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Proteins Involved in HDACi Induced Decay of ERBB2 Transcripts in Breast Cancer

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Proteins Involved in HDACi Induced Decay of ERBB2 Transcripts in Breast Cancer

A thesis submitted to the faculty of

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in partial fulfillment of the requirements

for the degree

Master of Science

In

Biology

By

Sadaf Z. Malik

San Rafael, California

May 2015

CERTIFICATION OF APPROVAL I certify that I have read *Proteins Involved in HDACi Induced Decay of ERBB2 Transcripts in Breast Cancer* by Sadaf Malik, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and the Buck Institute of Aging.

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ABSTRACT

ERBB2-positive breast cancer is an aggressive disease form that can result in rapid tumor growth and metastasis driven by the overexpression of ERBB2 growth factor receptors present on the cell surface. Currently, approved agents can target and disable ERBB2 receptor function, but as few as 30% of patients with disseminated ERBB2-positive breast cancer will respond to these targeted therapeutics when given as single agents. Even then, many initial responders will soon develop resistance to these agents. Histone deacetylase inhibitors (HDACi) are a powerful new class of epigenetic therapeutics that have demonstrated potent antitumor activity. However, there are several concerns that need to be addressed regarding the clinical usage of pan-HDACi therapeutics, such as the standard clinical prototype hydroxamic acid, trichostatin-A (TSA). These include the many genomic and non-genomic targets of pan-HDACi, the off-target side effects, and the lack of proven HDAC-dependent cancer pathways predictive of tumor responsiveness. Early observations in our laboratory have shown that *in vitro* TSA treatment of breast cancer cell line models promotes accelerated decay of polyribosomally associated ERBB2 mRNA, calling attention to a previously unrecognized transcript decay mechanism that could be exploited to develop new cancer therapeutics. Further studies have found that U-rich sequences in the 3'untranslated region (UTR) of ERBB2 transcripts are an important component of this transcript decay pathway. Based on these observations, we hypothesize that polyribosomally associated proteins, subject to regulation by HDAC-modulated protein acetylation, bind to the 3'UTR of ERBB2 mRNA within hours of TSA treatment, and mediate its rapid degradation. Using the ERBB2-overexpressing human breast cancer cell line SKBR3 and an ERBB2 3'UTR mRNA construct and assay designed to precipitate protein complexes, we attempt to identify proteins physically associated with and potentially mediating the polysome-associated ERBB2

mRNA decay pathway. Progress in this effort is expected to lead to a cancer treatment strategy more effective and selective than the use of pan-HDACi for the treatment to of ERBB2 overexpressing malignancies.

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ABBREVIATIONS

ER: Estrogen Receptor

ERBB2: Avian Erythroblastic Leukemia Viral Oncogene Homolog 2; also referred to as HER2 (human epidermal growth factor receptor-2)

GADPH: Glyceraldehyde 3-phosphate dehydrogenase

HDAC: histone deacetylase

HDACi: histone deacetylase inhibitor

hnRNP: heteronuclear riboprotein

IP: immunoprecipitation

PR: Progesterone receptor

SKBR3: HER2 overexpressing breast adenocarcinoma cell line

TSA: trichostatin A

U: uracil

UTR: untranslated region

INTRODUCTION

I. Breast Cancer Defined

Breast cancer affects an estimated 220,000 women each year in the United States alone (DeSantis et al., 2011). As the second leading cause of death among women in the United States, current projections predict that one in eight women will be diagnosed with breast cancer in their lifetime (DeSantis et al., 2011). While the exact cause cannot be pinpointed, there are certain genetic mutations and aberrations thought to contribute to breast cancer development. Such aberrations can either activate oncogenes or inactivate tumor suppressor genes, causing normal cells to develop into cancerous cells (Heimann and Hellman, 2000). Today, the widespread use of screening mammograms aides in identifying breast cancer before symptoms develop and more widespread dissemination of metastatic disease takes place (Norman, 2005). However, breast cancer can develop in several different forms, each with varying phenotypic and molecular characteristics that determine both its clinical behavior and optimal treatment approach.

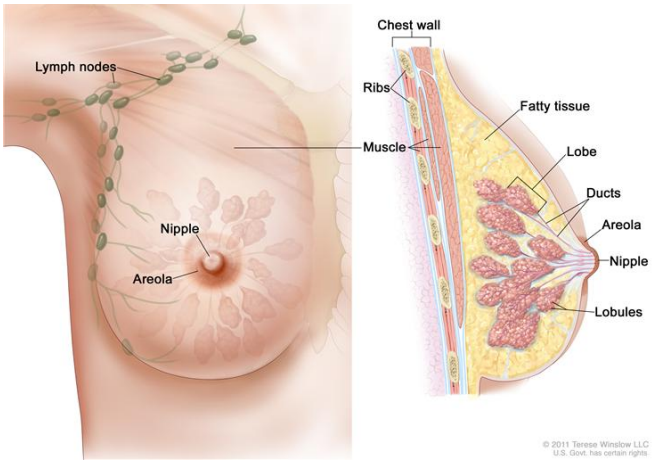

Figure 1. Anatomy of the Female Breast (Winslow, 2011)

The first description of breast cancer dates back between 3000-1500BC in Egyptian papyri that detailed observations fairly consistent with modern descriptions of breast cancer (Rayter and Manzi, 2008). Women affected with breast cancer may experience a variety of

symptoms, including retraction of nipple, atypical discharge from nipple, changes in skin texture, and atypical fullness or puckering of the breast (Fig. 1; Katz and Dotters, 2007). The most common symptom is a hard lump, usually in the upper outer corner of the breast (Katz and Dotters, 2007). The strongest risk factors in developing breast cancer are gender and increasing age, placing older women at higher risk for breast cancer (Emaus, 2009). Other risk factors include high age at first birth, early menarche, late menopause, genetic predisposition, high estrogen levels, and high breast density (Harald, 2007).

Breast cancer can be divided into clinical stages that are characterized by tumor size and extent of the body affected. Stage 0 is referred to as carcinoma *in situ*, where abnormal cells are found in the lining of a breast duct, lobules of the breast, or the nipple (Connolly et al., 2014). Currently, carcinomas *in situ* are not classified as cancerous (Connolly et al., 2014). Stage I is involvement of a tumor only within the breast, with the tumor's maximum dimension smaller than 2cm (Fig. 2; Chalasani et al., 2010). Stage II means that the breast tumor may have limited spread into axillary lymph nodes but its size remains smaller than 5cm (Fig. 2; Chalasani et al., 2010). These two stages are known as early breast cancer, which is considered highly curable because long-term patient survival rates are high if the tumor is completely excised and treated aggressively. The widespread use of screening mammography has been extremely important in detecting breast cancer in these early and clinically curable stages. Stage III breast cancer designates larger and more aggressive breast tumors that have spread to 4 or more axillary lymph nodes or invaded either the underlying chest wall or perforated the overlying skin (Fig. 2; Chalasani et al., 2010). Readily apparent physical changes in the composition and appearance of the breast occur by this later stage of breast cancer (Kataja and Castiglione, 2009; Koboldt et al., 2012). By stage IV, the breast tumor has also spread to distant organs such as the liver, lungs,

and bones (Fig. 2; Kataja and Castiglione, 2009). 5-year survival rates dramatically decrease from 100% for stage I breast cancers to 20% or less for newly diagnosed stage III or IV breast cancers (Chalasani et al., 2010).

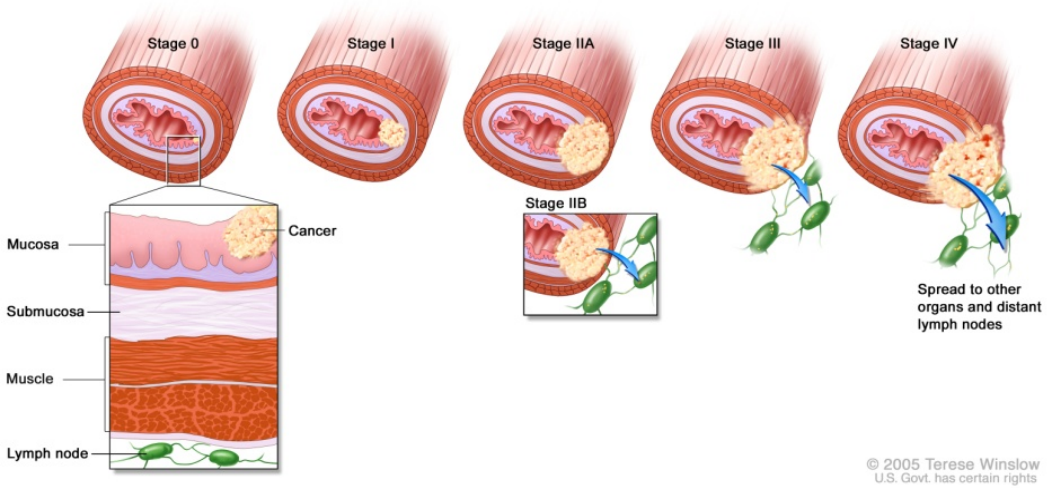

Figure 2. Illustration of the progression of breast cancer (Winslow, 2011)

II. History of Breast Cancer Treatment

Early stages of breast cancer can be locally treated using surgery to remove the cancerous cells (Chalasani et al., 2010). In 1757, Henri Le Dran, a French surgeon, was the first to suggest that surgical removal of the tumor could assist in the treatment of breast cancer, if infected lymph nodes were removed as well (Rayter and Mansi, 2008). In 1882, William Halsted tested Le Dran's theory at Johns Hopkins Hospital in Baltimore, creating a new surgical technique called radical mastectomy (Bloom and Harries, 1962). Radical mastectomy removed the breast, axillary nodes, and chest muscles in a single procedure (Fig. 3; Bloom and Harries, 1962). Although this treatment was the gold standard for the next century, it failed to help patients with metastatic (stage IV) breast cancer (Rayter and Mansi, 2008).

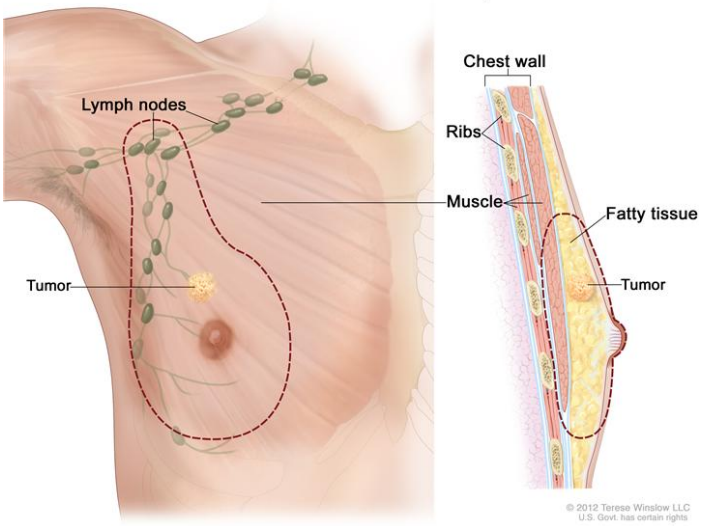

Figure 3. Anatomical Illustration of Radical Mastectomy (Winslow, 2011)

By the end of the nineteenth century, scientific and medical advances, such as the introduction of anesthetics, helped the advancement of breast cancer treatment (Rayter and Mansi, 2008). George Beatson was the first to demonstrate that a portion of breast cancer patients had a form that was hormonally dependent (Beatson, 1896). In 1895, he developed an alternative surgical method to treat breast cancer (Beatson, 1896). He performed a bilateral

oophorectomy, removal of the ovary, on a woman who was diagnosed with extensive soft tissue recurrent breast cancer, and then continued treatment with a thyroid extract (Beatson, 1896). His treatment was a combination of surgery and endocrine therapy, which was successful in helping this patient experience remission and 4 years of life after the procedure (Beatson, 1896). His contribution was significant in that it demonstrated a combination of surgery and regulation of ovarian hormones was capable of helping inhibit the development of breast cancer cells.

By the mid twentieth century, surgeons began to realize the limits in surgery alone as a viable means of curing patients of breast cancer (Rayter and Mansi, 2008). The deformities caused by the operations as well as the high risk of mortality made surgery an already daunting treatment for the disease (Rayter and Mansi, 2008). In light of Beatson's findings, physicians began to further investigate hormone dependent growth and behavior of breast tumors. The importance of the hormonal milieu was reinforced with development of therapies including adrenalectomy (Huggins and Bergenstal, 1952) and hypophysectomy (Luft and Olivecrona, 1955). In a third of patients who benefited, the mechanism responsible was speculated to be estrogen deprivation (Rayter and Mansi, 2008). This was confirmed with Jensen's discovery of the estrogen receptor in many breast tumors (Jensen and Jacobson, 1960). Thereafter, invasive endocrine surgeries were superseded by the development of endocrine therapies, such as the estrogen antagonist tamoxifen in place of oophorectomy (Jordan, 2003). In accordance, aromatase inhibitors ultimately replaced adrenalectomy, and luteinizing hormone releasing hormone agonists replaced hypophysectomy, resulting in the management of patients with metastatic breast cancer by medical rather than surgical treatments (Rayter and Mansi, 2008).

In addition to these findings, some surgeons began to experiment with radiotherapy as a form of treatment for breast cancer. Dr. Pfahler, a specialist in radiology in Philadelphia, used

radiotherapy on over a thousand patients with breast cancer, including many patients that were too frail for surgery (Case, 1955). Patients with early forms of the disease had a 5-year survival rate of 80%, demonstrating the effectiveness of this local radiotherapy approach (Case, 1955). Dr. McWhirter of England used a combination of mastectomy with radiotherapy to the supraclavicular, internal mammary and axillary lymph nodes in 759 patients (McWhirter, 1949). The results indicated that a combination of the therapies resulted in higher 5-year survival rates than either treatment alone (McWhirter, 1949). However, treatment for more aggressive forms of breast cancer still remained a mystery for much of the twentieth century.

III. ERBB2 (HER2) Positive Breast Cancer

Research and clinical evidence over the past two decades have pointed to the fact that breast cancer commonly develops into one of about 3 or 4 different clinical subtypes: luminal, hormone receptor (ER or PR)-positive; basal-like (often referred to as triple-negative because it lacks expression of ER, PR and ERBB2); and ERBB2 (also commonly called HER2)-positive breast cancers (Chaudhury et al., 2010). ERBB2-positive breast cancer makes up 20-25% of all breast cancer cases (Dent et al., 2013). ERBB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, and the overexpression of this cell surface growth factor receptor plays an important role in the pathogenesis of breast cancer (Fang et al., 2014). A non-malignant mammary epithelial cell expresses about 20,000 ERBB2 receptors, while an ERBB2-positive breast cancer cell may express 100 times the normal number of receptors on the cell surface due to the amplification of the *ErbB2* oncogene (King et al., 1985; Kumar and Badve, 2008; Turner and Leo, 2013). This gene amplification and overexpression of ERBB2-encoded mRNA and protein occurs early during the tumorigenic process and leads to rapid growth and division of the transformed epithelial cells and also confers other malignant characteristics including cell motility and invasiveness that facilitates metastatic dissemination (Moasser, 2007; Koboldt et al., 2012). Thus, women with the ERBB2-positive subtype of breast cancer are 11 times more likely to die from their disease (Chalasani et al., 2010).

Four tests have been developed to detect ERBB2-positive breast cancers and to distinguish this subtype from other breast cancer subtypes. The most common is immunohistochemistry (IHC), which measures ERBB2 protein expression on a 0 to 3+ scale: 0-1+ is negative, 2+ is borderline, and 3+ is ERBB2 positive (Ely and Vioral, 2007). Another common test is fluorescence *in situ* hybridization (FISH). This method assesses the degree of

ERBB2 gene amplification found in individual cancer cells, and results are indicated as positive or negative based on the presence of gene amplification (Ely and Vioral, 2007). A positive FISH test signifies the presence of at least double the normal level of the oncogene in each breast cancer cell (Kataja and Castiglione, 2009). Less common tests include the SPoT-Light ErbB2 CISH test and Inform ErbB2 Dual ISH test, which are similar to FISH in assessment of ERBB2 amplification as well as reporting a tumor as being either ERBB2/HER2 positive or negative (Ely and Vioral, 2007). It is important to note that while these tests are used to designate a tumor as ERBB2-positive and thereby identify the breast cancer patient as eligible to receive an anti-ERBB2 therapeutic, none of them can identify exactly which patients will benefit from that targeted therapeutic agent (Emaus, 2009; Harald, 2007). Not only are newer and more effective anti-ERBB2 therapies needed, but also needed are newer biomarkers predicting the clinical benefit (or not) of different ERBB2-targeted agents.

IV. Treatment for ERBB2 (HER2) Positive Breast Cancer

The first form of treatment for ERBB2-positive breast cancers was a monoclonal antibody, mumAb4D5, which bound specifically and tightly to the extracellular domain of the ERBB2 receptor (Fig. 4A; Carter et al., 1992). MumAb4D5 was shown to specifically inhibit the growth of breast tumor-derived cells line as well as facilitate immune attack against ERBB2-positive cancer cells (Slamon et al., 1987). The humanized form of that murine monoclonal was named trastuzumab, and marketed by Genentech as Herceptin; it went through a long process of clinical trials before finally being approved by the FDA in 1998 (Kumar and Badve, 2008). Many patients experienced an increase in quality and length of their lives with the use of trastuzumab; however, a significant percent of patients with ERBB2-positive breast cancer either did not respond to this antibody or developed resistance to it within a year (Jackisch, 2008).

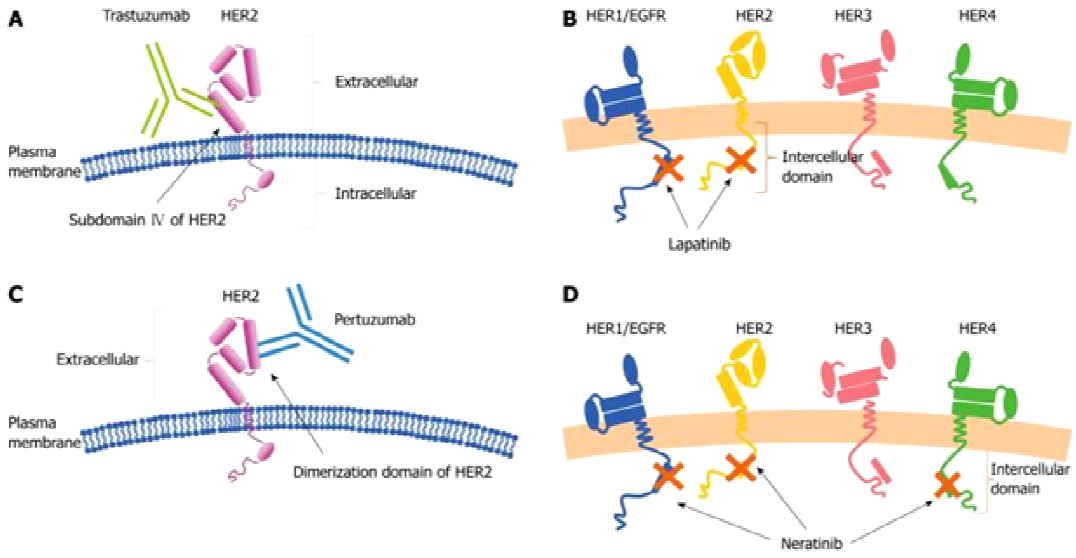

Figure 4. Various treatments for ERBB2-positive breast cancer and their mechanism of action. A) Trastuzumab interference with the extracellular domain of the ERBB2 receptor. B) Lapatinib interference with the intracellular domain of ERBB2 monomers and dimers. C) Pertuzumab inhibition of the dimerization of ERBB2 receptors. D) Neratinib interference with the intracellular domain of ERBB2 receptors. (Park et al., 2011)

To address clinical resistance to trastuzumab, Genentech developed another monoclonal antibody called pertuzumab (Jackisch, 2008). It targets another epitope on the extracellular domain of ERBB2 receptors to specifically inhibit the dimerization of ERBB2 receptors, resulting in a more comprehensive blockade of ERBB2 signaling (Fig. 4C; Park et al., 2011). Low molecular weight compounds have also been developed to inhibit the receptor's intracellular ATP binding site and thereby prevent its downstream signaling; two such agents include Lapatinib and Neratinib (Fig. 4B and 4D; Park et al., 2011). Even with the variety of treatments now available to disable the ERBB2 receptor function needed for the cancer cell's growth and metastatic spread, as few as 30% of patients with ERBB2-positive breast cancer respond clinically to these drugs and many others later develop clinical resistance after initial clinical response (Kute et al., 2004).

A common feature of all the above anti-ERBB2 therapeutics developed to date is that they directly target the surface receptor protein, not any of the key signaling and downstream tumorigenic pathways driven by overexpressed ERBB2. A novel mechanistic point of attack for the development of a newer anti-ERBB2 therapeutic would be to target the overexpressed ERBB2 mRNA that is translated into ERBB2 receptor protein—not simply inhibiting function of the already encoded protein—but accelerating the cytoplasmic decay of the normally long-lived and abundant ERBB2 mRNA produced off the amplified *ERBB2* gene. Initial insight into the feasibility of such a novel mechanistic point of attack came from our studies of histone deacetylase (HDAC) inhibitors in ERBB2-positive breast cancer cell line models.

V. HDACi as a New Therapeutic Tool

In general, the dysregulation of gene expression and function leads to a combination of genetic and epigenetic abnormalities that influence the development of breast cancer (Jones and Baylin, 2007). A very common epigenetic abnormality during tumor development is the altered expression of histone deacetylases (HDACs) (Ellis et al., 2009). HDACs are enzymes that regulate gene transcription and expression by removing **ε**-N-acetyl lysine residues off histone tails of chromatin and by the deacetylation of non-histone proteins (Fig. 5; Bolden et al., 2006). The basic mechanism of acetylating histones via histone acetyl transferases (HATs) removes the positive charge of the histones, which decreases its interaction with the negative phosphate groups of DNA. This in effect relaxes the chromatin structure to allow for transcription. However, HDACs oppose this activity and help maintain the closed structure of chromatin. To influence gene transcription in normal tissue and cancerous cell lines, HDACs do not directly bind DNA; instead, they require interaction with other proteins associated with large multi-protein complexes (Choudhary et al., 2009). Various studies have demonstrated the aberrant expression of HDACs during tumorigenesis as well as progression to metastatic phenotypes (Ellis et al., 2009; Glozak and Seto, 2007; Nakagawa et al., 2007; Scott et al., 2002).

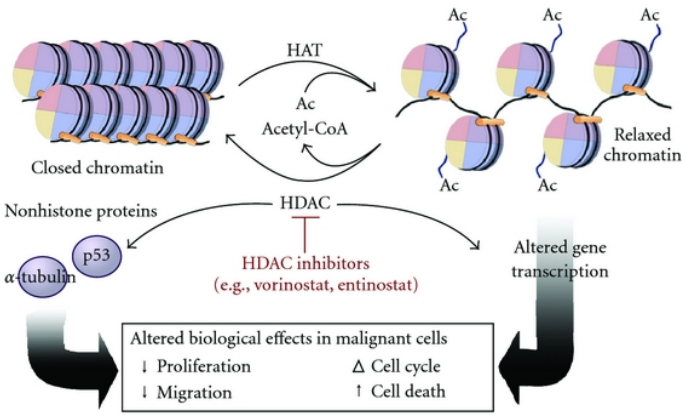

Figure 5. Schematic representation of HAT, HDAC, and HDACi mechanism of action. (Todd et al., 2012)

18 HDACs have been identified in humans and are classified into four groups (I, II, III, and IV) based on structural differences (Fig. 6; Glozak and Seto, 2007; Chun, 2015; Dokmanovic et al., 2007). Classes I, II, and IV require Zn^{2+} as a cofactor; however, class III HDACs are sirtuins that are Zn^{2+} independent, and will not be considered here (deRuijter et al., 2003; Gregoretti et al., 2004). Class I HDACs have a single catalytic site and are localized in the nucleus, whereas class II HDACs are found in both the nucleus and cytoplasm, containing zinc in their 1-2 catalytic sites (Dokmanovic et al., 2007). Knockout analyses of different class I and class II HDAC proteins indicate that class I HDACs play a role in cell survival and proliferation, while class II HDACs may have tissue-specific roles (Minucci and Pelicci, 2006). HDAC4, HDAC6 and HDAC11 were specifically shown to be overexpressed in breast cancer tissue (Nakagawa et al., 2007; Scott et al., 2002).

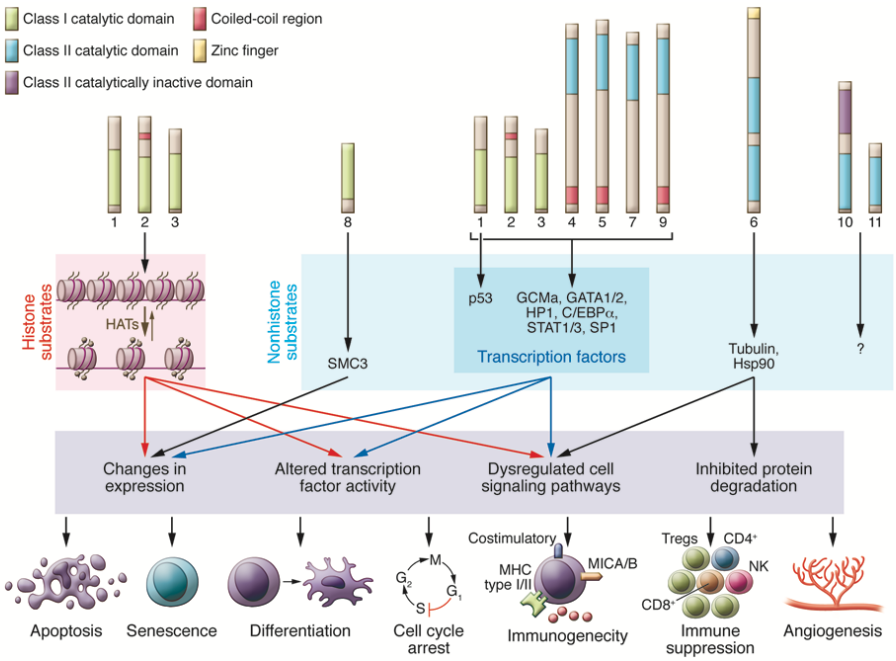

Figure 6. HDACs, their classifications, targets, and downstream signaling results as well as anticancer outcomes of HDAC inhibition. Class I HDACs (1-3 and 8) target histone substrates in addition to transcription factors like p53. Class II HDACs (4-11) target non-histone substrates such as SMC3 (structural maintenance of chromosomes 3), HP1 (heterochromatin protein 1), and tubulin resulting in dysregulated cell signaling and altered transcription activity. Implementation of HDACi results in apoptosis, senescence, cell cycle arrest, and other antitumor responses as highlighted in the lowermost portion of the diagram. (West and Johnstone, 2014)

Histone deacetylase inhibitors (HDACi) are a powerful new class of epigenetic therapeutics for the treatment of various malignancies such as breast cancer. Normal cells are relatively resistant to HDACi induced cell death; however, malignant cells have been shown to respond well with HDACi treatment (Milos et al., 2007). The most commonly used and earliest forms of HDACi include sodium butyrate, valproate, vorinostat, MS-275, romidepsin and trichostatin-A (Glozak and Seto, 2007). HDACi treatment results in increased histone acetylation, producing transcriptional activation of critical genes needed for tumor growth arrest (Glozak and Seto, 2007). The therapeutic value of HDACi comes from findings that normal cells are relatively resistant to HDAC inhibitor-induced cell death compared to tumor cells (Ungerstedt et al., 2005). HDAC inhibitors are also known to modify a number of tumor related, non-histone proteins in their pathway, producing an increase in acetylation that is associated with several molecular processes including the inhibition of angiogenesis and the induction of autophagy and apoptosis (Ellis et al., 2009).

HDACi can be categorized as either pan-HDACi, which target all HDACs, or class-selective HDACi, which target specific classes of HDACs. The difference lies in HDACi potency, isoform selectivity, and efficacy against various diseases (Berkley et al., 2012). In general, pan-HDACi are more potent, but class-selective HDACi may trade potency for greater selectivity, less toxicity and hence improved therapeutic index. However, with the many genomic and non-genomic targets of pan-HDACi independent of the specific transcript's function, the off-target side effects of pan-HDACi and the lack of other proven HDAC-dependent cancer pathways predictive of tumor responsiveness have been major concerns that have slowed the non-hematologic clinical development of pan-HDACi against solid tumors like breast cancer.

VI. Current HDACi Research

Previous findings in our laboratory have demonstrated that 6-hour pan-HDACi treatment (trichostatin-A or TSA) induces ERBB2 mRNA decay (Fig. 7 and 8A; Scott et al., 2002). Early observations in our laboratory (using TSA) not only inhibits ERBB2 transcription by promoter repression, but also induces ERBB2 protein destabilization and accelerated decay of ERBB2 transcripts (Fig. 7 and 8; Scott et al., 2002). Polysome profiling, normalized using GAPDH, revealed that ERBB2 transcripts are concentrated in fractions 17-23, which are polyribosomally associated (Fig. 8B and 8C). A 2-3 fold decrease in ERBB2 mRNA is observed in the same fractions when exposed to 6-hour treatment of TSA (Fig. 8C and 8D).

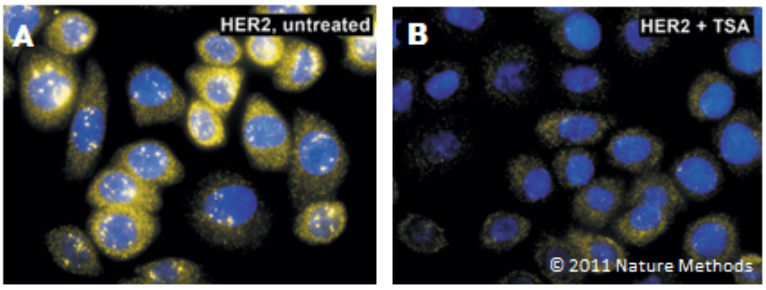

Figure 7. SKBR3 human breast cancer cells probed with Stellaris FISH probes to ERBB2/HER2 mRNAs show decrease in HER2 mRNA levels. A) Hundreds of cytoplasmic HER2 mRNA transcripts found in untreated breast cancer cells, with multiple bright spots of active transcription within the nuclei. B) HER2 transcript levels are markedly reduced with the addition of the HDACi TSA (trichostatin-A) as evidenced by the disappearance of both cytoplasmic and bright nuclear spots. (Orjalo et al., 2011)

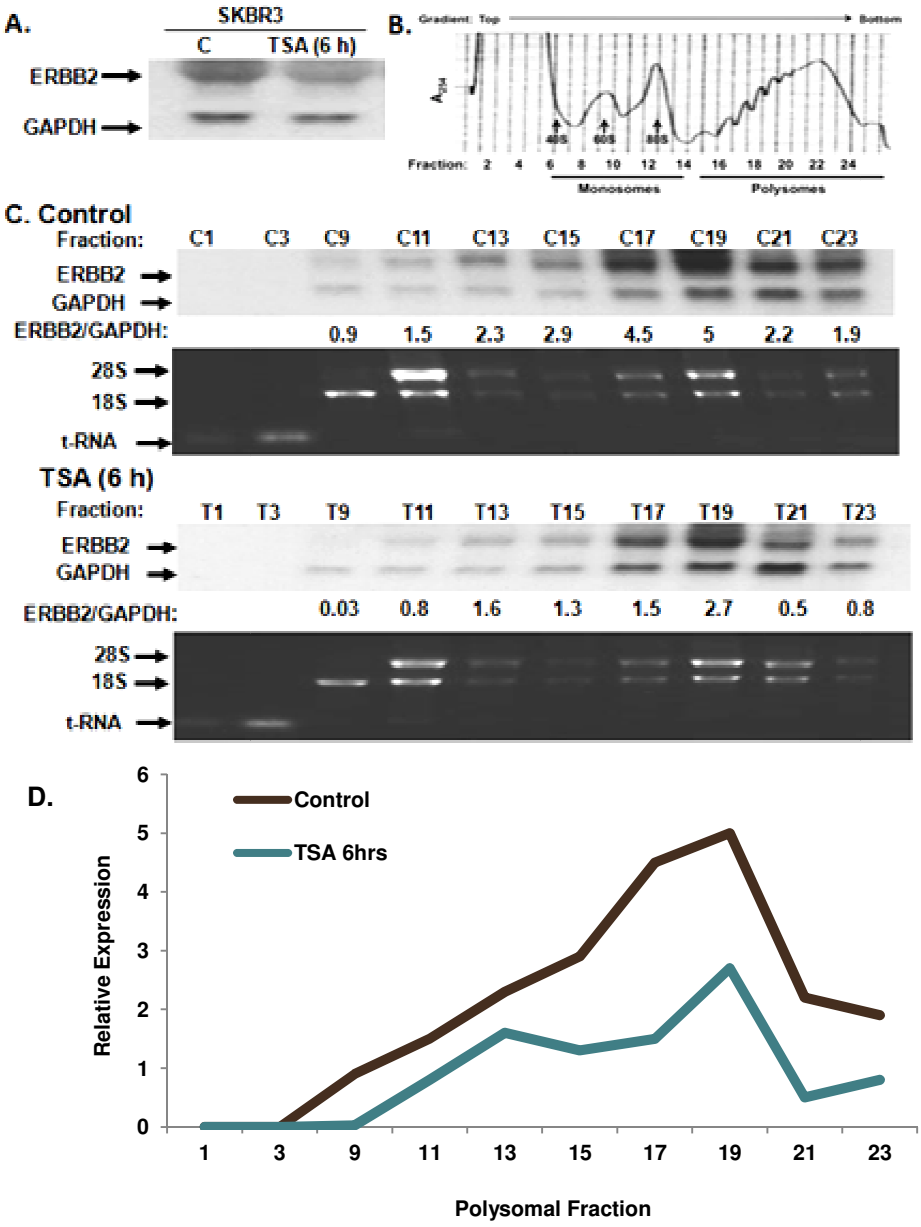

Figure 8. HDACi induced ERBB2 mRNA decay initiates on the polyribosome. A) Northern Blot analysis demonstrating 6-hour TSA treatment on SKBR3 cells results in decreased expression of ERBB2 mRNA relative to GAPDH transcripts. B) Sucrose gradient (15%-40%) profiles monitored by UV absorption (A_{254}) resolve total cytosolic lysates into free cytosol (#1-5), 40S, 60S and 80S monosome (#6-14), and polysome (#15-24) fractions for subsequent Northern and Western Blotting. C) Northern blots of RNA obtained from Control (C) and TSA (T) treated SKBR3 sucrose gradient fractions probed with ERBB2 and GAPDH relative to ethidium stained RNA gels showing 28S, 18S, and t-RNA bands. Reduced ERBB2/GAPDH transcript ratios in T versus C fractions confirm that HDACi induced ERBB2 mRNA decay originates on polysomes; no ERBB2 or GAPDH transcripts were detected in the free cytosol fractions. D) Graphical representation of normalized (ERBB2/GADPH) expression levels of ERBB2 transcripts under control and 6 hour TSA treatment conditions (Scott et al., 2002).

ERBB2 signaling activates the PI3K/Akt/mTOR pathway, which promotes cell survival and division, by inducing the phosphorylation of the 40s ribosomal subunit protein S6 (RPS6) and the eukaryotic initiation factor 4e binding protein 1 (4eBP1) (Wilson-Edell et Al, 2014). These events have been shown to stimulate polyribosome assembly and increase translation of tumorigenic mRNAs (Thoreen et al., 2012). Preliminary findings by our laboratory show that ERBB2 is associated with the polyribosome and that TSA induced decay of ERBB2 mRNA also primarily occurs at the polyribosome.

Since the mechanism responsible for ERBB2 mRNA decay is unknown, these findings led us to question the proteins involved in the ERBB2 mRNA decay pathway, specifically ones that are polyribosomally associated. Further studies have found that U-rich sequences in the 3'untranslated region (UTR) of ERBB2 transcripts are an important component of the decay pathway, since specific proteins are known to bind to the 3'UTR and modulate transcript stability (Abdelmohsen, 2012; Scott et al., 2007). Based on these observations, we hypothesize that polyribosomally associated proteins, subject to regulation by HDAC-modulated protein acetylation, bind to the 3'UTR of ERBB2 mRNA within hours of TSA treatment and mediate its rapid degradation. Using the ERBB2-overexpressing human breast cancer cell line SKBR3 and an ERBB2 3'UTR mRNA construct to bind and precipitate protein complexes, we attempt to identify proteins physically associated with and potentially mediating the polysome-associated ERBB2 mRNA decay pathway.

MATERIALS AND METHODS

- *Culture Growth, Treatment, and Harvesting of Human Breast Cancer Cell Line*

The ERBB2-positive human breast cancer cell line, SKBR3, was obtained from American Type Culture Collection and used in this study. The cells were grown in McCoy's medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Experimental cells were treated with commercially obtained (Sigma-Aldrich, St. Louis, MO) trichostatin-A (TSA) for 2, 4, or 6 hour time periods at a concentration of 1.5 μM. This specific concentration allows for a dynamic change in cellular activity without killing the cell. Various time points were chosen to allow for detection of differential binding of polyribosomal protein, with 6 hours being the maximum before cell death is induced.

Control and treated cells were harvested and dounced in a hypotonic buffer containing 50 mmol/L of Hepes (pH 7.4), 10 mmol/L of KCl, 0.3% NP40, 10 mmol/L of $MgCl_2$, 100 Units/mL of SUPERasein (Ambion, Grand Island, NY), and mini-complete protease inhibitors (Roche Diagnostics, Nutley, NJ). The cell lysate was then centrifuged at 16,000 rpm for 5 minutes to remove nuclei and other insoluble material. Cytosolic extracts were incubated with 1 to 2 mg/mL of poly[d(I-C) in 100 mmol/L of KCl, 10 mmol/L of Tris-HCl (pH 7.5), 2 mmol/L of DTT, and 20% vol/vol glycerol at 23□C.

- Polysome Profiling

Polysome profile analysis was employed on whole cell lysates collected as described above. The lysates were layered on top of a 10%-50% continuous sucrose gradient (Fig. 9). The samples were then spun at 38,000 rpm for 2 hours at 4 □C. The gradients were fractioned, and

polysome fractions were then collected using the Retriever 500 with ISCO Teledyne UV/Vis (UA6).

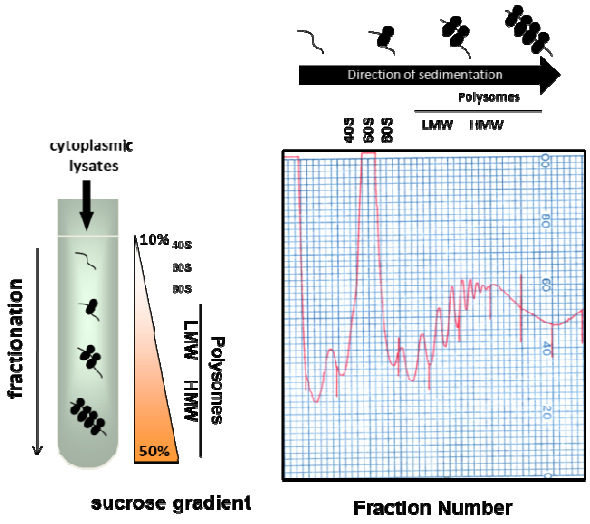

Figure 9. Schematic representation of polysome profiling from formation of continuous sucrose gradient to the polysome-profiling graph created by the ICO Teledyne UV/Vis. (Abdelmohsen, 2012)

❖ *ERBB2 3'UTR mRNA Construct and Digoxigenin Labeling*

EST clones containing 3'UTR sequence of ERBB2 were commercially obtained (Invitrogen, Grand Island, NY) and cloned into the XbaI site of the pBluescript KS(-) vector (Fig. 10; Stratagene, Santa Clara, CA). Blunt ends were used when inserting the 3'UTR to allow for the transcript to be inserted in both the forward and reverse orientation with equal probability—forward being the sense strand and reverse being the antisense strand. The vector was linearized using a HaeIII digest (Sigma Aldrich, St. Louis, MO). The 3'UTR of ERBB2 contains an internal BstXI site at 170 bp in addition to the BstXI site12 bp upstream of the XbaI site in the vector; thus, after the restriction enzyme disgest, the segment that is 183bp is in the sense strand orientation and the 430bp segment produced is in the antisense orientation. The RNA polymerase T7 used digoxigenin-labeled UTP as a ribonucleoside at a 1:15 ratio (~10 U's

in 3'UTR of ErbB2). The labeled mRNA was then purified by Nuc Spin columns (Ambion, Grand Island, NY) to remove any unincorporated digoxigenin. Reaction products were electrophoretically separated on an 1.5% formaldehyde-agarose gel (0.9g of agarose, 6mL of 10X MOPS, 43mL of ddH2O, and 11mL of formaldehyde), and bands were visualized by autoradiography to confirm orientation. RNA was subsequently denatured by a 5-minute incubation at 65 □C.

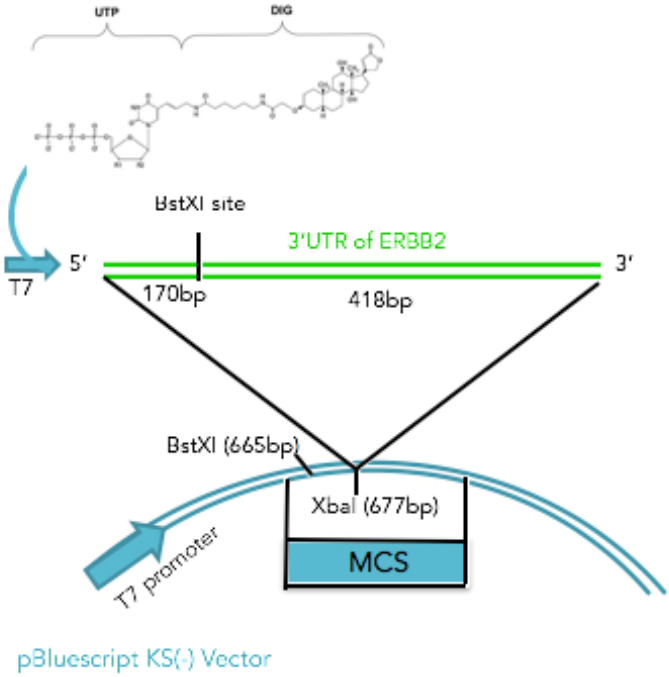

Figure 10. Schematic representation of ERBB2 3'UTR insertion into pBluescript KS(-) vector for polymerase T7 transcription using digoxigenin-labeled UTP as a ribonucleoside.

❖ *RNA Immunoprecipitation*

Denatured RNA probe was combined with different SKBR3 cytoplasmic extracts: control extract from untreated cells and HDACi-treated extracts from cells treated with trichostatin A (TSA) for 2, 4, or 6 hours. Cells were first heat shocked for 5 min at 37 □C and then cooled at room temp for 15 min. 0.2 µg of sheep anti-digoxigenin antibody and 12 µL of protein G

sepharose beads (GE Healthcare, Pittsburg, PA) per milliliter of lysate were added to cytoplasmic extracts with RNA, which were then rotated for 1.5 hours. The protein G beads from the were washed once at 4□C in wash buffer (composed of 20 mmol/L of Tris (pH 7.4), 50 mmol/L of KCl, 10 mmol/L of $MgCl_2$, and 0.3% NP40), resuspended in 1X loading buffer [20 mmol/L of Tris (pH 6.9), 2% SDS, 1% 2-mercaptoethanol, 50 mmol/L of NaCl, and 10% glycerol], heated to 95□C for 10 min, and stored in a -80 □C freezer (Fig. 11).

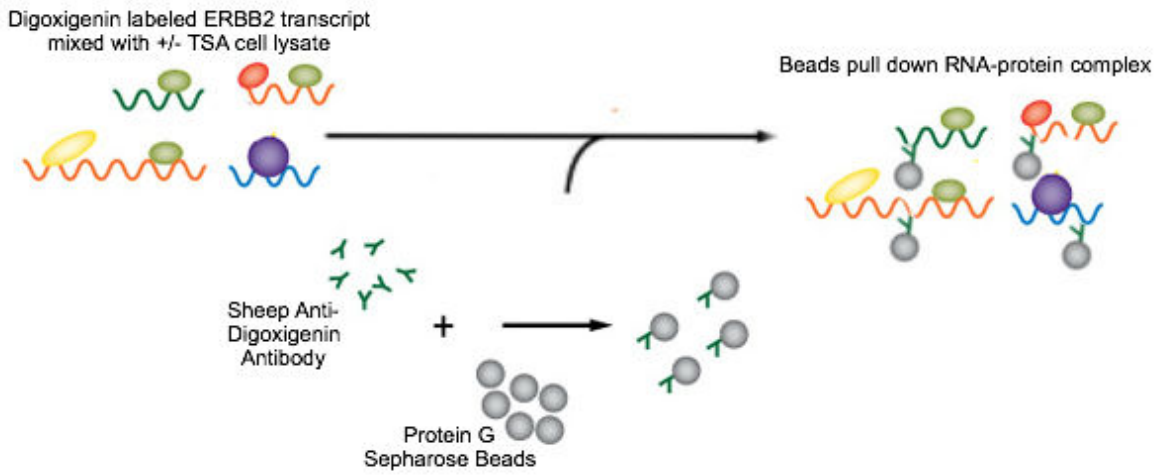

Figure 11. Schematic representation of immunoprecipitation reaction pulling down digoxigenin-labeled 3'UTR of ERBB2 mRNA protein probe bound with complexes using Protein G beads coated in anti-digoxigenin antibody.

❖ *Western Blotting*

Equal aliquots of immunoprecipitated material were electrophoresed using NuPAGE 4% gradient polyacrylamide gel (Invitrogen, Grand Island, NY) in 10% MES buffer for 30 minutes, transferred at 200V to nitrocellulose (Amersham Bioscience, Pittsburgh, PA) and probed with various antibodies (see below) in hybridization buffer (20 mmol/L of Tris (pH 7.5), 130 mmol/L of NaCl, and 0.05% Tween 20) with 5% nonfat milk. Hybridized antibodies were visualized using chemiluminescence, since antibodies used are labeled with chemiluminescent substrate horseradish peroxidase (HRP). Blots were treated with SuperSignal® West Pico Stable Peroxide

Solution and West Pico Luminol/ Enchancer solution (Cell Signaling, Beverly, MA) at a 1:1 ratio to visualize results on chemiluminescent film. The antibodies tested included HuR (mouse 3A2; Santa Cruz Biotechnology, Santa Cruz, CA), RNH1 (mouse H-135; Santa Cruz Biotechnology, Santa Cruz, CA), CyPA (rabbit C-14; Santa Cruz Biotechnology, Santa Cruz, CA), hnRNP K (rabbit A300-675A; Bethyl Labs, Montgomery, TX), p68 (rabbit H-144; Santa Cruz Biotechnology, Santa Cruz, CA), and RACK1 (mouse B-3; Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

I. Identification of Differential Protein Binding to 3'UTR of ERBB2 mRNA

In order to identify protein associated with the 3'UTR of ERBB2 mRNA, we created an assay where a 3'UTR construct of ERBB2 mRNA (see Material and Methods) was placed in a whole cell lysate to immunoprecipitate associated protein complexes. The immunoprecipitation reaction consisted of a control cytosolic lysate and a 2-hour TSA-treated cytosolic lysate, and both lysates were combined with the digoxigenin-labeled 3'UTR construct of ERBB2 mRNA in both the sense and antisense orientations. The reaction pulled down digoxigenin labeled mRNA and any bound protein complexes with it. Products of the immunoprecipitation reactions (sense untreated, sense 2-hour TSA treated, antisense untreated, and antisense 2-hour TSA treated) were loaded in the first four lanes of a polyacrylamide gel. Total cell lysate that was untreated as well as treated with TSA for 2 hours was loaded in the last two lanes as indication of total protein present. The resultant gel, stained with SyphroRuby (Invitrogen, Grand Island, NY), revealed a multitude of protein bands in both sense and antisense stands as well as in treated and non-treated lanes (Fig. 12). The lysate lanes show a difference in total protein acetylation with 2 hour TSA treatment. However, visual inspection could not discriminate protein bands with differential intensity in the treated and non-treated lanes that were also specifically bound to the sense strand. Due to this lack of sensitive resolution, specific bands were excised from the gel (Fig. 12, red boxes) and analyzed by mass spectrometry.

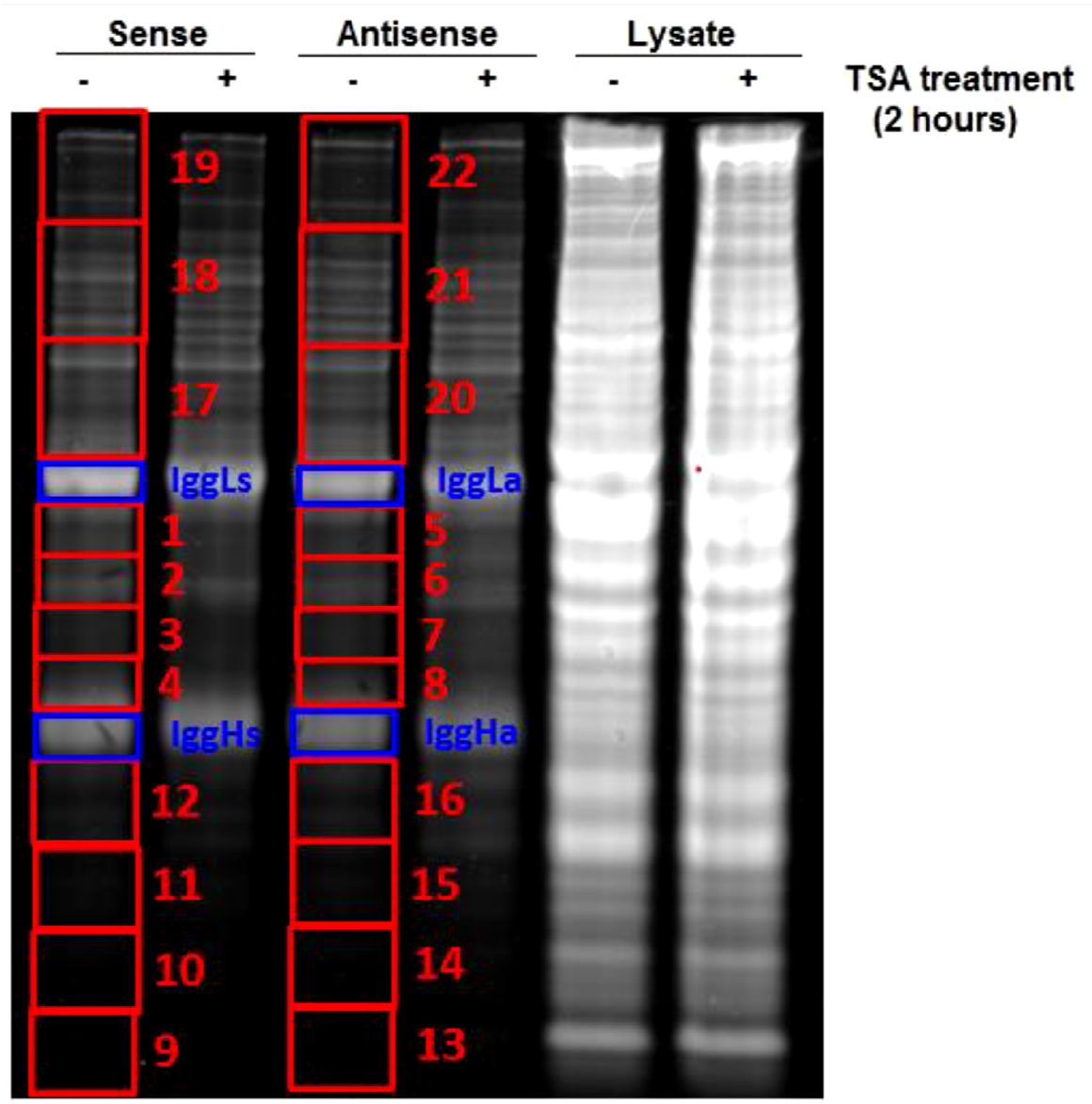

Figure 12. SyphroRuby stained gel reveals abundance of protein binding to 3'UTR of ERBB2 mRNA but with lack of resolution to determine specific protein bands with high specificity to the sense strand versus the antisense strand. Red boxes indicate sections of the gel excised and analyzed by mass spectrometry to identify strand specific protein binding, while blue boxes indicate light chain and heavy chain interaction of the antibody used in the immunoprecipitation reaction.

Only segments of the control sense and control antisense lanes were analyzed by mass spectrometry to help identify proteins with sense (coding) strand binding specificity to the 3'UTR of ERBB2 mRNA. Mass spectrometry identified several proteins bound to the 3'UTR of ERBB2 in both a sense strand-specific and non-strand specific manner (Appendix 2). In order to distinguish protein candidates for further analysis, we looked for proteins that showed at least a two-fold preferential binding to the sense strand using a sense to antisense strand ratio. Of the 502 proteins that were identified by mass spectrometry, only 42 protein candidates met this criterion (Appendix 1). Several of these proteins were heterogeneous nuclear ribonucleoproteins (hnRNP), which are complexes of RNA and protein present in the cell nucleus during gene transcription and subsequent transcriptional modification of pre-mRNA (Chaudhury et al., 2010; Table 1). Most of these hnRNPs showed a two-fold difference in specificity to the sense strand versus the antisense strand.

Table 1: Heteronuclear Proteins Discovered from MS Analysis

Protein	Gene	Name	Ratio (sense/antisense)
ELAV1	ELAVL1	ELAV-like protein 1 (HuR) [CONTROL]	2.1
ROAA	HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	>1
ROA1	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	2.8
RA1L2	HNRNPA1L2	Heterogeneous nuclear ribonucleoprotein A1-like 2	2.8
ROA3	HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	2.9
HNRCL	HNRNPCL1	Heterogeneous nuclear ribonucleoprotein C-like 1	1.9
HNRPD	HNRNPD	Heterogeneous nuclear ribonucleoprotein D0	3
HNRDL	HNRNPDL	Heterogeneous nuclear ribonucleoprotein D-like	3
HNRPF	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	1.2
HNRH1	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H	2.2
HNRH3	HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3	1
ROA2	HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	4
HNRPC	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	1.9
HNRPU	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	3.8
HNRPK	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	2.7
HNRL1	HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	13.4

There were also a few strong candidates, other than hnRNP's, present in multiple isoforms and that had strong sense-strand specificity as identified by mass spectrometry (Table 2). The strongest non-hnRNP candidate was peptidylprolyl isomerase A (PPIA), which catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerates the folding of proteins (Pliyev and Gurvits, 1999). PPIA demonstrated a 42.7times stronger specificity to the sense strand over the antisense strand, on average of the various isoforms detected. The other non-hnRNP candidates were probable ATP-dependent RNA helicases, specifically DEAD box proteins characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD) (Andreou and Lkostermeier, 2014). They are responsible for catalyzing the ATP-dependent unwinding of RNA duplexes and accompany RNA molecules throughout their cellular life (Andreou and Lkostermeier, 2014). Mass spectrometry analysis demonstrated, on average of the various isoforms detected, a 4.7 fold difference in specificity to the sense strand over the antisense strand (Table 2).

Table 2: Non-hnRNP Protein Candidates from MS Analysis (Present in Multiple Isoforms)

Precursor (molecular weight)	**Protein**	**Ratio (sense/antisense)**	**Average Ratio**
528.27	Peptidyl-prolyl cis-trans isomerase A	27.5	42.7
577.79	Peptidyl-prolyl cis-trans isomerase A	47.9	
624.32	Peptidyl-prolyl cis-trans isomerase A	58.9	
684.87	Probable ATP-dependent RNA helicase DDX5	11.1	4.7
547.78	Probable ATP-dependent RNA helicase DDX5	6.4	
648.33	Probable ATP-dependent RNA helicase DDX5	3.4	
468.77	Probable ATP-dependent RNA helicase DDX5	2.5	
613.86	Probable ATP-dependent RNA helicase DDX5	4.2	
565.33	Probable ATP-dependent RNA helicase DDX5	9.9	
492.76	Probable ATP-dependent RNA helicase DDX5	2.2	
493.29	Probable ATP-dependent RNA helicase DDX5	4.1	
426.72	ATP-dependent RNA helicase DDX3X	2.1	2.6
547.28	ATP-dependent RNA helicase DDX3X	2.1	
582.30	ATP-dependent RNA helicase DDX3X	1.3	
434.22	ATP-dependent RNA helicase DDX3X	6.4	
584.86	ATP-dependent RNA helicase DDX3X	3.3	

II. Establishing Sense-Strand Specificity with HuR as a Positive Control

Previous data from our laboratory established ERBB2 expression is regulated by the transcript stability factor HuR (Human Antigen R) (Scott et al., 2008). An *in vitro* binding assay demonstrated that HuR is capable of binding to a radiolabelled probe consisting of the conserved U-rich element of the ERBB2 3'UTR mRNA, established by the supershift induced in the HuR-probe complex (Fig. 13B). In addition, polysome fractions used in earlier experiments to demonstrate decaying levels of ERBB2 mRNA upon TSA treatment were probed with HuR to confirm that HuR was polyribosomally associated (Fig. 13D). As mentioned earlier, ERRB2 decay was shown to initiate on the polyribosome; therefore, we are searching for protein candidates that could also associate with the polyribosome. Western blot analysis revealed that high concentrations of HuR are found in the cytosol and notable amounts were found to be associated with the polyribosomal fractions (Fig. 13E).

Results from mass spectrometry showed that HuR, also known as ELAV1, binds with a 2-fold higher specificity to the sense strand in comparison to the antisense strand (Table 1). We used Western blot analysis to confirm that stability factors bind with greater affinity to the coding strand of ERBB2 mRNA. As shown in Figure 13C, HuR bound to the sense strand in both untreated and treated immunoprecipitates, but not to the antisense strand in the untreated and treated immunnoprecipitates (Fig.13C). Due to the stable and non-differential expression of HuR in both the treated and non-treated lanes, it serves as a positive control for the rest of the immunoprecipitate data.

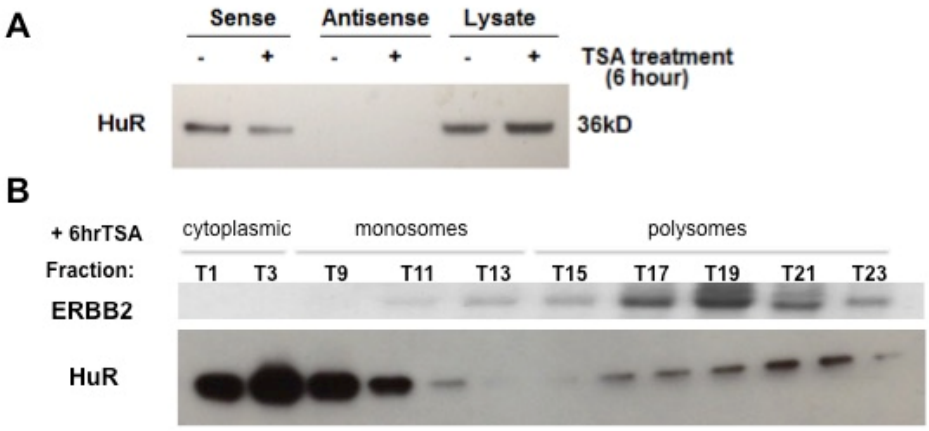

Figure 13. ERBB2 expression is regulated by the transcript stability factor, HuR, which binds a conserved U-rich element in the sense strand of the 3' UTR of ERBB2 mRNA. A) Western Blot analysis, using immunoprecipitates with sense vs. antisense 3'UTR ERBB2 mRNA constructs, reveals that HuR specifically binds to the sense strand of the 3'UTR of ERBB2. B) 6-hour TSA-treated SKBR3 cell fractions demonstrating expression of ERBB2 mRNA in fractions associated with the polysome. Same treated fractions in are probed with HuR on a Western Blot and shown at the bottom. High concentrations are found in the cytosol and notable amounts are found in the polysomally-associated fractions.

III. Western Blot Analysis of Protein Candidates Identified by Mass Spectrometry

Peptidylprolyl isomerase A (PPIA), also known as cyclophilin A, was the strongest protein candidate identified by mass spectrometry showing the greatest affinity to the sense strand (Table 2). Studies have demonstrated its overexpression in several cancers, including breast cancer (Hathout et al., 2002; Zheng et al., 2008). However, Western blot analysis could not confirm sense-strand specificity as seen by the lack of PPIA in the sense and antisense regardless of treatment (Fig. 14). Furthermore, Western blot analysis only recognized PPIA in the cytoplasmic lysates but not the immunoprecipitates containing the ERBB2 mRNA construct (Fig. 14A). Polysome profiling analysis also revealed that PPIA is not associated with the polyribosome (Fig. 14B).

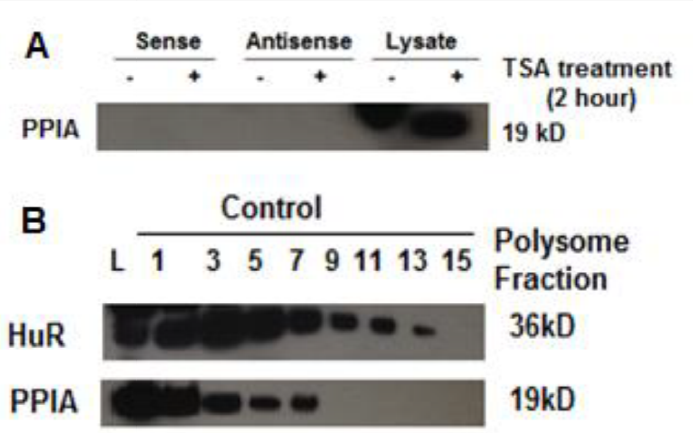

Figure 14. Western blot analysis does not confirm mass spectrometry results for PPIA. A) Western blot analysis using the CyPA antibody shows that PPIA is absence in both sense or antisense strands for control and 2-hour TSA-treated immunoprecipitates. B) Western blot analysis shows that PPIA is not polyribosomally associated in comparison to HuR, with the last notable signal ending in fraction 7 for PPIA.

Probable ATP-dependent RNA helicase DDX5, also known as p68, was another strong protein candidate identified by mass spectrometry with a higher affinity to the sense strand (Table 2). Studies have identified DDX5 as a required factor for cell proliferation in certain

breast cancer cells (Mazurek et al., 2012). Western blot analysis identified DDX5 binding to the 3'UTR of ERBB2 mRNA but specificity for the sense strand over the antisense could not be confirmed (Fig. 15A). The non-treated sense and antisense strands did show a difference in intensity, with greater binding to the sense strand; however, the same difference in intensity was not noted in the treated sense and antisense lanes. Looking at polysome profile analysis, DDX5 did show to be closer to the polyribosomal fractions than PPIA; however, there is a significant drop off in signal after fraction 9 (Fig. 15B).

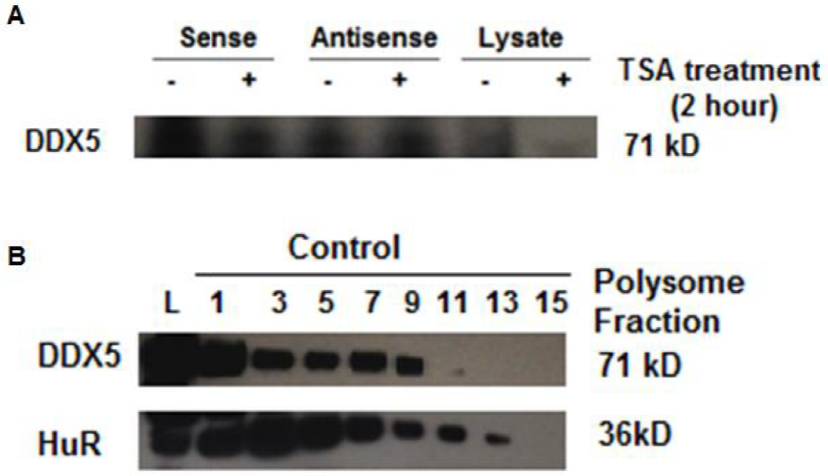

Figure 15. Western blot analysis partially confirms mass spectrometry results for DDX5. A) Western blot analysis using the p68 antibody shows that DDX5 is present in both sense and antisense strands for control and 2-hour TSA-treated immunoprecipitates. B) Western blot analysis shows that DDX5 may be polyribosomally associated in comparison to HuR, with the last notable signal ending in fraction 9.

Another weaker candidate, ribonuclease inhibitor 1 (RNH1), was tested using Western blot analysis due to its role in HDACi resistance in certain cancers and its role in mRNA turnover (Zhu et al., 2014). RNH1 was detected in our mass spectrometry analysis with a 1.8 fold preferential binding to the sense strand. RACK1 is used to normalize for loading error in further Western blots due to it strong association with the polysome and absence in cytoplasmic fractions (Wilson-Edell, 2014). However, like PPIA, no signal could be detected in sense and antisense lanes of the immunoprecipitates of both control and 2-hour TSA treatment (Fig. 16A). We briefly looked at possible differential expression resultant of TSA treatment in polysomal fractions since we were unable to investigate this in our pulldown. However, polysome profile analysis confirmed that RNH1 is not associated with the polyribosome and is mainly present in cytosolic fractions (Fig. 16B).

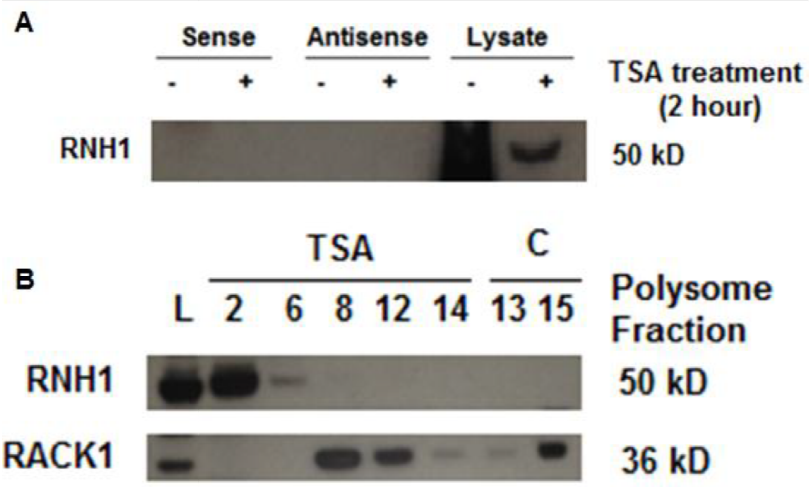

Figure 16. Western blot analysis confirms that RNH1 is a weak protein candidate involved in the ERBB2 decay pathway. A) Western blot analysis shows that RNH1 is not present in the sense or antisense strands for control and 2-hour TSA-treated immunoprecipitates. B) Western blot analysis shows that RNH1 is not polyribosomally associated in comparison to RACK1, with the last notable signal ending in fraction 6 of the treated fractions.

IV. hnRNP K as a Strong Candidate Mediating ERBB2 mRNA Stability

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is an essential RNA- and DNA-binding protein involved in gene expression and signal transduction including DNA transcription, RNA splicing, RNA stability, and translation (Zhou et al., 2010). Several studies have shown overexpression of hnRNP K in breast cancer and its importance in tumor cell viability (Van Domselaar et al., 2012; Zhou et al., 2010). Mass spectrometry identified hnRNP K with a three-fold difference in its specificity to the sense strand over the antisense strand (Table 1). Western blot analysis showed hnRNP K was present in both sense and antisense strands for control and 2-hour TSA-treated immunoprecipitates (Fig. 17A). However, there was a notable difference in intensity when comparing the sense strand of the control to that of the 2-hour TSA-treated lane. This was further investigated in Western blot analysis of polysome fractions (Fig. 17B). The results demonstrated that in addition to hnRNP K being polyribosomally associated, it bound 3-times more strongly to TSA-treated fractions than control fractions (Fig. 17B). These findings indicate that hnRNP K is a strong protein candidate mediating ERBB2 mRNA decay.

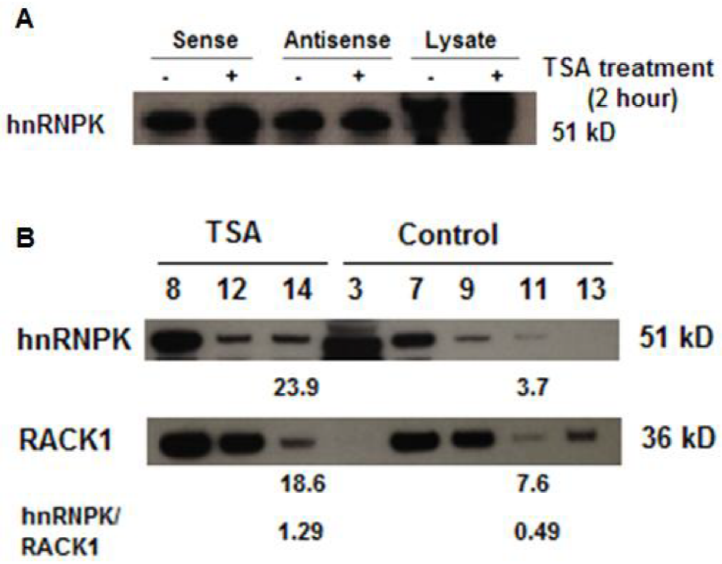

Figure 17. Western blot analysis reveals hnRNP K as a strong protein candidate that shows differential binding to the 3'UTR of ERBB2 mRNA following TSA treatment and is polyribosomally associated. A) Western blot analysis shows hnRNP K is present in both sense and antisense strands for control and 2-hour TSA-treated immunoprecipitates. However, a notable difference in intensity in the sense strand of the control and treated lanes suggests that hnRNP K is involved in ERBB2 degradation following HDACi treatmet B) Western blot analysis of polysome fractions demonstrates hnRNP K is polyribosomally associated, shows 3 times greater binding to treated fractions in comparison to control fractions as indicated by quantified expression levels below the blot.

Based on this data, we wanted to further investigate expression patterns of hnRNP K in extended polysome fractions to confirm its ribosomal association and differential expression with TSA treatment. When comparing later polysome fractions (16+), there is a noticable difference in the expression levels of hnRNP K in the control and 2-hour TSA-treated fractions (Fig. 18). In the absence of treatment, hnRNP K expression drops rapidly before the polyribosomal fractions; however, in the presence of 2-hour treatment with TSA, hnRNP K expression sustains itself to later polyribosomally-associated fractions (Fig. 18A and C). When charting the expression levels, the difference in expression levels between the control and treated fractions are similar in early fractions, but after fraction 16, the expression patterns are significantly different (Fig. 18 B and D).

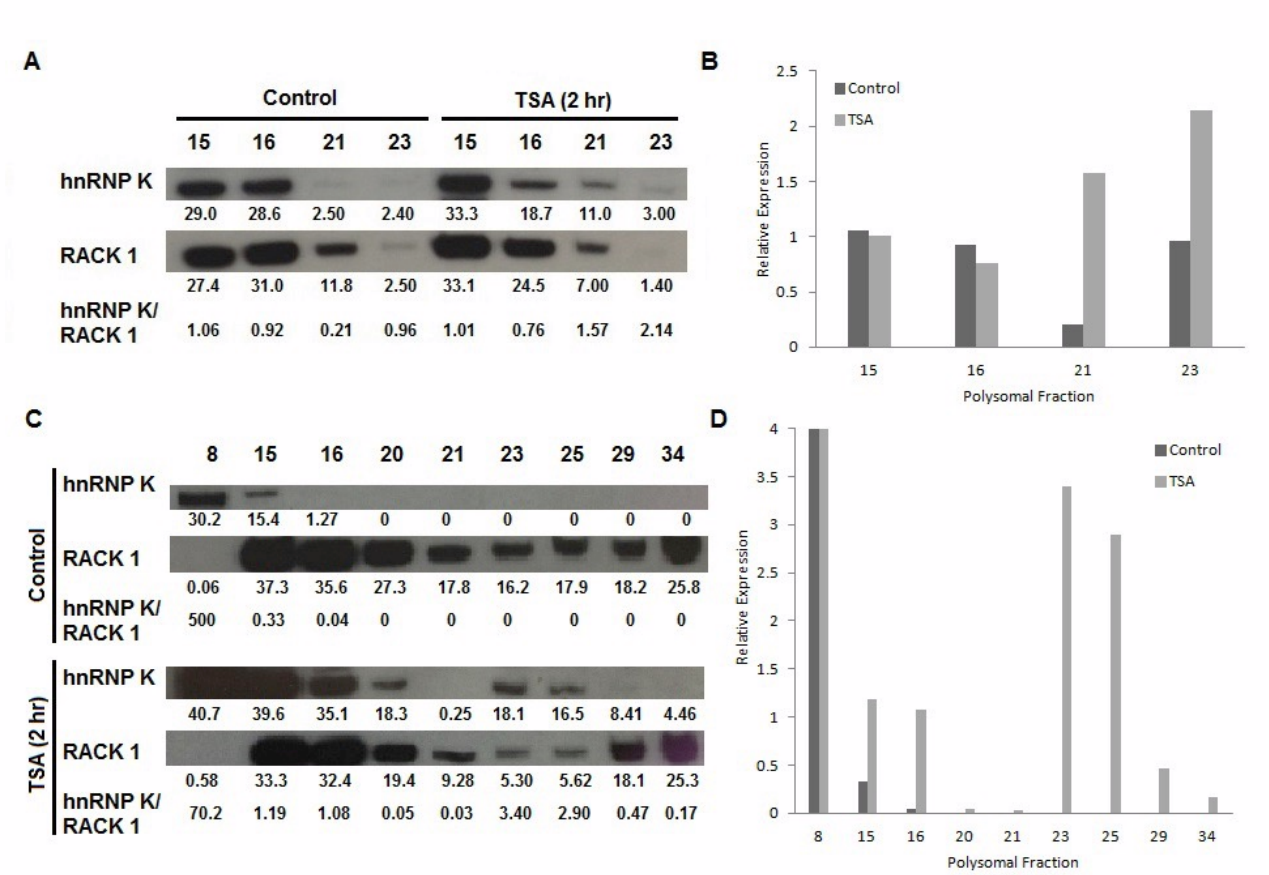

Figure 18. 2-hour TSA treatment induces stronger hnRNP K expression in polyribosomally associated fractions. A) Single Western blot analysis reveals relative expression of hnRNP K is similar in earlier monosomal fractions regardless of treatment, but a 2 fold or greater expression is indicated in fractions treated with TSA that are polyribosomally associated. B) Graphic analysis of data in part A uses hnRNPK/RACK1 expression ratio on the y-axis to visualize the significant difference of hnRNP K expression in fractions 21 and 23 following 2-hour treatment with TSA. C) Extended Western blot analysis using later fractions measures the extent of hnRNP K in later polyribosomal fractions. D) Graphic analysis of data in part C visualizes the stark drop-off of hnRNP K expression in control fractions after 16, whereas 2-hour TSA-treated fractions sustain expression until fraction 34.

DISCUSSION

We were able to identify differentially bound polyribosomal proteins to the coding-strand sequence of the 3'UTR of ERBB2 mRNA, which helped uncover some candidates possibly mediating HDACi induced decay of endogenously overexpressed ERBB2 transcripts in breast cancer. hnRNP K demonstrated the most promising results in that its binding was significantly altered after 2-hour TSA treatment in comparison to the control (Fig. 17 and 18). Previous studies have illustrated that hnRNP K can be methylated at arginine residues, which plays a key role in coordinating transcriptional responses to DNA damage as a cofactor for p53 (Chen et al., 2008). Upon DNA damage, hnRNP K is shielded from MDM2-mediated proteasomal degradation and then cooperates with p53 in transcriptional activation of cell-cycle arrest genes (Moumen et al., 2005). Additionally, hnRNP K can be modified by both acetylation and phosphorylation, strengthening the argument that HDACi treatment potentially induces hnRNP K acetylation as part of the ERBB2 transcript decay mechanism (Enge et al., 2009). However, further analysis of acetylation patterns of hnRNP K needs to be investigated.

With the amount of hnRNPs that were identified by mass spectrometry, it is likely that hnRNPs form a complex, initiated by hnRNP K binding, to mediate ERBB2 mRNA decay. hnRNPs are complexes of RNA and protein present in the cell nucleus during gene transcription and subsequent post-transcriptional modification of mRNA (Han et al., 2013). They are known to bind RNA polymerase II transcripts to form hnRNP particles, and these play functional roles in DNA repair, telomere biogenesis, cell signaling, and gene expression regulation (Han et al., 2013). The role of hnRNPs in mRNA stability and oncogene splicing are particular functions of interest. Each hnRNP protein contains at least one RNA-binding motif and the adenylate plus the uridylate-rich element (ARE) (Carpenter et al. 2006). They are commonly found in the 3'

untranslated region (UTR) of mRNAs and mediate the degradation of cytokine and proto-oncogene mRNAs (Carpenter et al. 2006). In our case, these findings suggest that with the abundance of hnRNPs present in our mass spectrometry analysis, several of these proteins may form a complex and cooperate in mediating ERBB2 transcript decay. hnRNP K itself has been shown to be part of several complexes that bind to the ERBB2 promoter, regulate metastasis at the translational level, and promote tumorigenicity (Gumireddy et al., 2013; Sugimasa et al., 2015; Zhang et al., 2012).

Nonetheless, when taking into account the abundance of data generated through mass spectrometry, initial reports of sense-strand specific protein binding by mass spectrometry in many cases, such as PPIA and RNH1, could not be confirmed by Western blot analysis. The quality of the sample is the primary determinant for success in native mass spectrometry, and sample heterogeneity due to partial protein degradation or the binding of adducts can severely restrict the outcome of this technique (Van Duijn, 2010). Nonetheless, the use of mass spectrometry in the future can also allow us to identify acetylation patterns of protein in addition to their strand specificity. Another source of discrepancy for the Western blot results could include the epitope specificity of antibodies used to detect the specific protein during Western blot analysis coupled with the basic assumption that an exogenously supplied ERBB2 mRNA construct can function similarly to an endogenously produced ERBB2 transcript. Additionally, the *in vitro* results obtained in Figures 10-15 need to be verified by *in vivo* experiments to confirm their reproducibility and significance as well as to evaluate the utility of a ERBB2 mRNA construct used in this study.

In order to translate the present findings into some therapeutic value in treating ERBB2-positive breast cancers, further experiments will need to be conducted using an HDAC inhibitor

that can be tested clinically instead of prototype tools, like TSA which is commonly used experimentally due to its commercial availability but lacks good bioavailability so cannot be used clinically. Currently, Panobinostat, developed by Novartis, is a pan-HDACi with the highest inhibitory potency among clinically used hydroxamic acids, and clinical trials are looking at its effectiveness in combination with transtuzumab to treat HER2-positive breast cancer (Dent et al., 2009). In one study, patients who received 10 mg of Panobinostat three times weekly or 15 mg of Panobinostat on days 1 and 8 of a 21-day cycle in combination with weekly Trastuzumab have responded well to the treatment, and two of the patients have even shown a reduction in tumor size (Conte et al., 2009). A structurally similar compound, Dacinostat, inhibits HDACs in submicromolar concentrations and has demonstrated the ability to inhibit cell growth in addition to inducing apoptosis (Dent et al., 2009). Also known as LAQ824, it has been shown to down-regulate HER2 by depleting HER2 transcripts and promoted its degradation by the proteasome (Fuino et al., 2003). Investigating the effects of these agents in place of TSA will help verify the mechanism of HDACi induced ERBB2 decay in breast cancer patients.

Furthermore, development of transcript-targeted treatments coupled with small molecule and receptor-disabling agents could lead to a highly successful strategy for overcoming resistance to currently available receptor-targeted agents. Dacinostat was also shown to sensitize human breast cancer cells to transtuzumab and other receptor disabling agents (Fuino et al., 2003). Another study focused on the combination of Vorinostat, a pan-HDACi, and Tamoxifen in breast cancer patients who progressed regardless of prior hormone therapy (Munster et al., 2009). Hormone receptor signaling was disrupted with HDACi treatment, giving reason that resistance to hormone receptor modulators is reversible (Dent et al., 2009). Studies have shown that HDACi sensitizes ER-negative cell lines to Tamoxifen by inducing the release of HDAC1

from the ERα-promoter, and in turn, restoring expression of ERα (Yang et al., 2001; Zhou et al., 2007) or by activation of ERβ (Hodges-Gallagher et al., 2006), involving an upregulation or translocation of ERβ (Duong et al., 2006; Jang et al., 2004). Six patients in this phase II study responded well to the treatment with no resistance to the combination for longer than 6 months, suggesting that this drug combination may help restore hormone sensitivity (Munster et al., 2009). Progress in this effort could potentially lead to a cancer treatment strategy that is more effective and selective, and less toxic, than the use of currently available pan-HDACi for the treatment of ERBB2-overexpressing malignancies. Ultimately, advances in this research will greatly improve the survival likelihood of patients suffering from ERBB2-positive breast cancer.

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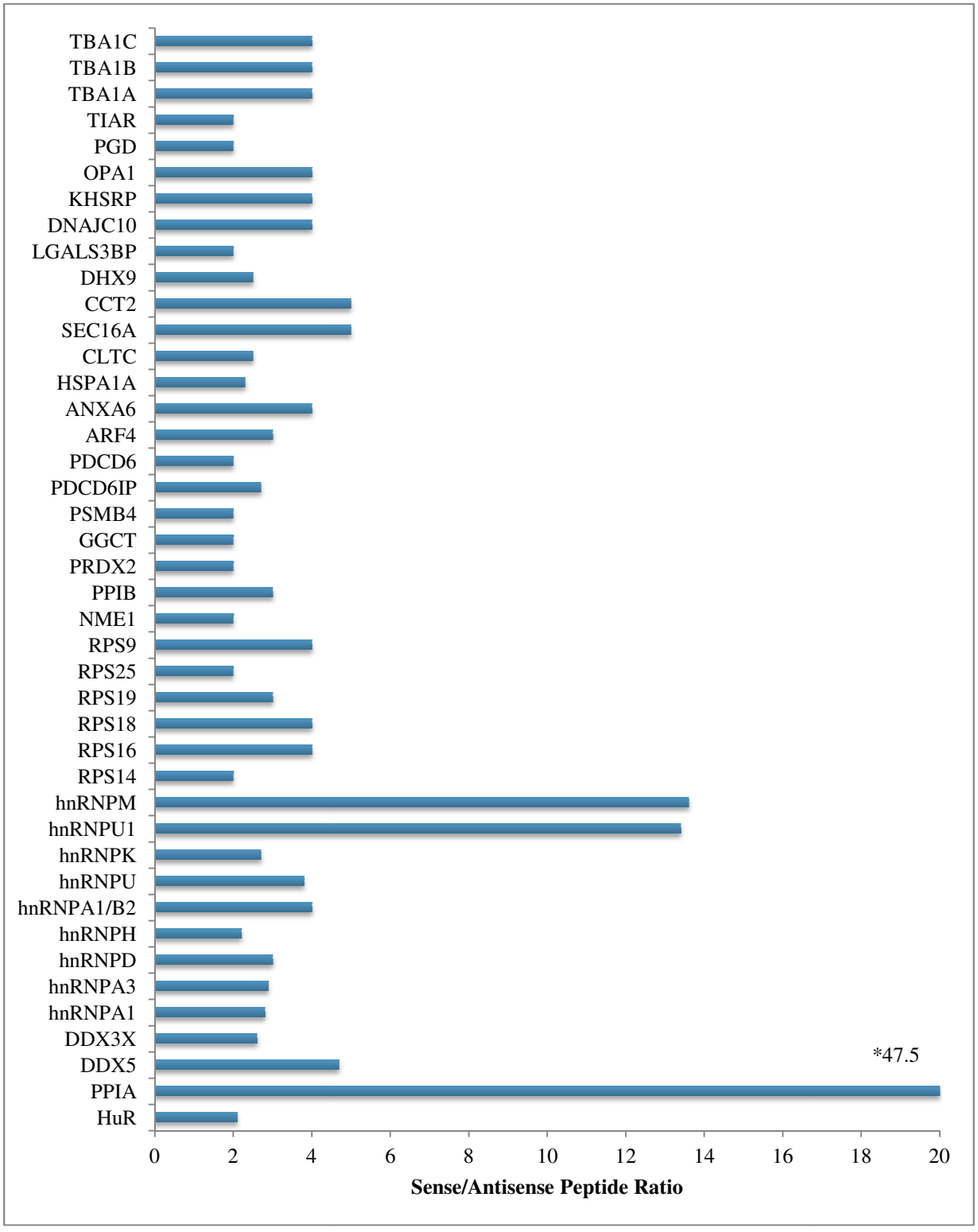
APPENDIX 1

List of 42 protein candidates that displayed a 2 fold or greater binding to the sense strand over the antisense strand.



APPENDIX 2

Complete list of protein binding to the 3'UTR of ERBB2 discovered during mass spectrometry.

Protein	**Gene**
10 kDa heat shock protein, mitochondrial	HSPE1
14-3-3 protein epsilon	YWHAE
2,4-dienoyl-CoA reductase, mitochondrial	DECR1
26S proteasome non-ATPase regulatory subunit 11	PSMD11
26S proteasome non-ATPase regulatory subunit 13	PSMD13
26S proteasome non-ATPase regulatory subunit 3	PSMD3
28S ribosomal protein S16, mitochondrial	MRPS16
28S ribosomal protein S18b, mitochondrial	MRPS18B
28S ribosomal protein S22, mitochondrial	MRPS22
28S ribosomal protein S26, mitochondrial	MRPS26
28S ribosomal protein S27, mitochondrial	MRPS27
28S ribosomal protein S29, mitochondrial	DAP3
28S ribosomal protein S5, mitochondrial	MRPS5
28S ribosomal protein S6, mitochondrial	MRPS6
3-ketoacyl-CoA thiolase, mitochondrial	ACAA2
3-ketoacyl-CoA thiolase, peroxisomal	ACAA1
4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1
40S ribosomal protein S10	RPS10
40S ribosomal protein S11	RPS11
40S ribosomal protein S14	RPS14
40S ribosomal protein S15a	RPS15A
40S ribosomal protein S16	RPS16
40S ribosomal protein S18	RPS18
40S ribosomal protein S19	RPS19
40S ribosomal protein S2	RPS2
40S ribosomal protein S20	RPS20
40S ribosomal protein S21	RPS21
40S ribosomal protein S25	RPS25
40S ribosomal protein S28	RPS28
40S ribosomal protein S3	RPS3
40S ribosomal protein S3a	RPS3A
40S ribosomal protein S4, X isoform	RPS4X
40S ribosomal protein S6	RPS6
40S ribosomal protein S7	RPS7
40S ribosomal protein S9	RPS9
40S ribosomal protein SA	RPSA

4F2 cell-surface antigen heavy chain	SLC3A2
6-phosphofructokinase type C	PFKP
6-phosphofructokinase, liver type	PFKL
6-phosphogluconate dehydrogenase, decarboxylating	PGD
60 kDa heat shock protein, mitochondrial	HSPD1
60S acidic ribosomal protein P0	RPLP0
60S acidic ribosomal protein P0-like	RPLP0P6
60S acidic ribosomal protein P2	RPLP2
60S ribosomal protein L11	RPL11
60S ribosomal protein L12	RPL12
60S ribosomal protein L17	RPL17
60S ribosomal protein L18a	RPL18A
60S ribosomal protein L22	RPL22
60S ribosomal protein L24	RPL24
60S ribosomal protein L26-like 1	RPL26L1
60S ribosomal protein L27	RPL27
60S ribosomal protein L27a	RPL27A
60S ribosomal protein L28	RPL28
60S ribosomal protein L3	RPL3
60S ribosomal protein L30	RPL30
60S ribosomal protein L31	RPL31
60S ribosomal protein L34	RPL34
60S ribosomal protein L35	RPL35
60S ribosomal protein L38	RPL38
60S ribosomal protein L4	RPL4
60S ribosomal protein L5	RPL5
60S ribosomal protein L6	RPL6
60S ribosomal protein L7	RPL7
60S ribosomal protein L7a	RPL7A
60S ribosomal protein L8	RPL8
60S ribosomal protein L9	RPL9
78 kDa glucose-regulated protein	HSPA5
Acetyl-CoA acetyltransferase, mitochondrial	ACAT1
Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A
Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B
Acidic leucine-rich nuclear phosphoprotein 32 family member C	ANP32C
Acidic leucine-rich nuclear phosphoprotein 32 family member D	ANP32D
Aconitate hydratase, mitochondrial	ACO2
Actin-related protein 2/3 complex subunit 4	ARPC4
Actin-related protein 3	ACTR3
Actin-related protein 3B	ACTR3B

Actin, alpha skeletal muscle	ACTA1
Actin, cytoplasmic 1	ACTB
Actin, cytoplasmic 2	ACTG1
Activated RNA polymerase II transcriptional coactivator p15	SUB1
Acyl-coenzyme A thioesterase 13	ACOT13
Acylamino-acid-releasing enzyme	APEH
Adenosine kinase	ADK
Adenosylhomocysteinase	AHCY
ADP-ribosylation factor 1	ARF1
ADP-ribosylation factor 4	ARF4
ADP-ribosylation factor-like protein 8B	ARL8B
ADP/ATP translocase 1	SLC25A4
ADP/ATP translocase 2	SLC25A5
ADP/ATP translocase 3	SLC25A6
ADP/ATP translocase 4	SLC25A31
Alanine--tRNA ligase, cytoplasmic	AARS
Alcohol dehydrogenase [NADP(+)]	AKR1A1
Aldehyde dehydrogenase, mitochondrial	ALDH2
Alpha-2-HS-glycoprotein	AHSG
Alpha-enolase	ENO1
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	AIMP1
Annexin A11	ANXA11
Annexin A2	ANXA2
Annexin A5	ANXA5
Annexin A6	ANXA6
Annexin A7	ANXA7
Anterior gradient protein 2 homolog	AGR2
AP-1 complex subunit beta-1	AP1B1
Apolipoprotein D	APOD
Arginase-1	ARG1
Arginine--tRNA ligase, cytoplasmic	RARS
Argininosuccinate synthase	ASS1
Aspartate aminotransferase, cytoplasmic	GOT1
Aspartate aminotransferase, mitochondrial	GOT2
ATP synthase subunit alpha, mitochondrial	ATP5A1
ATP synthase subunit beta, mitochondrial	ATP5B
ATP-citrate synthase	ACLY
ATP-dependent RNA helicase A	DHX9
ATP-dependent RNA helicase DDX1	DDX1
ATP-dependent RNA helicase DDX3X	DDX3X
Basic leucine zipper and W2 domain-containing protein 2	BZW2

Bifunctional glutamate/proline--tRNA ligase	EPRS
Biliverdin reductase A	BLVRA
C-1-tetrahydrofolate synthase, cytoplasmic	MTHFD1
CAD protein	CAD
Calmodulin-like protein 5	CALML5
Calnexin	CANX
Calpain-2 catalytic subunit	CAPN2
Calumenin	CALU
Carbamoyl-phosphate synthase [ammonia], mitochondrial	CPS1
Carbonyl reductase [NADPH] 1	CBR1
Caspase-14	CASP14
Cathepsin D	CTSD
Citrate synthase, mitochondrial	CS
Clathrin heavy chain 1	CLTC
Clathrin light chain A	CLTA
Clathrin light chain B	CLTB
Coatomer subunit alpha	COPA
Cofilin-1	CFL1
Constitutive coactivator of PPAR-gamma-like protein 1	FAM120A
Copine-3	CPNE3
Creatine kinase B-type	CKB
Creatine kinase U-type, mitochondrial	CKMT1A
CTP synthase 1	CTPS1
Cyclin-dependent kinase 1	CDK1
Cyclin-dependent kinase 12	CDK12
Cyclin-dependent kinase 13	CDK13
Cyclin-dependent kinase 14	CDK14
Cyclin-dependent kinase 15	CDK15
Cyclin-dependent kinase 16	CDK16
Cyclin-dependent kinase 17	CDK17
Cyclin-dependent kinase 18	CDK18
Cyclin-dependent kinase 2	CDK2
Cyclin-dependent kinase 20	CDK20
Cyclin-dependent kinase 3	CDK3
Cyclin-dependent kinase 4	CDK4
Cyclin-dependent kinase 5	CDK5
Cyclin-dependent kinase 6	CDK6
Cyclin-dependent kinase 9	CDK9
Cytosolic non-specific dipeptidase	CNDP2
D-3-phosphoglycerate dehydrogenase	PHGDH
D-dopachrome decarboxylase	DDT

Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	ECH1
Dermcidin	DCD
Desmoplakin	DSP
Dihydrolipoyl dehydrogenase, mitochondrial	DLD
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	DLAT
DNA-dependent protein kinase catalytic subunit	PRKDC
DNA-directed RNA polymerase II subunit RPB1	POLR2A
DNA-directed RNA polymerase II subunit RPB2	POLR2B
DNA-directed RNA polymerases I, II, and III subunit RPABC1	POLR2E
DnaJ homolog subfamily C member 10	DNAJC10
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	DDOST
Dynamin-like 120 kDa protein, mitochondrial	OPA1
E3 ubiquitin-protein ligase CHIP	STUB1
E3 ubiquitin-protein ligase TRIM21	TRIM21
ELAV-like protein 1	ELAVL1
Elongation factor 1-alpha 1	EEF1A1
Elongation factor 1-alpha 2	EEF1A2
Elongation factor 1-delta	EEF1D
Elongation factor 1-gamma	EEF1G
Elongation factor 2	EEF2
Elongation factor Tu, mitochondrial	TUFM
Endoplasmic reticulum resident protein 44	ERP44
Endoplasmic reticulum-Golgi intermediate compartment protein 1	ERGIC1
Endoplasmin	HSP90B1
Epoxide hydrolase 1	EPHX1
Eukaryotic initiation factor 4A-I	EIF4A1
Eukaryotic initiation factor 4A-III	EIF4A3
Eukaryotic peptide chain release factor subunit 1	ETF1
Eukaryotic translation initiation factor 2 subunit 1	EIF2S1
Eukaryotic translation initiation factor 2 subunit 2	EIF2S2
Eukaryotic translation initiation factor 3 subunit A	EIF3A
Eukaryotic translation initiation factor 3 subunit C	EIF3C
Eukaryotic translation initiation factor 3 subunit E	EIF3E
Eukaryotic translation initiation factor 3 subunit F	EIF3F
Eukaryotic translation initiation factor 3 subunit I	EIF3I
Eukaryotic translation initiation factor 3 subunit J	EIF3J
Eukaryotic translation initiation factor 3 subunit L	EIF3L
Eukaryotic translation initiation factor 4 gamma 1	EIF4G1
Exportin-2	CSE1L

Ezrin	EZR
F-actin-capping protein subunit alpha-1	CAPZA1
F-actin-capping protein subunit alpha-2	CAPZA2
Far upstream element-binding protein 1	FUBP1
Far upstream element-binding protein 2	KHSRP
Farnesyl pyrophosphate synthase	FDPS
Fatty acid synthase	FASN
Fibroblast growth factor receptor 1	FGFR1
Fibroblast growth factor receptor 2	FGFR2
Fibroblast growth factor receptor 3	FGFR3
Fibroblast growth factor receptor 4	FGFR4
Fructose-bisphosphate aldolase A	ALDOA
Fumarate hydratase, mitochondrial	FH
Fumarylacetoacetase	FAH
Galectin-3-binding protein	LGALS3BP
Galectin-7	LGALS7
Gamma-glutamylcyclotransferase	GGCT
Gasdermin-A	GSDMA
Geranylgeranyl transferase type-2 subunit beta	RABGGTB
Glucose-6-phosphate 1-dehydrogenase	G6PD
Glucose-6-phosphate 1-dehydrogenase	G6PD
Glucose-6-phosphate isomerase	GPI
Glutamine synthetase	GLUL
Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1
Glutamine--tRNA ligase	QARS
Glutathione synthetase	GSS
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Glycine--tRNA ligase	GARS
Glycogen debranching enzyme	AGL
Glycogen phosphorylase, brain form	PYGB
Glyoxylate reductase/hydroxypyruvate reductase	GRHPR
GTP-binding protein SAR1b	SAR1B
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	GNB3
Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1
Guanine nucleotide-binding protein subunit beta-4	GNB4
Heat shock 70 kDa protein 1A/1B	HSPA1A
Heat shock cognate 71 kDa protein	HSPA8
Heat shock protein 105 kDa	HSPH1
Heat shock protein 75 kDa, mitochondrial	TRAP1

Heat shock protein HSP 90-alpha	HSP90AA1
Heat shock protein HSP 90-beta	HSP90AB1
Heterogeneous nuclear ribonucleoprotein A/B	HNRNPAB
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1
Heterogeneous nuclear ribonucleoprotein A1-like 2	HNRNPA1L2
Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3
Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1
Heterogeneous nuclear ribonucleoprotein D-like	HNRNPDL
Heterogeneous nuclear ribonucleoprotein D0	HNRNPD
Heterogeneous nuclear ribonucleoprotein F	HNRNPF
Heterogeneous nuclear ribonucleoprotein H	HNRNPH1
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3
Heterogeneous nuclear ribonucleoprotein K	HNRNPK
Heterogeneous nuclear ribonucleoprotein L	HNRNPL
Heterogeneous nuclear ribonucleoprotein M	HNRNPM
Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP
Heterogeneous nuclear ribonucleoprotein R	HNRNPR
Heterogeneous nuclear ribonucleoprotein U	HNRNPU
Heterogeneous nuclear ribonucleoprotein U-like protein 1	HNRNPUL1
Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRNPUL2
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1
Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC
Hexokinase-1	HK1
Histidine--tRNA ligase, cytoplasmic	HARS
Histone-arginine methyltransferase CARM1	CARM1
Hypoxia up-regulated protein 1	HYOU1
Ig gamma-1 chain C region	IGHG1
Ig gamma-3 chain C region	IGHG3
Immunoglobulin lambda-like polypeptide 5	IGLL5
Importin subunit beta-1	KPNB1
Inorganic pyrophosphatase	PPA1
Inorganic pyrophosphatase 2, mitochondrial	PPA2
Inosine-5'-monophosphate dehydrogenase 1	IMPDH1
Inosine-5'-monophosphate dehydrogenase 1	IMPDH1
Inosine-5'-monophosphate dehydrogenase 2	IMPDH2
Interleukin enhancer-binding factor 2	ILF2
Interleukin enhancer-binding factor 3	ILF3
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDH3A
Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1
Isocitrate dehydrogenase [NADP], mitochondrial	IDH2
Isoleucine--tRNA ligase, cytoplasmic	IARS

Keratinocyte proline-rich protein	KPRP
L-lactate dehydrogenase A chain	LDHA
L-lactate dehydrogenase B chain	LDHB
La-related protein 1	LARP1
Lamin-B2	LMNB2
LanC-like protein 1	LANCL1
Leucine--tRNA ligase, cytoplasmic	LARS
Lupus La protein	SSB
Lysine--tRNA ligase	KARS
Lysosome-associated membrane glycoprotein 1	LAMP1
Malate dehydrogenase, cytoplasmic	MDH1
Malate dehydrogenase, mitochondrial	MDH2
Methionine--tRNA ligase, cytoplasmic	MARS
mRNA cap guanine-N7 methyltransferase	RNMT
Mucin-like protein 1	MUCL1
Multifunctional protein ADE2	PAICS
Myosin light polypeptide 6	MYL6
Myosin regulatory light polypeptide 9	MYL9
Myosin-9	MYH9
N-terminal kinase-like protein	SCYL1
NAD(P)H dehydrogenase [quinone] 1	NQO1
NADP-dependent malic enzyme	ME1
Nascent polypeptide-associated complex subunit alpha	NACA
Neuroblast differentiation-associated protein AHNAK	AHNAK
Neutral alpha-glucosidase AB	GANAB
Nicotinate phosphoribosyltransferase	NAPRT1
Nitric oxide-associated protein 1	NOA1
Non-POU domain-containing octamer-binding protein	NONO
Nuclear migration protein nudC	NUDC
Nuclear protein localization protein 4 homolog	NPLOC4
Nuclease-sensitive element-binding protein 1	YBX1
Nucleolin	NCL
Nucleolysin TIA-1 isoform p40	TIA1
Nucleolysin TIAR	TIAL1
Nucleophosmin	NPM1
Nucleoside diphosphate kinase A	NME1
Obg-like ATPase 1	OLA1
Ornithine aminotransferase, mitochondrial	OAT
Peflin	PEF1
Peptidyl-prolyl cis-trans isomerase A	PPIA
Peptidyl-prolyl cis-trans isomerase B	PPIB

Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4
Peroxiredoxin-1	PRDX1
Peroxiredoxin-2	PRDX2
Peroxiredoxin-5, mitochondrial	PRDX5
Peroxisomal acyl-coenzyme A oxidase 1	ACOX1
Phenylalanine--tRNA ligase alpha subunit	FARSA
Phenylalanine--tRNA ligase beta subunit	FARSB
Phosphatidylethanolamine-binding protein 1	PEBP1
Phosphoglucomutase-1	PGM1
Phosphoglycerate kinase 1	PGK1
Phosphoserine aminotransferase	PSAT1
Plasminogen activator inhibitor 1 RNA-binding protein	SERBP1
Plastin-2	LCP1
Plastin-3	PLS3
Poly [ADP-ribose] polymerase 1	PARP1
Poly(rC)-binding protein 1	PCBP1
Polyadenylate-binding protein 1	PABPC1
Polyadenylate-binding protein 1-like	PABPC1L
Polyadenylate-binding protein 4	PABPC4
Polyadenylate-binding protein 5	PABPC5
Polypyrimidine tract-binding protein 1	PTBP1
POTE ankyrin domain family member E	POTEE
Pre-mRNA-processing factor 39	PRPF39
Prelamin-A/C	LMNA
Probable ATP-dependent RNA helicase DDX17	DDX17
Probable ATP-dependent RNA helicase DDX5	DDX5
Profilin-1	PFN1
Programmed cell death 6-interacting protein	PDCD6IP
Programmed cell death protein 6	PDCD6
Proliferating cell nuclear antigen	PCNA
Proliferation-associated protein 2G4	PA2G4
Proliferation-associated protein 2G4	PA2G4
Proteasome subunit beta type-1	PSMB1
Proteasome subunit beta type-2	PSMB2
Proteasome subunit beta type-4	PSMB4
Proteasome subunit beta type-6	PSMB6
Protein arginine N-methyltransferase 1	PRMT1
Protein diaphanous homolog 1	DIAPH1
Protein disulfide-isomerase	P4HB
Protein disulfide-isomerase A3	PDIA3
Protein disulfide-isomerase A4	PDIA4

Protein disulfide-isomerase A6	PDIA6
Protein DJ-1	PARK7
Protein kinase C and casein kinase substrate in neurons protein 2	PACSIN2
Protein S100-A6	S100A6
Protein S100-A8	S100A8
Protein S100-A9	S100A9
Protein SEC13 homolog	SEC13
Protein SET	SET
Protein SETSIP	SETSIP
Protein Shroom3	SHROOM3
Protein TFG	TFG
Protein transport protein Sec16A	SEC16A
Protein transport protein Sec23B	SEC23B
Protein transport protein Sec24A	SEC24A
Protein transport protein Sec24B	SEC24B
Protein transport protein Sec24C	SEC24C
Protein transport protein Sec24D	SEC24D
Protein transport protein Sec31A	SEC31A
Prothymosin alpha	PTMA
Purine nucleoside phosphorylase	PNP
Putative 40S ribosomal protein S26-like 1	RPS26P11
Putative annexin A2-like protein	ANXA2P2
Putative ATP-dependent RNA helicase DHX30	DHX30
Putative elongation factor 1-alpha-like 3	EEF1A1P5
Putative elongation factor 1-alpha-like 3	EEF1A1P5
Putative heat shock protein HSP 90-beta 2	HSP90AB2P
Putative protein FAM10A4	ST13P4
Putative protein FAM10A5	ST13P5
Putative synaptogyrin-2 like protein	None
Pyrroline-5-carboxylate reductase 1, mitochondrial	PYCR1
Pyrroline-5-carboxylate reductase 2	PYCR2
Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	PDHA1
Pyruvate kinase PKLR	PKLR
Pyruvate kinase PKLR	PKLR
Pyruvate kinase PKM	PKM
Rab GDP dissociation inhibitor beta	GDI2
Ras GTPase-activating-like protein IQGAP1	IQGAP1
Ras-related protein Rab-1B	RAB1B
Ras-related protein Rab-7a	RAB7A
Receptor tyrosine-protein kinase erbB-2	ERBB2

Replication protein A 14 kDa subunit	RPA3
Replication protein A 32 kDa subunit	RPA2
Replication protein A 70 kDa DNA-binding subunit	RPA1
Replication protein A 70 kDa DNA-binding subunit	RPA1
Reticulon-4	RTN4
Ribonuclease inhibitor	RNH1
Ribose-phosphate pyrophosphokinase 1	PRPS1
Ribose-phosphate pyrophosphokinase 2	PRPS2
RNA-binding protein EWS	EWSR1
RNA-binding protein FUS	FUS
RuvB-like 1	RUVBL1
Serine hydroxymethyltransferase, mitochondrial	SHMT2
Serine/threonine-protein kinase ICK	ICK
Serine/threonine-protein kinase MAK	MAK
Serpin B4	SERPINB4
Serum albumin	ALB
Single-stranded DNA-binding protein, mitochondrial	SSBP1
Small nuclear ribonucleoprotein E	SNRPE
Small nuclear ribonucleoprotein Sm D2	SNRPD2
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1
Sorcin	SRI
Sorting nexin-9	SNX9
Spermidine synthase	SRM
Spliceosome RNA helicase DDX39B	DDX39B
Squamous cell carcinoma antigen recognized by T-cells 3	SART3
Src substrate cortactin	CTTN
Staphylococcal nuclease domain-containing protein 1	SND1
Stress-70 protein, mitochondrial	HSPA9
Stress-induced-phosphoprotein 1	STIP1
Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	SUCLG2
Superoxide dismutase [Cu-Zn]	SOD1
Synaptogyrin-2	SYNGR2
T-complex protein 1 subunit alpha	TCP1
T-complex protein 1 subunit beta	CCT2
T-complex protein 1 subunit delta	CCT4
T-complex protein 1 subunit epsilon	CCT5
T-complex protein 1 subunit eta	CCT7
T-complex protein 1 subunit gamma	CCT3
T-complex protein 1 subunit theta	CCT8
T-complex protein 1 subunit zeta	CCT6A
Tax1-binding protein 1	TAX1BP1

Thioredoxin	TXN
Thioredoxin domain-containing protein 5	TXNDC5
Thioredoxin-dependent peroxide reductase, mitochondrial	PRDX3
Thiosulfate sulfurtransferase/rhodanese-like domain-containing protein 1	TSTD1
Threonine--tRNA ligase, cytoplasmic	TARS
Thymidine phosphorylase	TYMP
Transaldolase	TALDO1
Transcription activator BRG1	SMARCA4
Transcription elongation factor SPT5	SUPT5H
Transcriptional activator protein Pur-alpha	PURA
Transcriptional activator protein Pur-beta	PURB
Transcriptional adapter 2-beta	TADA2B
Transcriptional adapter 2-beta	TADA2B
Transferrin receptor protein 1	TFRC
Transgelin-2	TAGLN2
Transitional endoplasmic reticulum ATPase	VCP
Transketolase	TKT
Trifunctional enzyme subunit alpha, mitochondrial	HADHA
Tripartite motif-containing protein 3	TRIM3
tRNA (cytosine(34)-C(5))-methyltransferase	NSUN2
tRNA selenocysteine 1-associated protein 1	TRNAU1AP
tRNA-splicing ligase RtcB homolog	RTCB
Tubulin alpha-1A chain	TUBA1A
Tubulin alpha-1B chain	TUBA1B
Tubulin alpha-1C chain	TUBA1C
Tubulin alpha-3E chain	TUBA3E
Tubulin beta chain	TUBB
Tubulin beta-1 chain	TUBB1
Tubulin beta-2A chain	TUBB2A
Tubulin beta-2B chain	TUBB2B
Tubulin beta-3 chain	TUBB3
Tubulin beta-4A chain	TUBB4A
Tubulin beta-4B chain	TUBB4B
Tubulin beta-6 chain	TUBB6
Tubulin beta-8 chain	TUBB8
Tubulin-specific chaperone A	TBCA
Tyrosine-protein kinase Blk	BLK
Tyrosine-protein kinase Fgr	FGR
Tyrosine-protein kinase Fyn	FYN
Tyrosine-protein kinase HCK	HCK
Tyrosine-protein kinase Lck	LCK

Tyrosine-protein kinase Lyn	LYN
Tyrosine-protein kinase Yes	YES1
U1 small nuclear ribonucleoprotein A	SNRPA
Ubiquitin carboxyl-terminal hydrolase 13	USP13
Ubiquitin-like modifier-activating enzyme 1	UBA1
UDP-glucose:glycoprotein glucosyltransferase 1	UGGT1
UTP--glucose-1-phosphate uridylyltransferase	UGP2
Vacuolar protein sorting-associated protein 35	VPS35
Valine--tRNA ligase	VARS
Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADVL
Vesicle-trafficking protein SEC22b	SEC22B
Vinculin	VCL
WD repeat-containing protein 1	WDR1
WD repeat-containing protein 61	WDR61
X-ray repair cross-complementing protein 5	XRCC5
X-ray repair cross-complementing protein 6	XRCC6
YTH domain-containing family protein 2	YTHDF2
Zinc-alpha-2-glycoprotein	AZGP1