MB-231, MDA-MB-468); HER2+ breast cancer cells lines were treated with Trastuzumab (BT474 Trastuzumab sensitive, BT474 Trastuzumab resistant); ER+ breast cancer cell lines were treated with 4-Hydroxytamoxifen, Fulvestrant or were deprived of Estrogen (MCF-7). After seven days of treatment, cells were washed with PBS, trypsinized and counted. The total volume of the resuspended cells was recorded to calculate the total cell count and measure the fold change.

Table 5. Number of Cells Seeded per Well for Each Cell Line in Growth Assays.

Cell Line	Number of Seeded Cells
MDA-MB-231	16,000
MDA-MB-468	32,000
BT474 Trastuzumab Sensitive	55,000
BT474 Trastuzumab Resistant	45,000
MCF-7	32,000

Real-Time Polymerase Chain Reaction (PCR)

RNA Extraction. Cells were collected in 300µL of Ambio TRIzol Reagent, either scraped directly from tissue culture plates or spun down and resuspended in the reagent. At this point, cells can either be stored at -80°C or the RNA can be extracted immediately. To the cells suspended in TrIzol, 50µL of 1-Bromo-3-chloropropane (BCP) were added to the suspension and vortexed for approximately 15 seconds. The suspensions were then incubated at room temperature for 5 minutes and then centrifuged for 15 minutes at 14,000rpm at 4°C. At this point, the Zymo Research Direct-zol RNA Miniprep Plus kit was followed (with a few modifications to the protocol in the kit). The clear, aqueous phase was removed and placed in a new 1.5mL Eppendorf tube. Equal parts of 200 proof molecular grade ethanol were added to each tube and the entire sample was placed in a Zymo-Spin IIICG Column within a collection tube. The samples were centrifuged at top speed for 30 seconds. 200µL of Direct-zol RNA PreWash were added to the column and centrifuged for the same time and speed as previously described. This was done twice to the column. Then 500µL of RNA Wash Buffer were added to the column and centrifuged at top speed for 2 minutes. An additional 30 seconds of centrifuging the column were done to ensure that it was completely dry

of any wash buffers. Then 50µL of DNase/RNase-Free Water were added directly to the column matrix and centrifuged for 30 seconds at top speed to elute the RNA. The samples were placed immediately on ice and the concentration of RNA was measured using the NanoDrop 2000.

Reverse Transcription (RT). To generate 25ng/µL of cDNA, 0.5µg of RNA are needed per reaction. From the concentration of RNA measured with the NanoDrop 2000, the amount of RNA needed per reaction to generate that amount of cDNA was calculated and diluted with water if necessary. The components for each reaction are listed in the table below. Once every component was mixed together, a BioSystems 2720 Thermal Cycler was used to reverse transcribe the RNA over a series of temperatures and times that will anneal, polymerize the DNA and deactivate the reverse transcription enzyme to make several copies of cDNA.

Table 6: Components for a 50µL RT-PCR Reaction

Components	Amount (µL)	Final Concentration
10X RT Buffer	5	1X
25mM $MgCl_2$	11	5.5mM
dNTPs	10	500µM/dNTP
Random Hexamers	2.5	2.5µM
Rnase Inhibitor	1	0.4U/L
RT Enzyme	1.25	1.25U/L
RNA + Nuclease Free Water	19.25	0.5µg RNA

Quantitative Real Time PCR (qRT-PCR). cDNA generated as described in the method above was then used in a qRT-PCR analysis, which uses the SYBR Green Master Mix and primers to target and amplify certain transcripts in the cDNA sample and quantifies the relative quantity of gene transcripts expressed over the course of the reaction. The components and quantity to set up the reaction are listed in the table below. Once each reaction was prepared in a 96-well plate,

the Applied Biosystems Step One Plus Real-Time thermocycler was used to initiate the reaction. From the reaction, the instrument recorded cycle threshold (CT) values, which are the number of cycles required for the fluorescent signal to cross a specific threshold. Using these values, the $2^{-\Delta\Delta CT}$ calculation was performed to achieve relative fold change of a gene transcript normalized to HPRT and compared to a control sample.

Table 7: Components For a 12.5µL qRT-PCR Reaction

Components	Amount (µL)	Final Concentration
Nuclease Free Water	4.5	-
2X SYBR Green Master Mix	6.25	1X
50µM Forward Primer	0.25	12.5µM
50µM Reverse Primer	0.25	12.5µM
25ng/µL cDNA	1.25	2.5ng/µL

Cell Cycle

MDA-MB-231 cells were seeded at a density of 400,000 cell in 6cm dishes and transfected with siRNA or expression plasmids (refer to Transfection methods). After transfection, cells were tripsinized and seeded into two plates, under each condition (control siRNA vs. DAXX siRNA and pCMV6 vs. DAXX overexpression vector). Remaining cells were centrifuged and lysed to collect protein to confirm DAXX knockdown or overexpression (refer to Western Blotting methods). Cells were treated with either DMSO or 5µM Carboplatin for one day. On the day of the assay, cells were washed, trypsinized and individualized. All media and PBS were kept, collecting all cells regardless if they were dead or alive. The cells were centrifuged at 1200rpm for 3 minutes and washed with 2mL of cold FACS buffer (5% FBS in PBS). Cells were then resuspended in 250µL RNase A, that was diluted 1:1000 in PBS, and incubated first for 15 minutes

at 37°C and then for 5 minutes at room temperature. In the dark, 250µL of Propidium Iodine (PI), diluted 1:10 in PBS, were added to the cells, and they were left to incubate in the dark for at least an hour before running flow cytometry on the BD FACS Canto II instrument.

Cell Fractionation

Cells were seeded in a 10cm tissue culture dish and treated over the course of seven days, collecting cells at an initial (no treatment), three-day, and seven-day time point. A protease inhibitor solution (PIS) was made by adding 50µL of a protease inhibitor cocktail and 20µL of PMSF to 200µL PBS. The medium was aspirated from the cells and they were washed with PBS. 750mL of PBS were added to each plate and using a rubber cell scraper, cells were scraped off the plate and placed in 1.5mL Eppendorf tubes on ice. The cells were then centrifuged at 2,300rpm for 5 minutes at 4°C and the PBS was aspirated. 500µL of CER I, made from adding 10µL PIS to 500µL of CER I buffer from the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit, were added to the pellet and vortexed twice for 10 seconds. The suspension was incubated on ice for 10 minutes. Then, 25µL of the CER II Buffer was added to the suspension, vortexed for 10 seconds, incubated for 1 minute on ice and vortexed again. The suspension was pelleted by centrifuging for 5 minutes at 14,000rpm at 4°C. The cytoplasmic fraction, the supernatant, was collected and placed on ice. To the remaining pellet, 250µL of working NER Buffer were added, made by adding 20µL pf PIS to 1mL of NER Buffer from the kit. The new suspension was vortexed for 10 seconds twice and placed back on ice. This was repeated every 10 minutes for 40 minutes. During the 40 minutes, the suspension was also pipetted vigorously to forcefully break the nuclear membrane during this time. Then, the suspension was centrifuged for 10 minutes at 14,000rpm at 4°C and the remaining supernatant, the nuclear fraction, was collected and placed on ice. BCA assay (refer to Western Blotting Methods above) was performed on the

different fractions to determine the concentration of protein present—except only 5μL of sample and 4X sample buffer were used instead of 10μL and 2X sample buffer.

CHAPTER IV

RESULTS

Specific Aim 1: Determine if DAXX is a Suppressor of Breast Cancer Cells of Distinct Subtypes by Regulating the Notch Pathway.

Notch signaling regulates diverse several cellular processes including cell survival, proliferation, and cell death. Preliminary data show that DAXX may be limiting overall bulk cell proliferation in various subtypes of breast cancer to different degrees. Preliminary data also show that DAXX is upregulated in response to GSI when combined with endocrine therapy in ER+ MCF-7 cells and is required for the efficacy of the GSI to inhibit cancer stem cells derived from this subtype. In HER2+ and triple negative cell lines, expression of the DAXX protein is necessary for the GSI to inhibit bulk cell proliferation. The preliminary data suggest that DAXX is necessary for the Notch inhibitor to effectively inhibit proliferation of at least three cell lines representing three breast cancer subtypes. Therefore, the goal of this first aim is to determine if DAXX acts upstream or downstream of the Notch signaling pathway to inhibit breast cancer cell survival.

DAXX Regulates, HES1, a Notch Target Transcription Factor, Possibly Independent of the Signaling Pathway.

To determine if DAXX expression regulates the Notch pathway, DAXX was depleted in the triple negative cell line, MDA-MB-231, and treated with a GSI. Measurement of Notch

target gene transcripts (HES1, HEY1, CMYC, HEY2, NOTCH3, and NOTCH4) was performed by RT-qPCR. If DAXX is a regulator of Notch signaling, then depletion of DAXX would lead to an increase or a decrease in Notch target gene transcripts. Upon measuring the expression of several Notch target transcripts, DAXX depletion by siRNA results in decreased expression of HES1, HEY1, and CMYC (***figure 12A***), but increased expression of other genes, HEY2, NOTCH3, and NOTCH4 (***figure 12B***). Thus, it appears that DAXX expression is required for both the activation and repression of the Notch pathway. Of the gene transcripts measured, only HES1 expression was significantly decreased upon DAXX depletion. These results suggest that DAXX is required for expression of the Notch target gene, HES1, a transcriptional repressor. Thus, it is possible that DAXX inhibits cell proliferation of MDA-MB-231 and other cells by activating HES1.

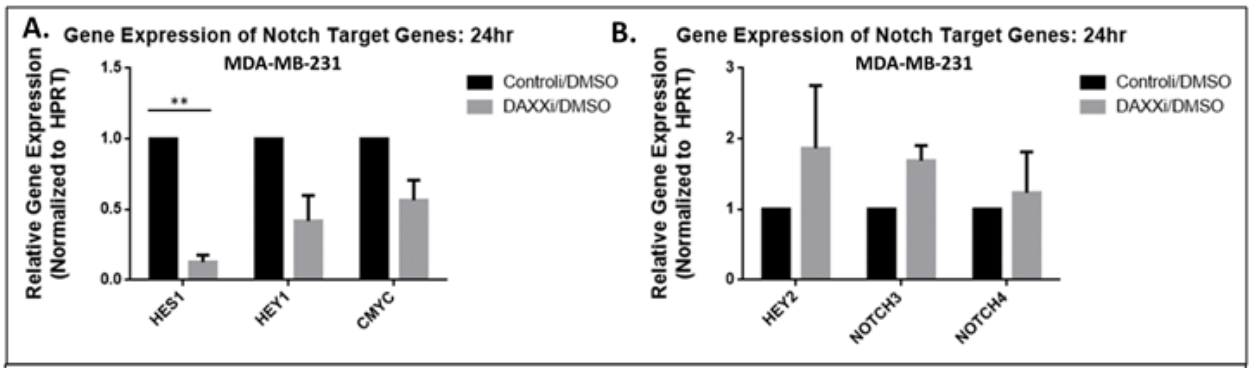

Figure 13: Expression of DAXX Affects the Expression of Several Notch Target Genes. MDA-MB-231 cells were transfected with control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% FBS IMEM in 6-well plates and treated for 24 hours with two different GSIs. After treatment, RNA was extracted from the cells and cDNA was synthesized. The relative gene expression of various Notch targets was measured by RT-qPCR. Genes that require DAXX expression to be **(A)** activated or **(B)** suppressed are shown.

To determine if DAXX inhibits growth of MDA-MB-231 cells through HES1, HES1 was either depleted or overexpressed and cell proliferation was measured. If DAXX is inhibiting proliferation of breast cancer cells through the activation of HES1, then depleting HES1 will result in an increase in proliferation in a similar manner as when DAXX is depleted. Additionally, if this is the case, then overexpression of HES1 should limit proliferation of these cells, regardless of DAXX expression.

When cells are depleted of HES1 (***figure 13B)***, bulk cell proliferation of MDA-MB-231 cells significantly increases (***figure 13A)***. However, when HES1 is overexpressed (***figure 13D and E)***, there is little effect on bulk cell proliferation (***figure 13C, second column***). In fact, bulk cell proliferation is increased when DAXX is depleted, even when HES1 is overexpressed, contrary to what was expected (***third and fourth column***). Together, these data demonstrate that the effects of DAXX on cell proliferation of MDA-MB-231 cells may be independent of HES1, one of the major targets of Notch signaling.

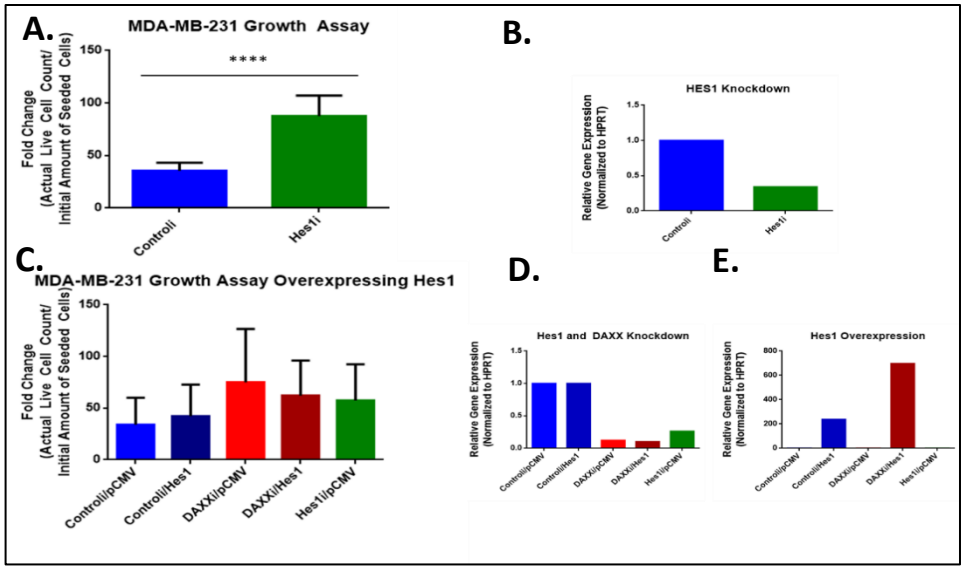

Figure 14: Expression and Regulation of DAXX May Be Notch-Independent. MDA-MB-231 cells were transfected with either control, DAXX or HES1 siRNA. Additionally, cells were also transfected with an empty vector or an expression vector containing a CMV promoter and HES1 gene sequence. After transfection, cells were grown in 5% FBS IMEM for 7 days. The fold change in proliferation of cells in HES1 knockdown conditions (**A.**) or in various combinations of HES1 or DAXX depletion along with HES1 overexpression (**C.**) was measured after 7 days. Knockdown of HES1 alone or HES1 and DAXX with either expression vector was confirmed by gene expression via RT-qPCR (**B.** and **D.**, respectively). Overexpression of HES1 was confirmed by gene expression as well via RT-qPCR (**E.**)

Specific Aim 2: Determine if DAXX Expression is Modulated by Current Standard of Care Therapy and is Required for Drug Sensitivity.

Breast cancers that are treated with current standard of care eventually acquire resistance. What is not known is whether DAXX expression is a critical regulator of drug sensitivity. Therefore, the goals of this aim are to determine: 1. if current therapies regulates expression of DAXX and 2. if DAXX regulates sensitivity of these breast cancer cells to their subtype-specific therapy.

Sensitivity of ER+ Breast Cancer Cells to Endocrine Therapy is Not Dependent on DAXX.

To investigate the effects of DAXX expression on ER+ breast cancer therapy, MCF-7 cells were transfected with DAXX siRNA and treated with endocrine therapy that directly inhibits the activity of the ER for 7 days. If DAXX expression modulates sensitivity to endocrine therapy in MCF-7 cells, then depleting DAXX will either increase or decrease the concentration necessary to achieve 50% inhibition of proliferation (IC50). Proliferation as measured by fold increase of live cells at day 7 or % growth is decreased in a concentration dependent manner in response to either 4-hydroxytamoxifen (4-OHT) (***figure 14A and B***) or fulvestrant (***figure 14C and D***), two forms of endocrine therapy. DAXX expression has very little effect on drug sensitivity, since the proliferation continues to decrease in response to 4-OHT or fulvestrant regardless of DAXX depletion. The negative control for this experiment was the vehicle, ethanol.

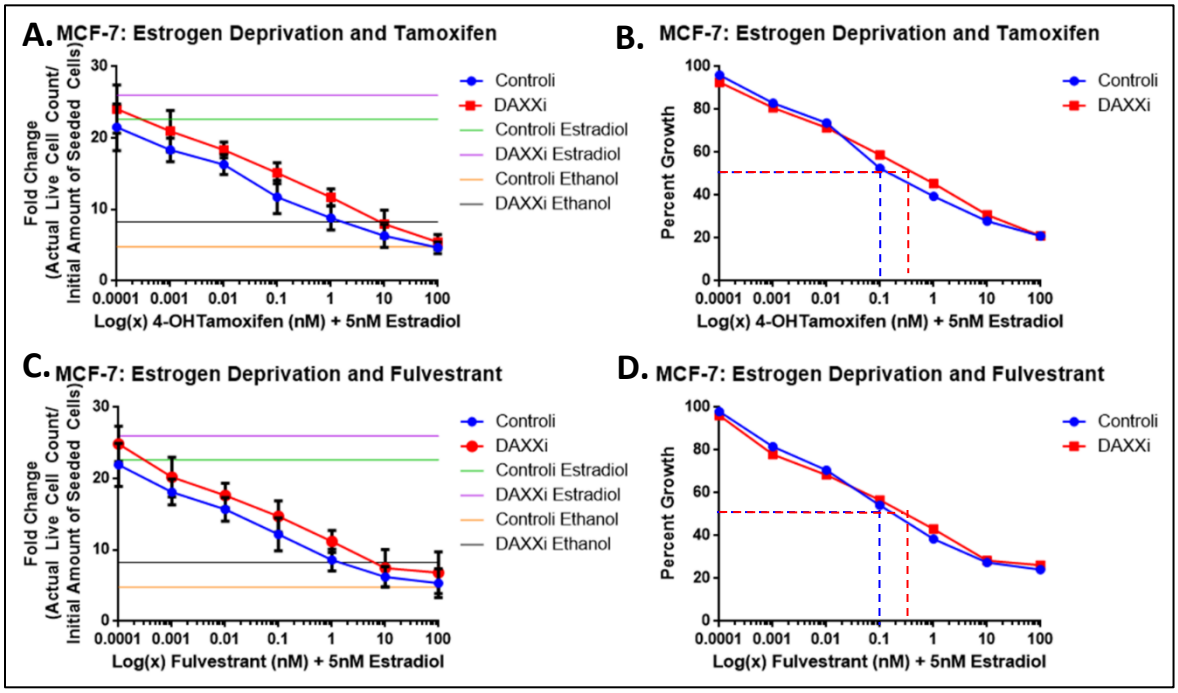

Figure 15: DAXX is Required to Limit Proliferation but Only has a Slight Effect on the Sensitivity of ER+ Breast Cancer Therapies. MCF-7 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were then grown in 10% FBS RPMI without phenol red and treated daily with either 4-hydroxytamoxifen or fulvestrant for 7 days, daily. After 7 days, the fold change in proliferation in those cells treated with 4-OHT (**A.**) of fulvestrant (**C.**) was measured as well as the percent growth (**B., D. respectively**) were measured.

Table 8: Statistical Significance P-values for Proliferation of DAXX-Depleted versus DAXX-Expressing MCF-7 Cells Treated with 4-Hydroxytamoxifen.

[4OH-Tamoxifen]	P-value	Significance
0	< 0.0001	****
0.0001	0.062	**
0.001	0.016	*
0.01	0.0014	**
0.1	< 0.0001	****
1.0	0.0063	**
10	0.0008	***
100	0.0103	*

Table 9: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing MCF-7 Cells Treated with Fulvestrant.

[Fulvestrant]	P-value	Significance
0	< 0.0001	****
0.0001	0.0159	*
0.001	0.0024	**
0.01	0.0001	***
0.1	0.0015	**
1.0	0.026	**
10	0.0911	ns
100	0.0698	ns

Differential Expression of DAXX in Response to Endocrine or Targeted Therapies

To investigate if 4-OHT or fulvestrant modulates DAXX protein expression in ER+ breast cancer, MCF-7 cells treated daily for three days with either the vehicle control (ethanol), estradiol (positive control), 4-OHT, or fulvestrant. The expression of DAXX protein was detected by Western blot analysis. If endocrine therapy modulates DAXX protein expression, then DAXX expression will either increase or decrease in response to the therapy. A DAXX siRNA was used to confirm the detection of the DAXX protein and confirm knockdown for other studies described above. Estradiol increases DAXX protein expression compared to the control ethanol-treated group (***figure 15, first column to third column***). Three forms of endocrine therapy, estrogen deprivation (***figure 15***, ***third column to first column***), 4-OHT (***figure 15***, ***fifth column to first column***), or fulvestrant ***(figure 15***, ***seventh column to first column)*** decrease expression of the DAXX protein to varying degrees. Also, the Western blot confirms that the DAXX siRNA was effective at decreasing DAXX levels.

Together, these data show that DAXX protein expression is increased by estrogen but is not required for cell proliferation nor drug sensitivity of an ER+ breast cancer cell line.

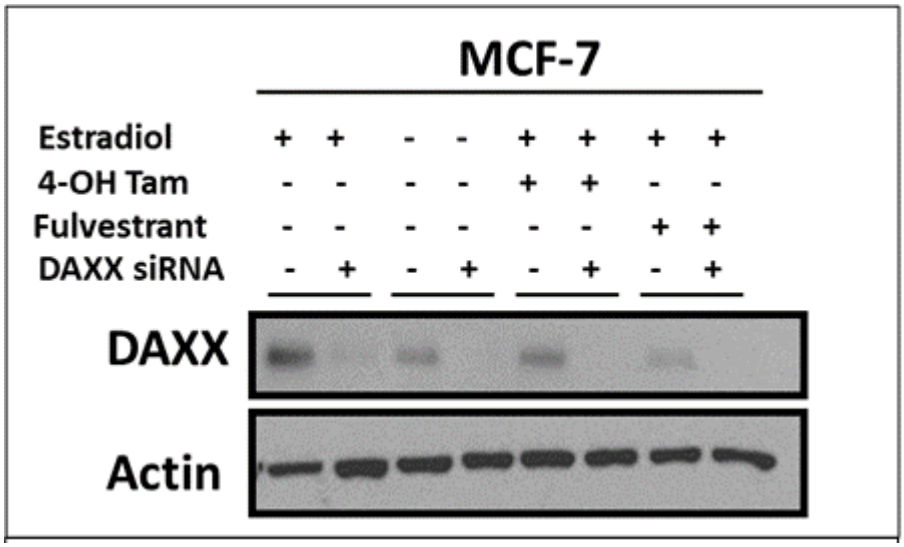

Figure 16: DAXX Expression is Modulated Differently Based on the Form of Endocrine Therapy Given. MCF-7 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% FBS RMPI without phenol red for three days, while treating with the drugs listed above daily. Cells were then lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

DAXX Limits Proliferation of Both Sensitive and Resistant HER2+ Cell Lines but has Little Effect on Sensitivty to Anti-HER2 Therapy.

The Osipo lab showed previously that trastuzumab increases Notch1 expression to maintain resistance in HER2+ breast cancer cells (Osipo et al., 2008; Baker at al., 2018). Further, it was shown that Notch-1 directly regulates DAXX expression (Albain et al., 2014). To determine if DAXX is the mediator of resistance to trastuzumab, BT474 trastuzumab sensitive and resistant cells were transfected with DAXX siRNA and treated daily for 7 days with increasing concentrations of trastuzumab. Proliferation was assessed by measuring the fold change in live cells at 7 days and calculating the percent growth. If DAXX expression modulates sensititvity to trastuzumab, then depleting DAXX in either cell line should result in an increase in cell proliferation regardless of the concentration. DAXX depletion resulted in increased proliferation

of both trastuzumab sensitive (***figure 16A***) and resistant (***figure 16C***) cells regardless of trastuzumab treatment. The percent growth of each cell line showed that the sensitive cells remained sensitive to trastuzumab (***figure 16B)***, while the resistant cells remained resistant (***figure 16D)***, independent of DAXX expression.

These data suggest that while DAXX limits the proliferation of HER2+, BT474 breast cancer cells, this function of DAXX does change sensitivity to trastuzumab.

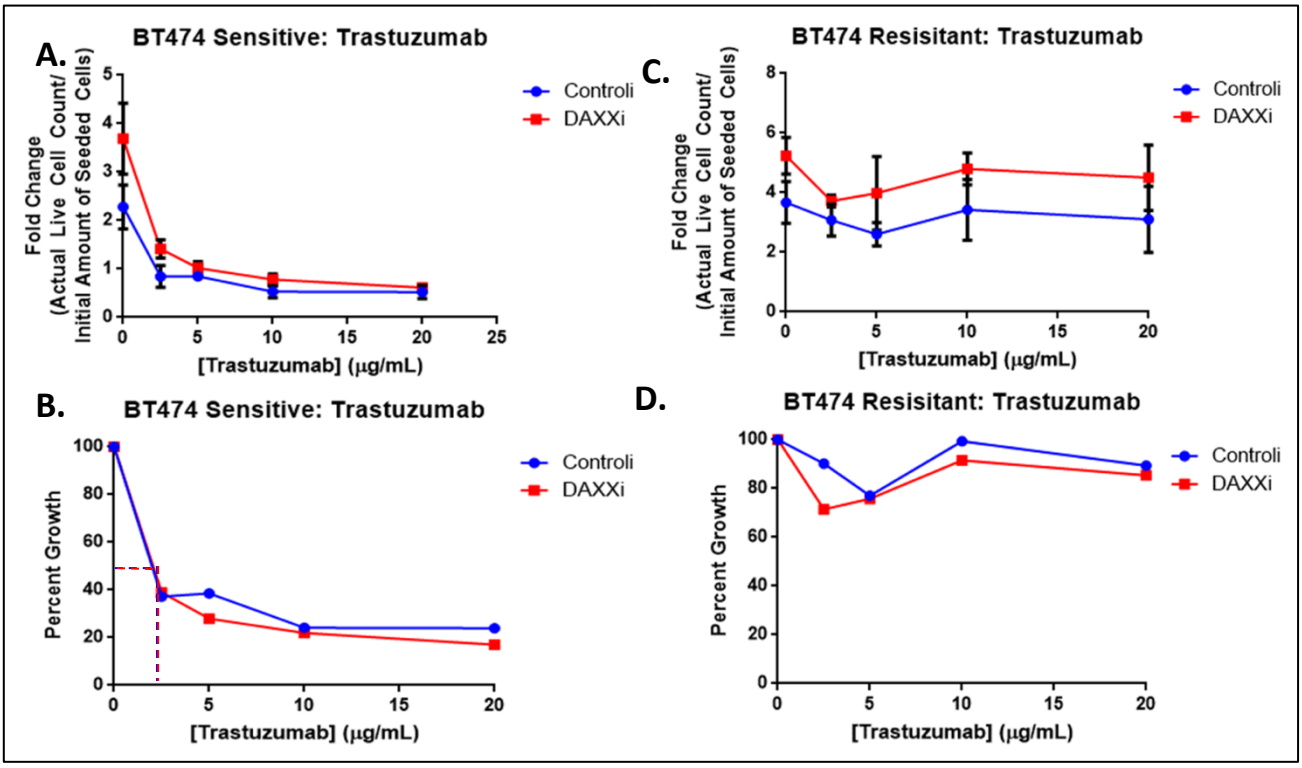

Figure 17: DAXX Expression is Modulated Differently Based on the Form of Endocrine Therapy Given. MCF-7 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% FBS RMPI without phenol red for three days, while treating with the drugs listed above daily. Cells were then lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

Table 10: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing BT474 Sensitive Cells.

[Trastuzumab]	P-value	Significance
0	0.003	***
2.5	< 0.0001	****
5.0	0.0084	**
10.0	0.0004	***
20.0	0.993	ns

Table 11: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing BT474 Resistant Cells.

[Trastuzumab]	P-value	Significance
0	0.002	***
2.5	0.0189	*
5.0	0.0160	*
10.0	0.0038	**
20.0	0.0160	*

Differential Effects of Trastuzumab on DAXX Protein Expression in HER2+ Sensitive and Resistant Cell Lines.

To assess whether trastuzumab modulates DAXX protein expression, both BT474 sensitive and resistant cell lysates were treated with trastuzumab and subjected to Western blotting to detect DAXX. A Notch inhibitor (GSI) was used as a positive control as previous work has shown Notch modulates DAXX protein expression. DAXX protein levels were decreased by the GSI in BT474 sensitive cells (***figure 17A)***, while having little effect in resistant cells (***figure 17B).*** Similarly, trastuzumab decreased DAXX protein expression in sensitive cells (***figure 17A),*** but has little effect in resistant cells (***figure 17B)***. The figure below also confirms that DAXX has been depleted in these cells by siRNA. These data with the proliferation studies suggest that DAXX is required

to limit proliferation of HER2+ breast cancer cells but is not required for trastuzumab sensitivity. Further, these results indicate that while trastuzumab decreases DAXX expression in sensitive cells, this decrease in DAXX may not be linked to proliferation or sensitivity to trastuzumab.

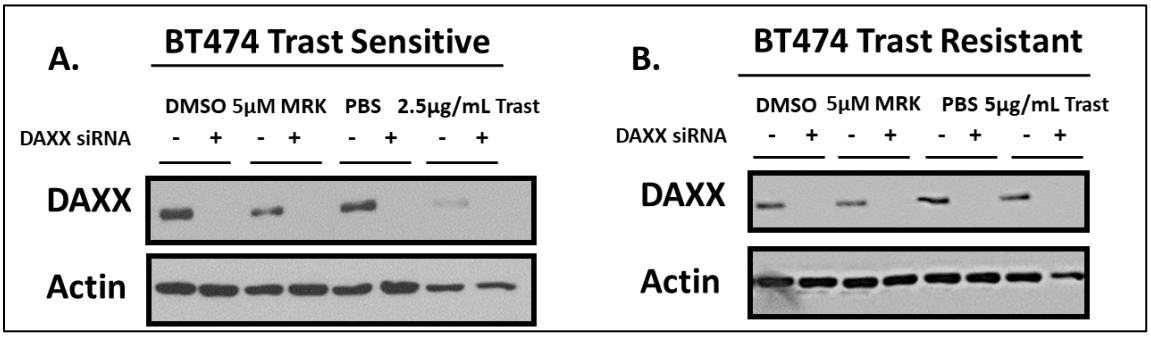

Figure 18: DAXX Expression Upon Treatment with Trastuzumab is Only Affected in a Sensitive HER2+ Cell Line. BT474 trastuzumab sensitive (**A.**) and resistant (**B.**) cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were growth in 10% FBS DMEM for three days and treated with either GSI or trastuzumab daily. The concentration of trastuzumab used were determined using the growth data. After treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

DAXX Limits Peoliferation in a Triple Negative Cell Line and Modulates Sensitivity to Some Chemotherapies.

In order to determine if DAXX is required to modulate sensitivity to chemotherapy such as carboplatin, in a triple negative cell line, MDA-MB-231 cells were transfected with DAXX siRNA and treated with carboplatin daily for 7 days. If DAXX expression is required for drug sensitivity, then sensitivity to carboplatin will increase when DAXX is expressed and decreased when DAXX is depleted. When DAXX is depleted in the absence of carboplatin, the proliferation of these cells significantly increases from 50 fold to over 75 fold (***figure 18A***). In addition, the Inhibitory concentration at 50% cell proliferation (IC50) of carboplatin is 2.5μM when DAXX is expressed compared to IC50 = 5μM when DAXX is depleted (***figure 18B***).

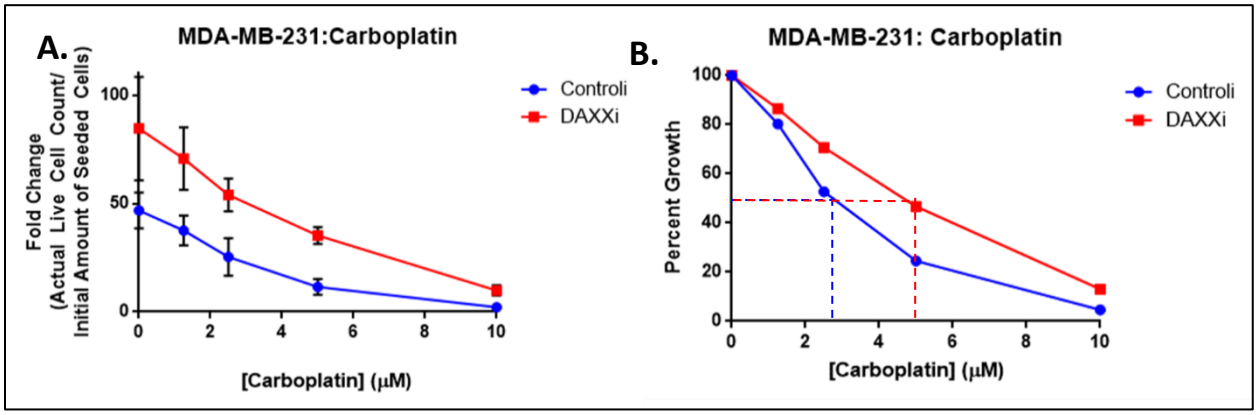

Figure 19: DAXX is Required for Both Limiting Cellular Proliferation Significantly and Drug Sensitivity in a Triple Negative Cell Line. MDA-MB-231 cells were transfected with either control siRNA or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% FBS IMEM for 7 days and treated with carboplatin daily. After treatment the fold change in proliferation (**A.**) and percent growth (**B.**) were calculated.

Table 12: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing MDA-MB-231Cells.

[Carboplatin]	P-value	Significance
0	0.012	**
1.25	< 0.0001	****
2.5	< 0.0001	****
5.0	< 0.0001	****
10.0	< 0.0001	****

Carboplatin Increases DAXX Protein Expression.

To determine if carboplatin modulates DAXX expression in the MDA-MB-231 cells, cells were transfected with DAXX siRNA and treated with either a GSI or carboplatin daily for three days. If DAXX expression if required for sensitivity to carboplatin, then treating the cells with carboplatin will increase the expression of DAXX protein. Western blot (***figure 19***) of DAXX

shows that the Merck GSI has little effect on the expression of the DAXX protein. DAXX protein expression increases in response to carboplatin. The figure below also confirms that DAXX was efficiently via siRNA.

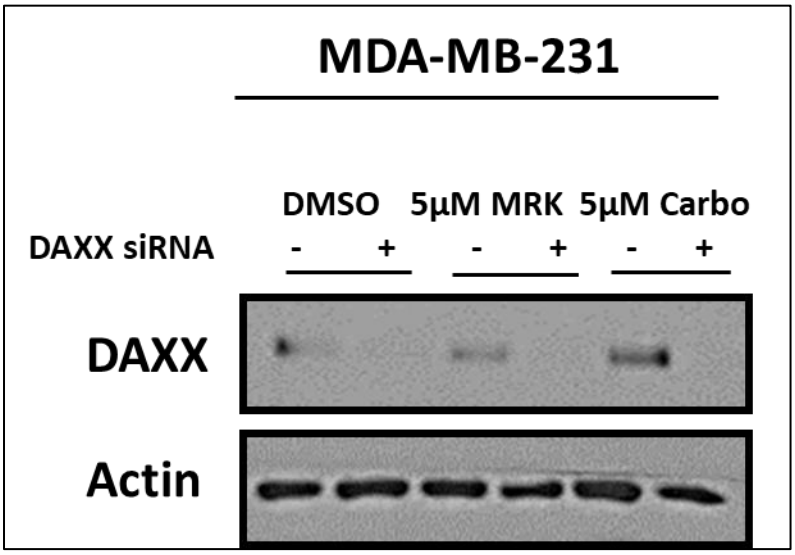

Figure 20: Standard of Care Therapy for Triple Negative Breast Cancer Increases DAXX Expression. MDA-MB-231 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% IMEM and treated daily with GSI or carboplatin. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

Together, these data suggest that DAXX expression is required to increase the sensitivity of triple negative breast cancer cells to carboplatin. Also, it appears that the DNA damaging agent is also increasing the expression of DAXX, correlating to the bulk cell proliferation data, where high DAXX expression increases sensitivity.

DAXX Modestly Limits Proliferation of a Second Triple Negative Cell Line but has Little Effect on Carboplatin Sensitivity.

To potentially establish if the phenotype seen in MDA-MB-231 cells is cell-type or subtype specific, a second triple negative cell line, MDA-MB-468, was also transfected with DAXX

siRNA and treated in a similar manner with carboplatin for 7 days. If the phenotype previously seen with MDA-MB-231 cells was subtype specific, then it would be expected that DAXX will 1) significantly limit the proliferation of these cells and 2) be required for the cells to be sensitive to carboplatin. As seen in ***figure 20A***, DAXX expression limits proliferation of MDA-MB-468 cells, but only modestly. When it comes to drug sensitivity however, DAXX expression has little effect; cells are sensitive to carboplatin, whether DAXX is expressed or depleted (***figure 20B***).

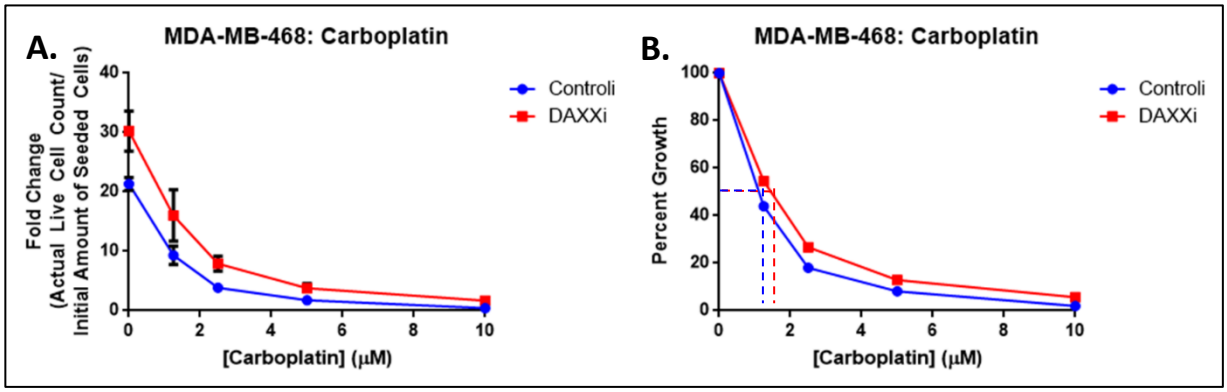

Figure 21: DAXX Expression Limits Proliferation but has No Effect on Drug Sensitivity in a Second Triple Negative Breast Cancer Cell Line. MDA-MB-468 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% FBS DMEM for 7 days and treated with carboplatin, daily. After treatment the fold change in proliferation **(A.)** and percent growth **(B.)** were calculated.

Table 13: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing MDA-MB-468 Cells.

[Carboplatin]	P-value	Significance
0	<0.0001	****
1.25	0.0014	**
2.5	<0.0001	****
5.0	<0.0001	****
10.0	<0.0001	****

DAXX Protein Expression is Decreased in MDA-MB-468 Cells.

If DAXX expression is modulated by standard of care therapy, and depletion of DAXX results in an increase sensitivity to therapy, then it is expected that DAXX expression will be decreased—at least in the presence of carboplatin. When MDA-MB-468 cells are treated with either a GSI or carboplatin, DAXX expression decreased (***figure 21***), opposite of what was seen in MDA-MB-231 cells, but consistent with the bulk cell proliferation results. The figure below also confirms that DAXX was depleted in these cells, via siRNA.

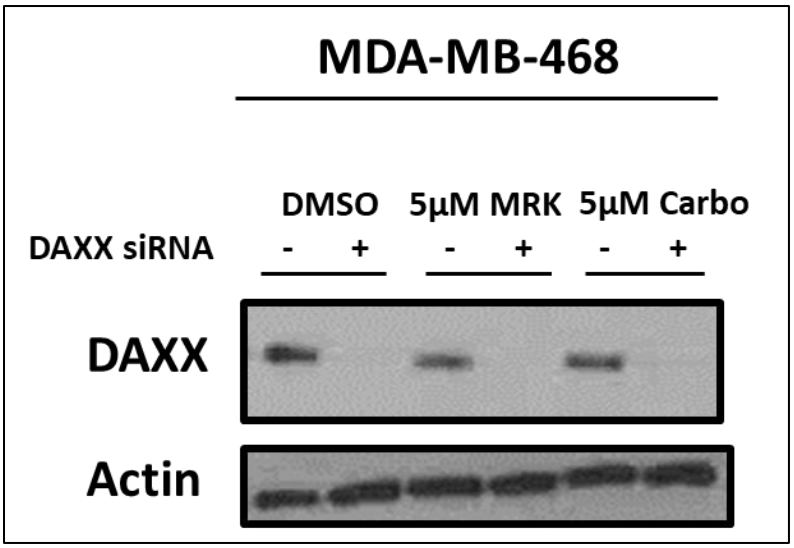

Figure 22: Standard of Care Therapy and Notch Inhibition has No Effect on DAXX Expression in Second Triple Negative Cell Line. MDA-MB-468 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% DMEM and treated daily with GSI or carboplatin. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

DAXX limits Proliferation of Cells in a Third Triple Negative Subtype but is Required for Resistance to Standard of Care Therapy.

To further determine if the previous phenotype was cell-type or subtype specific, a third triple negative subtype, BT549, were transfected as well with DAXX siRNA and treated with

carboplatin for a total of 7 days. Again, if the phenotype previously seen with MDA-MB-231 cells was subtype specific, then it would be expected that DAXX will both significantly limit the proliferation of these cells and be required for the cells to be sensitive to carboplatin. In ***figure 22A***, DAXX expression limits the proliferation of cells, more than in the MDA-MB-468 cells. In terms of drug sensitivity, it appears that low DAXX expression is required for sensitivity to carboplatin (***figure 22B***).

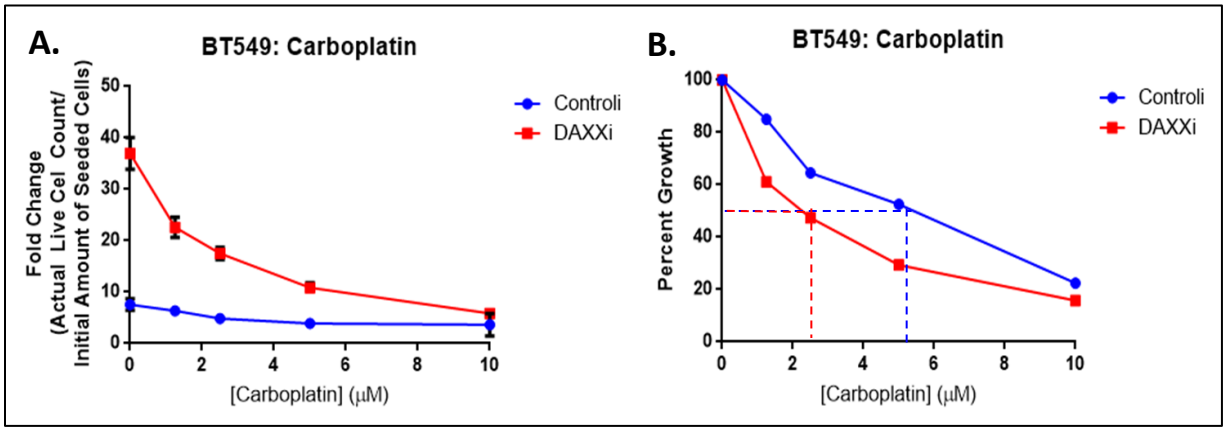

Figure 23: DAXX Expression Limits Proliferation but has No Effect on Drug Sensitivity in a Third Triple Negative Breast Cancer Cell Line. BT549 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% FBS RPMI for 7 days and treated with carboplatin, daily. After treatment the fold change in proliferation **(A.)** and percent growth **(B.)** were calculated.

Table 14: Statistical Significance P-values for Proliferation of DAXX-depleted versus DAXX-expressing BT549 Cells.

[Carboplatin]	P-value	Significance
0	<0.0001	****
1.25	<0.0001	****
2.5	<0.0001	****
5.0	<0.0001	****
10.0	0.0600	ns

Drug Treatments have No Effect on DAXX Protein Expression.

If current forms of therapy modulate DAXX expression, and low DAXX expression increases sensitivity to this therapy then it is expected that expression of DAXX will decrease upon treatment with carboplatin. In BT549 cells, DAXX expression remains unchanged, despite treatment with this therapy (***figure 23***).

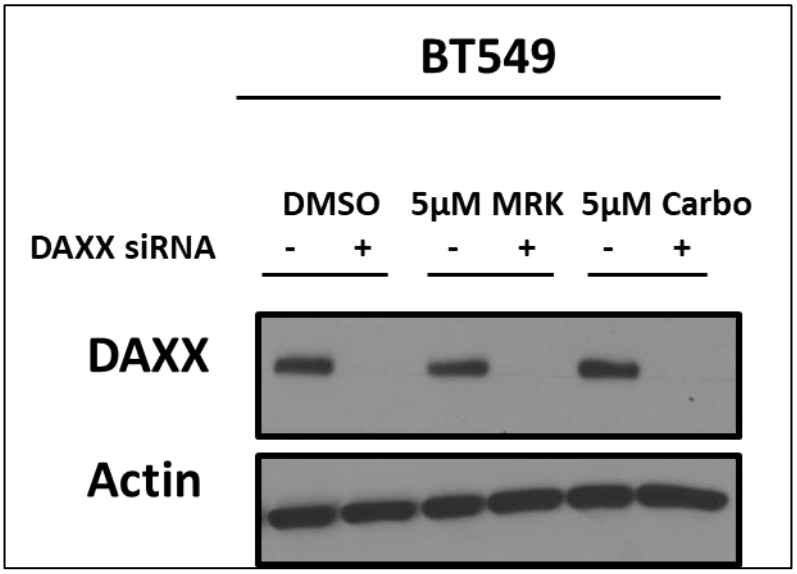

Figure 24: Standard of Care Therapy and Notch Inhibition has No Effect on DAXX Expression in Third Triple Negative Cell Line. BT549 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 10% RPMI and treated daily with GSI or carboplatin. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

Carboplatin Activates the JNK Signaling Pathway Through DAXX.

Since depletion of DAXX resulted in MDA-MB-231 cells being less sensitive to carboplatin, then it may be possible that DAXX-expressing cells undergo apoptosis in response to carboplatin. It is known that DAXX induces apoptosis through activation of JNK signaling (Salomoni & Khelifi, 2006). Therefore, if MDA-MB-231 cells are more sensitive to carboplatin due to DAXX-mediated activation of JNK resulting in apoptosis, then there should be an increase

in phosphorylated (or active) JNK in response to carboplatin when DAXX is expressed and not when DAXX is depleted. MDA-MB-231 cells were treated similarly as before for 3 days and the protein expression was detected via Western blot analysis. As seen in the data (***figure 24A***), samples that were treated with carboplatin show an increase in phosphorylated JNK. However, this finding is limited to those cells that are expressing DAXX because once DAXX is depleted, there is no detection of phosphorylated JNK. This would suggest that DAXX is being upregulated by carboplatin treatment, subsequently upregulating JNK activity. To further asses that upregulation of DAXX by carboplatin increases JNK activity, bulk cell proliferation studies were completed by treating MDA-MB-231 cells with carboplatin as well as SP600125, a JNK inhibitor (JNKi). Cells were transfected with DAXX siRNA and treated daily for 7 days prior to calculating the fold change in cell proliferation. If cells expressing DAXX are sensitive to carboplatin due to an increase in JNK activity, then treating cells with a JNK inhibitor should result in cells that are resistant to the effects of carboplatin when expressing DAXX. As seen in ***figure 24B***, proliferation of MDA-MB-231 cells increases when DAXX is depleted when treated with a vehicle or carboplatin. However, the increase of proliferation in DAXX-depleted cells is attenuated when JNK activity is inhibited, indicating that there may be a partial role for JNK in overall proliferation. The addition of carboplatin eliminates the effects seen with the JNKi, regardless of DAXX expression.

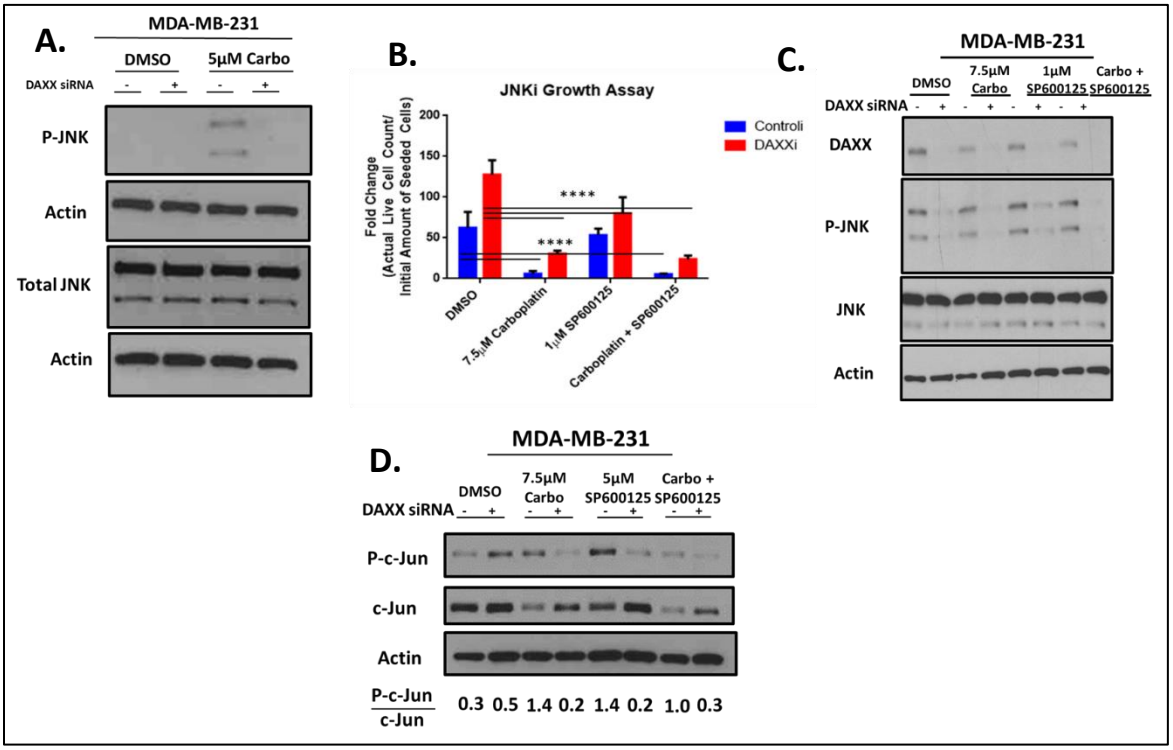

Figure 25: JNK Activity Regulation Through Carboplatin and DAXX Expression: MDA-MB-231 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% IMEM and treated daily with **(A.)** carboplatin for 3 days, **(B.,C.)** carboplatin and/or SP600125 for 7 days or **(D.)** carboplatin and/or SP600125 for 5 days. After the treatment, cells were either counted and the bulk cell proliferation fold changed calculated or were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

DAXX as a Potential Regulator of PARP Activity.

Because DAXX expression is upregulated in response to carboplatin, expression and cleavage of PARP-1 was measured in treat MDA-MB-231 cells to confirm that these cells fact were undergoing DNA damage. It is well known that PARP [poly(ADP-ribose) polymerase] is typically activated in response to DNA damage. Therefore, to determine that these cells were undergoing some type of DNA damage the expression of PARP-1 was measured. If the cells are treated with the vehicle, then it is expected that PARP-1 would be cleaved (inactivated) unlike

those samples that are treated with carboplatin. Additionally, if DAXX is upregulated when treated with carboplatin, then is the expression of PARP-1 affected by the expression of DAXX? To answer this and test the hypothesis, MDA-MB-231 cells transfected with DAXX siRNA and treated with carboplatin were analyzed via Western blot for PARP-1 expression. Interestingly enough, when DAXX is depleted PARP-1 is smeared, regardless of treatment with carboplatin (***figure 25***).

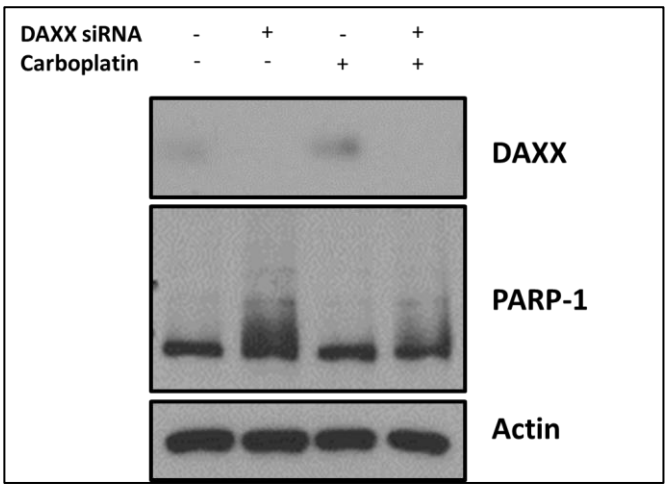

Figure 26: Depletion of DAXX Affects Expression of PARP-1. MDA-MB-231 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% IMEM and treated daily with carboplatin for 3 days. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

A recent publication summarized how cells that are under replication or mitotic stress, PARP activity is key in the re-stabilization of cells (Mcdermott, Buechelmaier, & Powell, 2019). Activity of PARP-1 includes synthesizing poly(ADP-ribose), or PAR, chains at sites of a single stranded DNA breaks onto acceptor proteins and to itself. The addition of these chains stabilizes the replication fork to signal repair factors. To restart the replication fork, another enzyme PARG [poly(ADP-ribose) glycohydrolase] degrades the PAR chains that have been added. Since depleting DAXX in MDA-MB-231 cells significantly increases their rate of proliferation and

causes a smearing effect in PARP-1, total PAR expression was measured in these samples to see how DAXX expression affects this modification. As seen in ***figure 25***, when DAXX is depleted, there is an increased smearing effect on the expression of PAR, with or without treatment of carboplatin—similar to what was seen in PARP-1 expression. Therefore, DAXX could potentially be regulating, in some way, this PARP/PARG cycle.

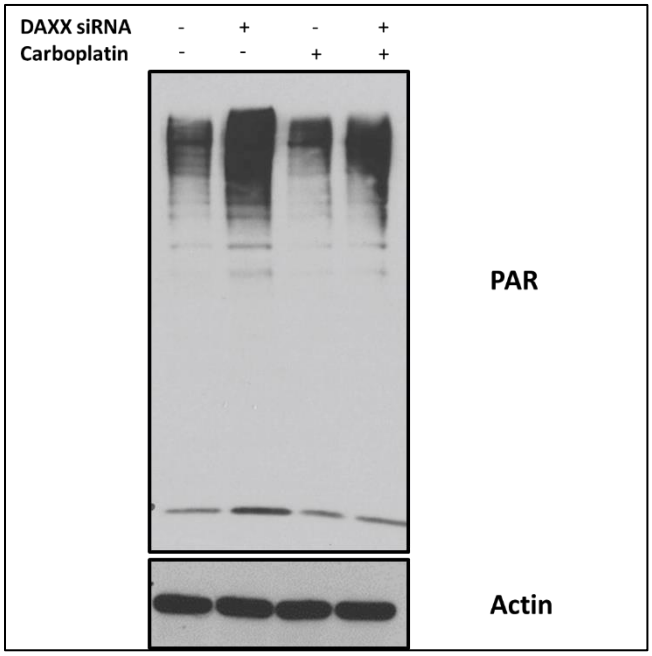

Figure 27: Depletion of DAXX Also Increases Smearing of PAR. MDA-MB-231 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% IMEM and treated daily with carboplatin for 3 days. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

To determine if DAXX is in fact a regulator of PARP/PARG activity, MDA-MB-231 cells depleted of DAXX were treated with a PARP-1 inhibitor, olaparib. If DAXX is a regulator of PARP-1 activity, then treatment with olaparib may reverse this smearing effect seen. As seen in ***figure 27***, as cells are treated with olaparib for more time, the smearing effect decreases, even

under conditions where DAXX is depleted. This indicates that there may be in fact a regulatory role for DAXX in PARP activity.

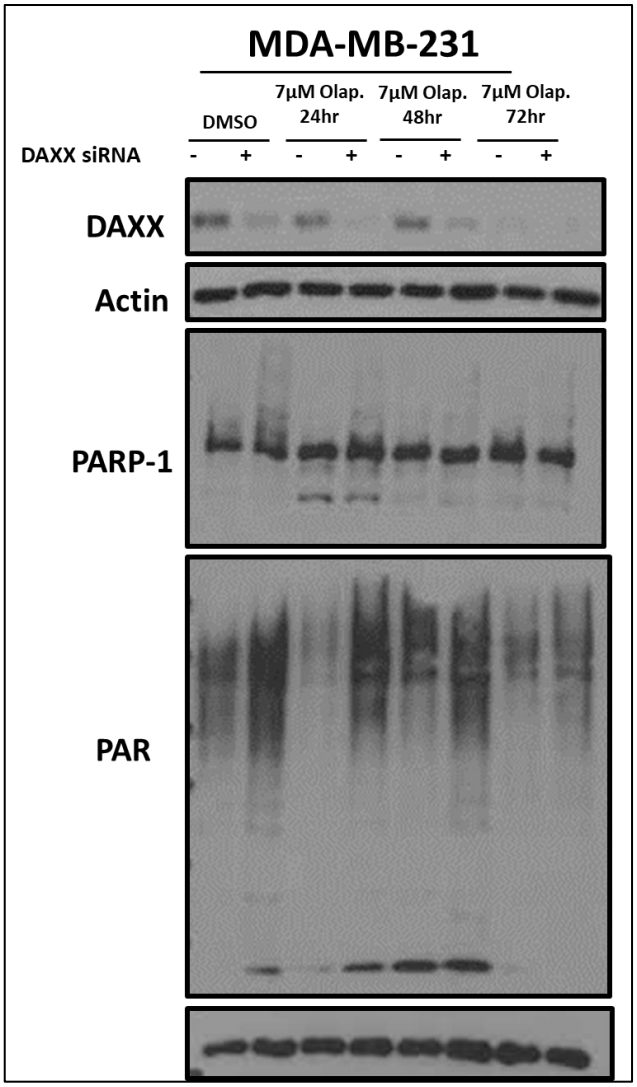

Figure 28: PARP Inhibitor Decreases Smear Effect in the Absence of DAXX. MDA-MB-231 cells were transfected with either control or DAXX siRNA for 48 hours. After transfection, cells were grown in 5% IMEM and treated daily with olaparib for 1, 2 or 3 days. After the treatment, cells were lysed, and protein was collected and measured to create samples for Western blotting. Protein was separated and visualized via Western blot analysis.

CHAPTER V

DISCUSSION

Out of all the breast cancer subtypes, TNBC is the most aggressive both in terms of treatment and outcome. Patients with TNBC have a higher risk of recurrence and metastasis, and unfortunately, do not have many therapeutic options other than chemotherapy. It is known that DAXX protein appears to be limiting the proliferation of breast cancer cells to some degree in all the subtypes and in the TNBC subtype does so, significantly. Cell cycle data supports this increase in proliferation, since there is almost a doubling effect in S-phase once DAXX is depleted (not shown). This indicates that DNA synthesis is reduced due to DAXX expression, therefore providing a potential way DAXX is limiting proliferation: via regulation of the cell cycle. It has also been seen that in a TNBC cell line, MDA-MB-231, DAXX is required for sensitivity to chemotherapy drug carboplatin. However, in other TNBC cell lines, such as MDA-MB-468 and BT549, DAXX expression does not limit proliferation or mediate sensitivity to the same extent. This only emphasizes how diverse and heterogeneous the triple negative subtype truly is, in terms of several factors such as genes expressed, sensitivity to certain drugs and rate proliferation rates (Bianchini et al., 2016). This can also be indicative of the extent by which DAXX affects cell cycling among different subtypes. However, this can only be concluded once the cell cycle of these other TNBC cell lines and even the other breast cancer subtypes is determined. Although there is a lot of evidence to support this role of DAXX, the exact mechanism by which DAXX mediates these processes is still unknown. For example, DAXX limits bulk cell proliferation but how this occurs exactly has yet to be identified. It was believed that this

mechanism may have been through Notch signaling; however, this hypothesis isn't strongly supported anymore.

It also known that DAXX expression is required for activation of JNK when cells are treated with carboplatin. Because JNK activation leads to apoptosis, it is possible that DAXX is required for carboplatin sensitivity by activating JNK mediated apoptosis. Cell fractionation studies primarily localized DAXX to the nucleus, even though it is known that the role DAXX in terms of JNK activity is in the cytoplasm when it interacts with the Fas death domain. Activated, or phosphorylated, JNK is also found in the nucleus, therefore it can be hypothesized that DAXX could be further activating JNK in the nucleus to activate apoptosis of the cell when treated with carboplatin. This could all indicate that DAXX may be an important biomarker that can potentially predict how resistant a patient's cancer or tumor may be to the same therapy.

It has also been seen that DAXX expression appears to be hindering PARP-1 and PAR expression. PARP-1 works to covalently modify acceptor proteins with PAR chains that stabilize replication forks in time of distress (i. e. DNA damage). Another enzyme, PARG, removes these PAR chains once there has been repair to restart the replication process of cells (Mcdermott et al., 2019). Cancerous cells have increased levels of spontaneous DNA damage and it was implicated that this PARP/PARG cycle could be a potential targeted therapy in cancer cells. It has been recently published that DAXX expression increases sensitivity to a PARP inhibitor in the same MDA-MB-231 cell line, thus inhibiting the ability of PARP-1 to mediate single strand DNA repair (Shi, Jin, Wang, Ji, & Guan, 2019). Although this is more in relation to DAXX's ability to mediate DNA damage response by binding to the promoter region of *RAD51*, a DNA damage response gene (Shi et al., 2019). This work is novel since similar results are produced, but in relation to standard of care therapy in TNBC, thus providing some evidence that a more effective therapy

may be through PARP inhibition in combination to the current standard of care. Before determining if this is the case, all elements of this PARP/PARG cycle must be explored. As seen in ***figure 28*** [adapted from (Mcdermott et al., 2019)], the data supports that DAXX could be inhibiting PARP-1 activity. However, there is a possibility that DAXX may be activating PARG activity as well. Data with olaparib, a known PARP-1 inhibitor, supports the hypothesis that DAXX is regulating PARP-1, but expression and inhibition of PARG has yet to be studied and could clarify the mechanism by which DAXX is regulating this cycle.

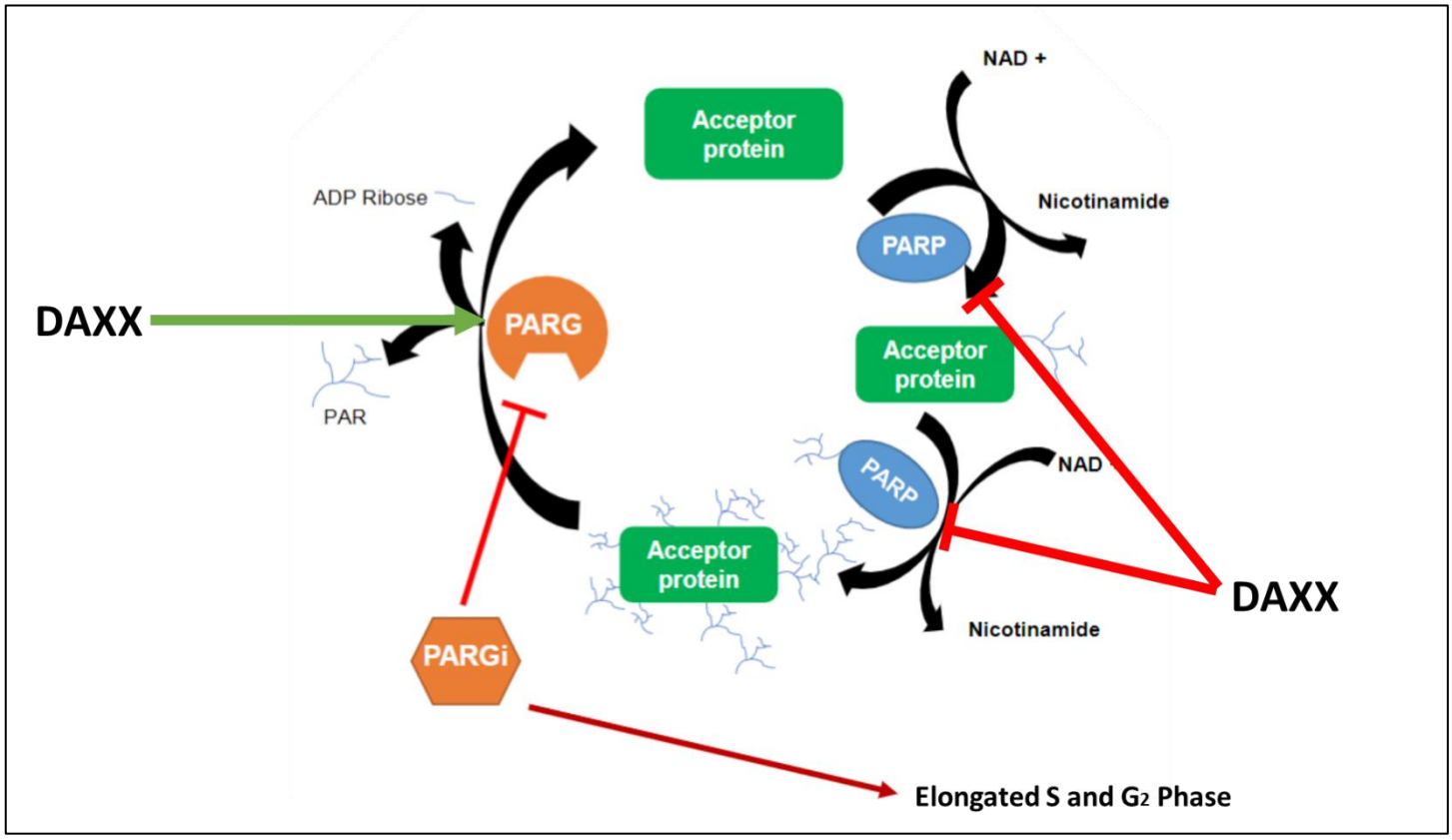

Figure 29: Proposed Role of DAXX Within PARP/PARG Cycle. When DNA is being replicated, PARP is recruited to sites of ss-DNA breaks to synthesize PAR chains to signal the recruitment of DNA-repairing enzymes such as DNA ligase III, DNA polymerase beta, and XRCC1 protein. Once the break is repaired, PARG is recruited to remove these PAR chains and promote the replication fork to restart and progress the cell cycle. Here, it is shown where and how DAXX is regulating this mechanism—either by inhibiting PARP activity or by activating PARG activity.

Determining where and how DAXX is functioning in this cycle, can not only elucidate yet another role for this multifunctional role and a clear mechanism by which it is acting by but can also provide a possible novel form of combinational targeted therapy in a very aggressive subtype. It has been previously described that cancer patients do not have effective forms of DNA damage repair mechanism and that TNBC is so aggressive due to a lack of targets. But there is a small percentage of TNBC patients with a *BRCA1* mutation that are responsive to olaparib. BRCA genes are human tumor suppressor genes that help either repair DNA, through homologous repair, or destroy cells if DNA cannot be repaired. When there is a mutation in this gene, it results in loss of function. When this mutation is present, cells cannot be repaired properly, and they die. Combining this mutation with a PARP inhibitor results in cells to undergo cell death making a PARP inhibitor an effective therapy for those patients with this mutation, and this can be seen summarized in ***figure 29*** (Dziadkowiec, Gąsiorowska, Nowak-markwitz, & Jankowska, 2016). Since it has been shown that DAXX plays a role in modulating sensitivity of MDA-MB-231 cells to DNA damaging agent carboplatin, and that DAXX could be potentially be playing a regulatory role within PARP/PARG, then this could allow for TNBC patients to be stratified into groups that will be responsive to this combinational targeted therapy, based off of their expression of DAXX as well as increasing the efficacy of carboplatin.

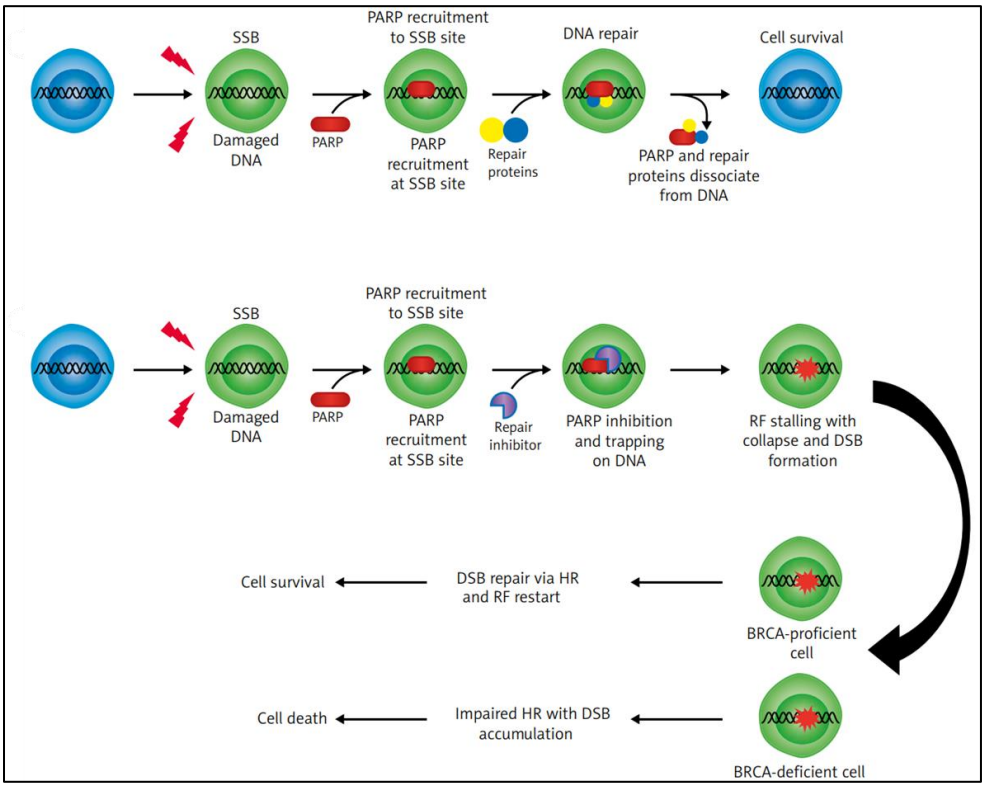

Figure 30: Relationship Between PARP Inhibition and BRCA1 Mutations. When there is a single stranded break (SSB) in the DNA, PARP functions to repair the site of damage (top). However, when PARP is inhibited and the site cannot be repaired, the SSB results in a double stranded break (DSB) in which a functional BRCA1 can repair. Loss of function mutations in this gene, like in some TNBC, result in a buildup of DSB ultimately causing cell death.

The effects of DAXX on the stem cell population is also unknown. In the cell line where the most significant data in terms of proliferation, effects of standard of care therapy, connection to JNK and effects on PARP/PAR has been studied, MDA-MB-231, the known stem cell assay, mammosphere formation assay has been challenging to say the least. Because these cells are mesenchymal, this assay, which is more for cells that are more basal-like, is not as effective in studying the stem cell population. Therefore, how DAXX expression affects these cells when treated with carboplatin remains largely unknown. This stem cell assay could be optimized to better predict mesenchymal stem cells but a simpler route to begin looking at this population of

cells could be to look at the expression of genes related to stemness first in these cells via RT-qPCR.

Being able to understand the mechanism by which DAXX is behaving can lie within the roles that have already been determined and characterized. Since DAXX has been localized to the nucleus, and it is known that in the nucleus DAXX functions as a repressor by associating with PML nuclear bodies (PML-NB), it is possible that some answers lie there as well. The work that has been presented does not directly look at DAXX's repressive role when interacting with these nuclear bodies. PML is rarely mutated in cancers, but it has been determined that its expression is lost in human tumors, making it seem like it is a tumor suppressor and is also a regulator of transcription (Salomoni, 2013). In tumor progression, chromatin levels are altered due to several factors such as 3D chromosome alterations and abnormal nuclear morphologies which result in varying levels of nuclear bodies present in the nucleus (Uhler & Shivashankar, 2018). Since there are 6 subtypes of TNBC alone, all with unique phenotypes and varying expression of genes, then it is possible that they also vary in the expression of nuclear bodies subsequently affecting the expression of PML-NB as well. Determining the expression of these PML-NB and how that expression is affected by DAXX could also provide some insight on how DAXX is mechanistically behaving differently in different TNBC subtypes or in breast cancer subtypes in general—which is another route to further progress the results presented.

It has been discussed that DAXX could be involved in several cellular functions such as proliferation, drug sensitivity, JNK activity, PARP/PARG regulation and hypothesized that there may be some role in stem like cells as well as PML-NB. But there are still several other cellular processes that could be responsible for the phenotypes seen, especially since DAXX is a multifaceted protein. For example, DAXX has been associated with histone modification and

maintaining heterochromatin and maintaining this function is hypothesized to be key in destroying cancer tumor cells. Because this is in the nucleus, where are cellular decisions are controlled and execute in some way, these different cellular functions it appears that DAXX is involved could be due to a direct involvement with heterochromatin that subsequently results in a number of pathways to be targeted or implicated in terms of DAXX expression. It is possible that DAXX is not directly linked to any of the functions listed before; the phenotypes seen are just a by-product of yet another disrupted process in these cells. Further experiments must be completed in terms of looking at histone modification to determine the effect of DAXX expression here and the repercussions of depleting the protein as well on the expression of genes that are heavily involved in the cellular pathways previously described.

Determining these mechanisms DAXX may be involved in can fully characterize DAXX to understand its potential as a biomarker. This knowledge can provide insight on how well a patient will respond to standard of care therapy, specifically in TNBC, to see if 1) said therapy would even be effective in the first place for them and 2) if it isn't effective, then what other therapy may be combined with it to combat the cancer. If the full extent of this protein is studied, then a new target in TNBC will have been identified that can improve outcome for those with TNBC. Since it appears that DAXX also limits proliferation in the other subtypes, then characterizing DAXX in such a way can also determine how a patient may respond to other therapies of the remaining subtypes. Ultimately, the data supports TNBC more therefore understanding the complex role of DAXX can allow for more targets to be utilized as therapeutics—such as JNK and PARP or PARG inhibitors. By improving therapeutics, especially that of TNBC, will greatly better overall outcome for those with this type of breast cancer, benefiting those that would have had a poor prognosis and who would suffer much longer the

effects of chemotherapies. Summarized in ***figure 30***, is a general overview of what has been learned thus far of DAXX, specifically in the context of TNBC, and the pathways it has been seen to be affecting.

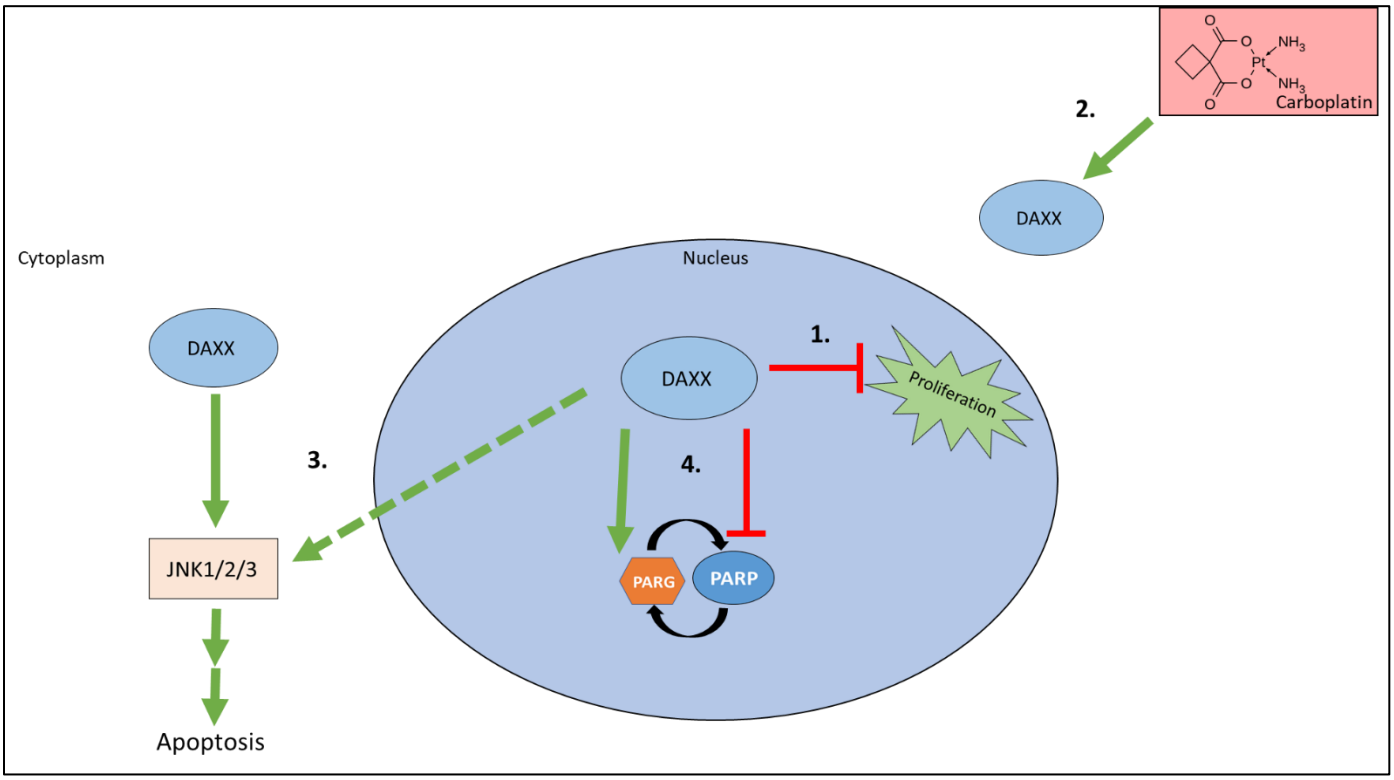

Figure 31: Final Model Highlighting Key Findings About DAXX. DAXX has been shown to be primarily localized in the nucleus of MDA-MB-231 cells, where it can be directly linked to its determined functions. **1.** DAXX is limiting the proliferation of cells. **2.** Chemotherapy drug, carboplatin upregulates the expression of DAXX. **3.** This upregulation of DAXX coincides with an increased in JNK activity. Which can be upregulated both in the cytoplasm, where DAXX is known to function, or even in the nucleus, where DAXX has been localized. And finally, **4.** DAXX expression regulates PARP/PARG cycle by either inhibiting PARP, which has been shown, or activating PARG, which still needs to be studied.

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VITA

Michelle Lucía Fernández was born on December 10th, 1994 to Marino Fernández and Magalys Abreu in Chicago, Illinois. She attended Lewis University in Romeoville, Illinois where she earned her Bachelor of Science in Biochemistry with a minor in Biology in May of 2017. During her undergraduate studies, she worked in the laboratory of Jason J Keleher, Ph.D., studying nanoparticle coated water filters with antimicrobial properties. After a year of this work, she initiated a project in Dr. Keleher's lab in conjunction with Sarah E Powers, Ph.D. studying the compounded protein structure of cyclin D3 and the significance this interacting interface has, specifically in the context of various cancers.

In late July of 2017, Michelle matriculated into the Biomedical Sciences Master's program at Loyola University Chicago in the Microbiology and Immunology department. She completed her graduate studies in the laboratory of Dr. Clodia Osipo, where she focused on investigating the role of DAXX as a potential biomarker in several breast cancer subtypes.

Upon completing her graduate studies, she will remain at Loyola as a Research Specialist for Dr. Osipo. Under Dr. Osipo's guidance, Michelle hopes to find work in industry studying cancer-related clinical therapies or translational research, prior to applying for PhD programs.