5-2018

Identification of substrate-selective histone deacetylases and their inhibitors that mediate HER2 transcript stability

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https://doi.org/10.33015/dominican.edu/2018.bio.06

Identification of substrate-selective histone deacetylases and their inhibitors that mediate HER2 transcript stability

By

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A culminating thesis submitted to the faculty of Dominican University of California in partial fulfillment of the requirements for the degree of Master of Science in Biology

San Rafael, California

May, 2018

This thesis, written under the direction of candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree of Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

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Abbreviations

ACY Acetylon

bp base pairs

CBP CREB-binding protein

cAMP cyclic adenosine monophosphate

cDNA complementary deoxyribonucleic acid

CREB cAMP response element-binding protein

Ent entinostat

ER estrogen receptor

ERE estrogen response element

FDA U.S. Food and Drug Administration

FISH fluorescence in situ hybridization

FK228 romidepsin (also referred to as istodax)

GAPDH glyceraldehyde 3-phosphate dehydrogenase

HAT histone acetyltransferase

HDAC histone deacetylase

HDACi histone deacetylase inhibitor

HER2 human epidermal growth factor receptor 2 (also referred to as ERBB2)

HRE hormone response element

IHC immunohistochemistry

IB immunoblot

IP immunoprecipitation

K lysine

mRNA messenger ribonucleic acid

MAPK mitogen-activated protein kinase

MS mass spectrometry

p/CAF p300/CBP-associated factor

PI3K phosphoinostide 3-kinase

PR progesterone receptor

PRE progesterone response element

RNA ribonucleic acid

siRNA small interfering ribonucleic acid

SKBR3 HER2 overexpressing breast adenocarcinoma mammalian cell line

TSA trichostatin A

UTR untranslated region

UV ultra violet

UV/Vis ultra violet visual

Abstract

Breast cancer is the most common malignancy diagnosed in women. 15-20% of these cancers overexpress the HER2 (ERRB2) oncoprotein. HER2-positive breast cancers are generally aggressive and are associated with poor prognosis. Unfortunately, only a mere 30% of HER2-positve patients respond to therapies when they are used as a single agent. Combining therapeutics can potentially lead to synergy and improved anticancer efficacy, and there is clearly a need for the development of new HER2-directed therapeutics. Newer approaches include the utilization of histone deacetylase (HDAC) inhibitors. It has been observed that HDAC inhibitors can induce the rapid decay of oncogenic transcripts such as the HER2 mRNA, though the mechanism underlying this process remains undefined. Earlier observations in our lab led to speculation that protein(s) binding the 3'-untranslated region (UTR) of HER2 mRNA become acetylated following HDAC inhibitor treatment and promote the HER2 mRNA decay while engaged in the translational machinery. Pursuing this speculation, a key ribosome- or polyribosome-associated candidate suspected of interacting with and mediating this HER2 mRNA decay became p300, identified by mass spectrometry in cytosolic fractions containing rapidly degrading HER2 mRNA as a protein appearing hyper-acetylated within hours of breast cancer cell exposure to a pan-HDAC inhibitor. Studies conducted using pan-HDAC inhibitors like Trichostatin A (TSA) as well as various class-selective HDAC inhibitors, including romidepsin (FK228), examine their comparative influences on the association of p300 with either the ribosome or polyribosome translational machinery, p300's protein levels and acetylation status before and after HDACi treatment, and their functional impact on HER2 mRNA by modulating

its expression levels. Understanding the mechanism by which optimal class-selective HDAC inhibitors modulate translation-associated p300 to facilitate HER2 mRNA degradation will allow for more effective use of HDAC inhibitor's antitumor activity to combat HER2-positive breast cancers.

Acknowledgements

There is a saying that floats around “it takes a village to raise a child.” This thesis is my baby and here I would like to extend my most sincere gratitude to “the village.” First, thank you to Dr. Christopher Benz for welcoming me into the Buck Institute for Research and Aging and especially his lab and allowing me to be a part of the legacy, none of this would have been possible without him. Next I would like to thank Dr. Gary Scott for his patience that and all the skills I’ve learned from him under his tutelage. I could not have asked for better mentors. I would also like to extend my appreciation to Dr. Birgit Schilling and her lab in the mass spectrometry core at the Buck, the analysis done by her lab provided a pivotal moment in my research findings. I also appreciate the instrumental roles that Dr. Shihyin Tsai, Dr. Christina Yau, Katya Frazier, Mauricio Ortega, Sana Khateeb, See Yang, Beatrice Becker, and Jacob Rose have played in making this project successful.

I would like to thank Dominican University of California for my second round of higher education. There is no community more supportive than the department of natural science and mathematics—specifically Dr. Maggie Louie who has given me so much guidance and her time and energy through the years. I’m thankful for Dr. Kristylea Ojeda and the outside perspective she had to offer in serving as a second reader for this thesis. I’d like to give a special thanks to Dr. Ekaterina Kalashnikova for inviting me to think critically in her classes.

Finally I want to thank my friends and family but most especially Mom, Dad, Paula, and Alex’e. I’m thankful for their unconditional love, unwavering support, and unlimited patience. We did it. This is for Bryson, my baby.

Introduction

Breast cancer is one of the most prominent malignancies diagnosed in women in the United States. One in eight women will be diagnosed with breast cancer over the course of her lifetime (DeSantis et al., 2015; DeSantis, Ma, Goding Sauer, Newman, & Jemal, 2017). In the United States, the American Cancer Society (ACS) predicts that 266,120 new cases of invasive breast cancer will be diagnosed in the year 2018. And of the women with existing cases of breast cancer, an estimated 40,920 women will die due to breast cancer (ACS, 2018). Breast cancer has the second highest cancer-related mortality rate after lung cancer (ACS, 2018; DeSantis et al., 2017), and it is now the most lethal cancer in females (ACS, 2018; Halpern et al., 2013; Lin, Zhang, Li, & Sun, 2017). Breast cancer susceptibility can be inherited or obtained through non-biological risk factors such as hormone exposure or other environmental factors.

Biomarkers of breast cancer

Estrogen receptor (ER) is a member of the super-family of nuclear hormone steroid receptors. Members of this family function as ligand-gated transcription factors to modulate expression of genes containing specific hormone response elements (HRE) (MacGregor & Jordan, 1998). Two main forms of ERs exist—ERα and ERβ which are encoded by separate genes located on different chromosomes (Nilsson et al., 2001). Although both ERα and ERβ are expressed in breast tissue, bind to estrogen with similar affinities, and share similar functions; ERα is the isoform necessary for normal mammary gland development (Platet, Cathiard, Gleizes, & Garcia, 2004).

ER-positive (ER+) breast cancers generally refer to the expression of ERα and are the most prevalent, accounting for approximately 70% of breast cancers diagnosed in women (Bodai & Tuso 2015, Lumachi et al 2013, Montemurro & Aglietta 2009). More than 50% of ER+ breast tumors are also progesterone receptor-positive (PR+) (Cui, Schiff, Arpino, Osborne, & Lee, 2005). In breast cancer, ER and PR are activated by their bona fide ligands, estrogen and progesterone respectively (Figure 1; Tanenbaum, Wang, Williams, & Sigler, 1998).

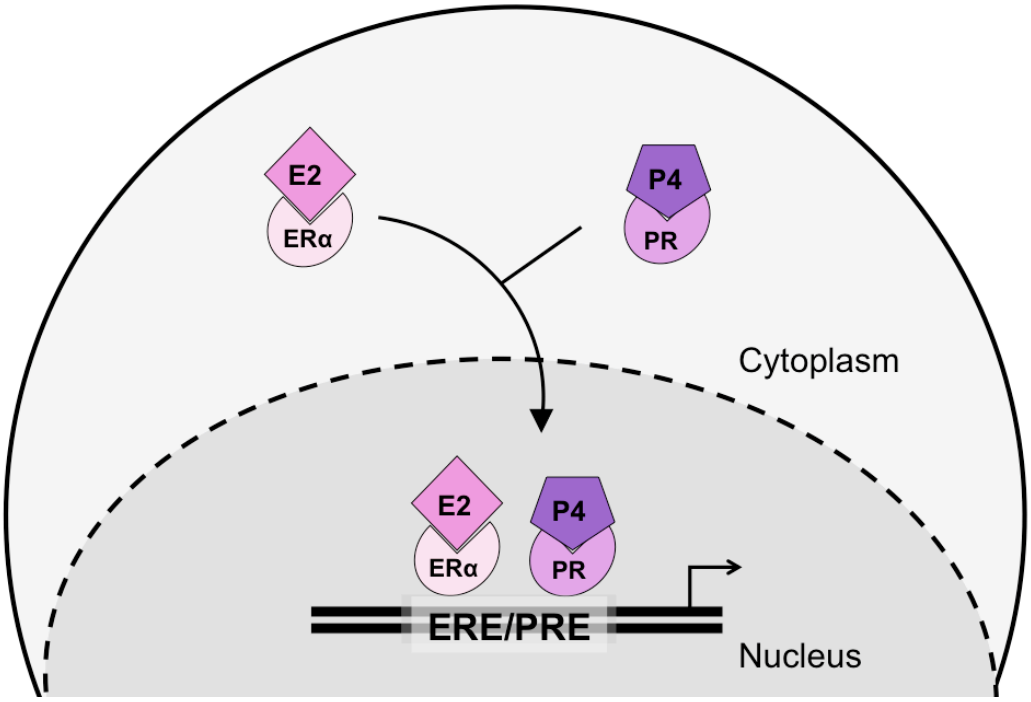


Figure 1. Steroid hormones estrogen (E2) and progesterone (P4) bind to their cognate receptors ER α and PR, respectively, activating translocation of the steroid-receptor complex to the nucleus. Each complex will bind to their DNA regulatory sites: estrogen response element (ERE) or progesterone response element (PRE) initiating transcription.

Nuclear receptors ERα and PR require co-regulators (co-activators and co-repressors) for efficient transcriptional regulation (ENCODE Project Consortium et al., 2007; O'Malley, 2006). Co-repressors and their associated complexes interact with nuclear receptors when bound to antagonists or un-liganded receptor to repress

transcription by binding to cis-acting elements within the promoters of target genes (ENCODE Project Consortium et al., 2007; Tetel, 2009). Co-activators significantly increase the transcriptional activity of ER and PR through epigenetic modifications such as acetylation, methylation, and phosphorylation, which are different forms of chromatin remodeling (ENCODE Project Consortium et al., 2007; Tetel, 2009). The p160 family of co-activators includes the steroid receptor co-activators: SRC-1 (NcoA-1), SRC-2 (NCoA-2, TIF2), and SRC-3 (A1B1, ACTR, RAC3) (Anzick et al., 1997; Tanenbaum et al., 1998; Tetel, 2009; Voegel, Heine, Zechel, Chambon, & Gronemeyer, 1996). SRCs physically interact with ER and PR in a ligand-dependent manner (O'Malley, 2006; Oñate et al., 1995; Tetel, 2009) and provide a means of recruiting other co-activators including CREB binding protein (CBP) and p300/CBP associated factor (p/CAF). CBP and p/CAF are each associated with histone acetyltransferase (HAT) activity and further assist in modifying the chromatin structure (Kamei et al., 1996; McKenna, Nawaz, Tsai, Tsai, & O'Malley, 1998).

PR exists as two isoforms: PR-A and PR-B. PR-A is the smaller of the two as it lacks 164 amino acids of the N-terminus (Shao, Markström, Friberg, Johansson, & Billig, 2003). In addition to the difference in size, the proteins are also transcribed from two different promoters located within the same gene in chromosome 11 (Kastner et al., 1990). PR-A and PR-B are capable of homo- or hetero- dimerization upon activation by the binding of progesterone (Humphreys, Lydon, O'Malley, & Rosen, 1997). Studies have also indicated that it is not the expression of one isoform or the other, but the ratio of the two isoforms that may be important for the development of breast cancer. Specifically, a high ratio of PR-A/B has been associated with poorer prognosis and

response to hormone therapy relative to ER+ breast cancers (Hopp et al., 2004). However, breast tumors expressing ER or PR are still associated with a greater chance of survival and overall decreased metastasis while the 15-20% of tumors that express HER2 (HER2 +) are associated with poorer prognosis and lower survival (Bardou, Arpino, Elledge, Osborne, & Clark, 2003; Knutson & Lange, 2014).

HER2 is a key member of the human epidermal growth factor receptor family that also consists of HER1, HER3, and HER4. With the exception of the kinase-dead HER3 receptor, each of these transmembrane family members contain an intracellular kinase domain that can either auto-phosphorylate and activate the receptor upon homodimerization or trans-phosphorylate and activate the other receptor upon heterodimerization (Connell & Doherty, 2017; Liu et al., 2017). Whether homo- or heterodimerization occurs depends on the availability of a variety of extracellular HER-binding ligands also known as heregulins (Mota et al., 2015). Once activated, the receptors activate a spectrum of downstream intracellular signaling cascades that culminate in the nuclear regulation of specific transcription factors and gene expression programs designed to control epithelial cell growth, survival, cell migration and invasion (Harari & Yarden, 2000; Mandal et al., 2001; Omar, Yan, & Salto-Tellez, 2015). When properly regulated and sequentially activated, this spectrum of HER-directed cellular responses are essential for the normal development of epithelial organs like the mammary gland. However, when constitutively activated by the oncogenic amplification and overexpression of the HER2 gene, as may occur in undifferentiated breast, gastrointestinal, bladder or ovarian epithelial cells, a HER2+ cancer develops, most commonly seen in the breast (Harari & Yarden, 2000; Mitri, Constantine, & O'Regan,

2012; Zardavas, Fouad, & Piccart, 2015). While membrane levels of HER1, HER3, and HER4 usually remain unaltered during HER2+ tumor formation, the amplified and overexpressed HER2 drives tumor formation and its progression (Connell & Doherty, 2017). This type of epithelial cancer is routinely clinically recognized as such by HER2 immunohistochemisty (IHC) and/or HER2 fluorescence in fluorescent in situ hybridization (FISH). Within each cell, the overexpressed HER2 membrane protein either homodimerizes with itself or heterodimerizes with the much less abundant HER3 protein. Consequently, even in the absence of any available extracellular ligand (Heregulin), these auto- and trans-phosphorylated HER2 dimers signal constitutively via MAPK and PI3K pathways, as shown in Figure 2, driving the uncontrolled growth and proliferation of that cell as well as stimulating its motility and invasiveness while also inhibiting any other intracellular attempts to activate programmed cell death (apoptosis). This accounts for the clinical aggressiveness and poor prognosis associated with HER2+ breast cancers (Gautrey et al., 2015; Yarden & Sliwkowski, 2001; Zardavas et al., 2015).

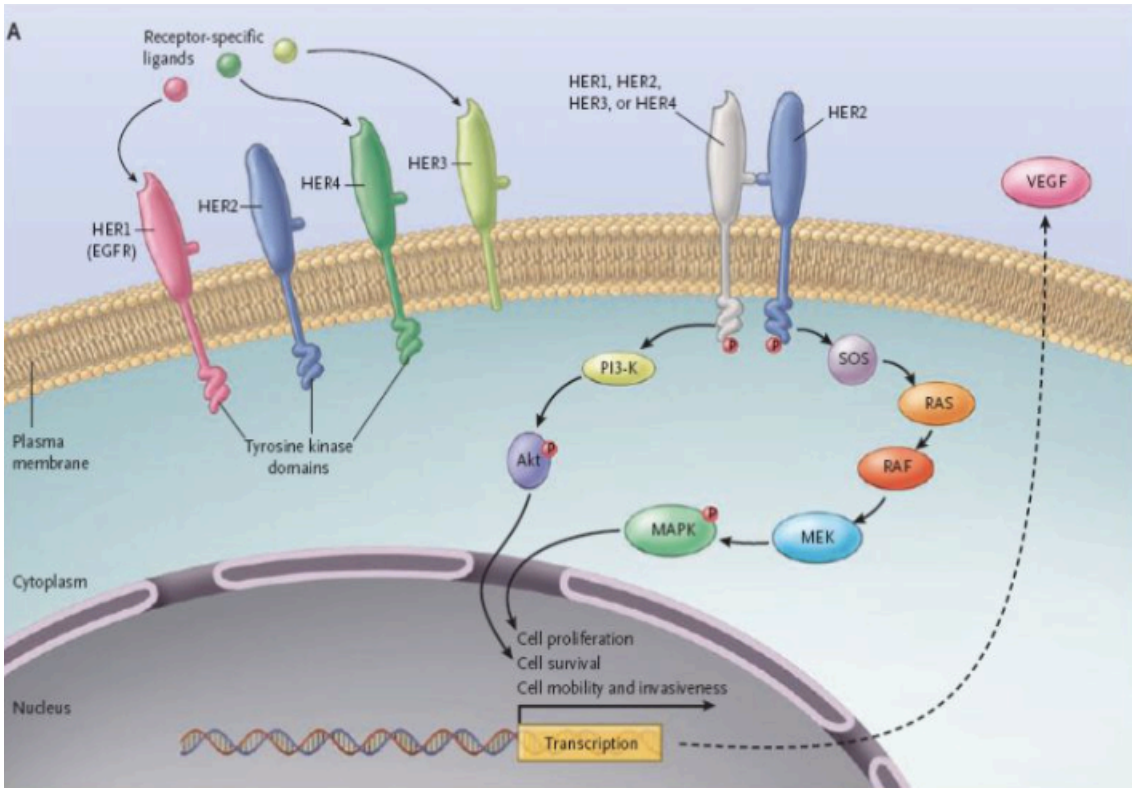


Figure 2. Ligand binding of HER1, HER3, or HER4 induces a conformational change in these transmembrane-tyrosine kinases that facilitate receptor dimerization. Upon activation, homo- or hetero-dimerization, two intracellular tyrosine kinase domains are joined and phosphorylation occurs. Downstream signaling proteins are recruited initiating a series of signaling cascades in pathways such as PI3K or MAPK which can lead to increased cell proliferation and cell survival. (Hudis, 2007)

Classifications of breast cancer

Breast cancer is classified clinically and at the molecular level based on the expression (or lack thereof) of three different breast cancer biomarkers: estrogen receptor-α (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2 or ERBB2) (Knutson & Lange, 2014; Manna & Holz, 2016; Patani, Martin, & Dowsett, 2013). In conjunction with mRNA expression (transcriptome) profiling, these three biomarkers define four primary intrinsic subclasses of breast cancer, as shown in Figure 3: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+,

HER2+), HER2-enriched (ER- and PR-, HER2+) and basal-like (ER-, PR- and HER2-) breast cancers (Cheang et al., 2008; Sørlie et al., 2001). Each of these four biologic subclasses of breast cancer is clinically distinct with respect to both patient prognosis and treatment approach. Since the focus of this study is solely on HER2+ breast cancers (whether or not they overexpress ER or PR), further description below will be confined to the molecular characteristics defining all HER2+ breast cancers.

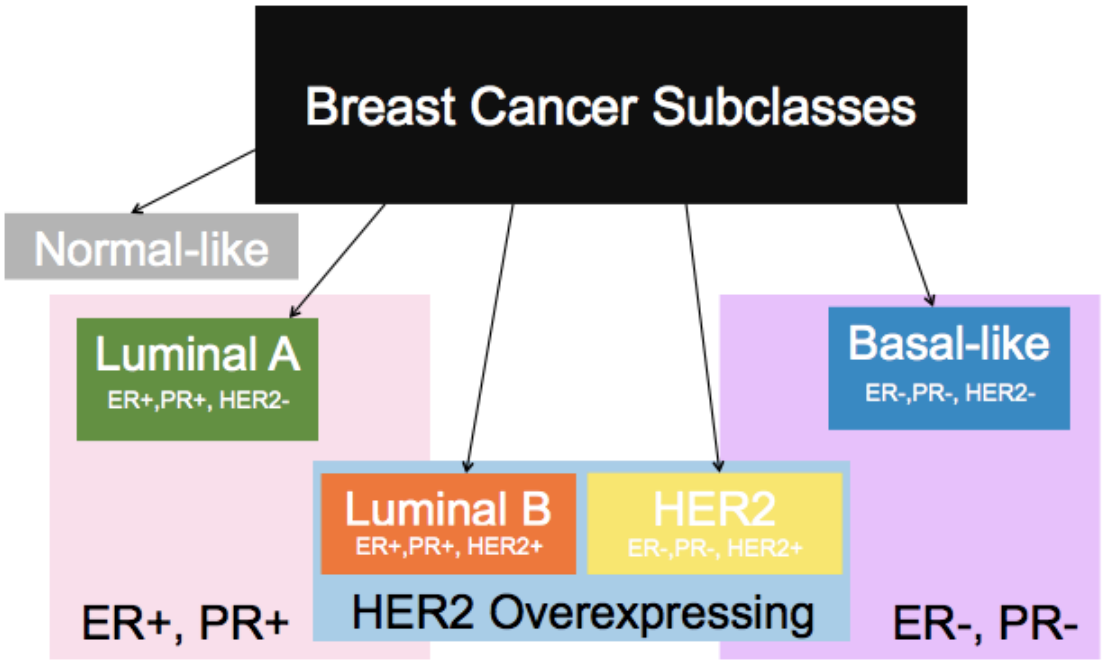


Figure 3. Diagram representation of the four primary molecular subclasses of breast cancer defined by overexpression (or not) of the three breast cancer biomarkers: ER, PR, HER2. Based on transcriptome subtyping, tumors overexpressing ER or PR but without HER2 overexpression may also be called Luminal A breast cancers. Those overexpressing HER2 (HER2+) may also be subtyped as either Luminal B (ER or PR+, HER2+) or HER2-enriched (ER and PR-, HER2+). Those lacking overexpression of ER, PR or HER2 may also be called basal-like breast cancers. Very rarely, breast tumors lacking overexpression of ER, PR or HER2 also appear to have a transcriptome most like that of normal breast epithelium, and may be referred to as normal-like breast cancers. The current study uses a cell line model (SKBR3) with biomarker and molecular properties of HER2-enriched breast cancers.

Current HER2-positive breast cancer therapeutics

HER2-positive breast cancers make up about 20% of all newly diagnosed breast cancers which is commonly studied using a cell line model (such as SKBR3) with biomarker and molecular properties of HER2-enriched breast cancers. Before the advent of HER2-targeted therapeutics in 1998, these were among the most aggressive and life-threatening of all breast cancers because of the subcellular mechanisms by which the amplified and overexpressed HER2 receptor induces breast tumor formation and drives its progression and metastatic potential (Carey et al., 2014; Howlader et al., 2014; Yersal & Barutca, 2014).

In 1998, the first HER2-targeted therapeutic, the monoclonal antibody Trastuzumab (Herceptin), became FDA-approved and forever changed the clinical prognosis and outcome of women suffering from advanced HER2+ breast cancers (Jeyakumar & Younis, 2012; Nahta & Esteva, 2006; Roche & Ingle, 1999). This first ever anti-HER2 therapeutic was followed several years later by a second FDA-approved anti-HER2 monoclonal antibody, Pertuzumab. While Trastuzumab binds to the receptor and impairs its ability to transduce downstream signals (Park, Neve, Szollosi, & Benz, 2008), Pertuzumab binds to a different part of the HER2 extracellular domain and prevents its dimerization (Harbeck et al., 2013; Le, Pruefer, & Bast, 2005). When used together, Trastuzumab and Pertuzumab provide a more comprehensive signaling blockade (Nahta, Hung, & Esteva, 2004; Scheuer et al., 2009; Swain et al., 2015). Today, these two monoclonal antibodies (both developed and marketed by Genentech) are not only commonly used together but also in combination with specific chemotherapeutic agents like taxanes in order to most effectively treat early or advanced stages of HER2+

breast cancers, dramatically improving the survival of HER2+ breast cancer patients (Swain et al., 2015; Tian, Ye, & Zhou, 2017).

Not withstanding these latest therapeutic developments and clinical advances, some early and many late-stage HER2+ breast cancers still fail to respond to any available HER2-targeted agent or their therapeutic combination with chemotherapy. This is even despite the fact that these same treatment-resistant tumors remain dependent on the constitutive signaling activity of the overexpressed membrane HER2 receptor and its intracellular downstream growth promoting consequences (Park et al., 2008), necessitating a search for newer therapeutic approaches to interrupt those critical oncogenic mechanisms driven by HER2. Toward this goal, the Benz Lab has long been exploring alternative interventions that would either silence the amplified and overexpressed HER2 gene or block its most downstream growth promoting effects on the cell's protein translational machinery. During a broad drug screening approach against HER2+ breast cancer cell lines to identify small, drug-like molecules capable of silencing HER2 gene expression, a top hit in this screen was found to be trichostatin-A (TSA), a broad-based inhibitor of histone deacetylases (HDACs). TSA was also shown to not only repress the nuclear transcription of HER2 but also to accelerate the decay of its cytosolic transcripts (Scott et al., 2008). TSA-like inhibitors of all HDAC classes (referred to as pan-HDACi) first emerged over 20 years ago as a new class of epigenetic, antineoplastic agents thought to primarily impact chromatin structure and function, with the first of these FDA approved agents showing potent clinical activity against various hematopoietic malignancies but thought to have little clinical potential against common solid tumor malignancies like breast cancer (Falkenberg & Johnstone, 2014).

HDACs and their inhibitors as anti-cancer agents

HDAC inhibitors (HDACi) are chemical compounds that impede the enzymatic activity of HDACs, which affect chromatin structure and gene expression that is more broadly and epigenetically regulated by post-translational enzymatic modifications to chromatin proteins like histones; these protein modifications include methylation, phosphorylation, ubiquitination, sumoylation, and acetylation. Collectively, these epigenetic protein modifications regulate chromatin condensation and control gene transcription (Rivera et al., 2017). With specific regard to histones, HDACs displace the ε-N-acetyl lysines of a given histone, allowing it to compact the chromatin structure around a gene and alter access to transcription factors (as illustrated in Figure 4), changing the expression of various genes, some of which may be driving tumor growth. Also shown in Figure 4 is how histone acetytransferases (HATs) are involved in the reversible process by catalyzing the neutralization of the positive charge of lysine residues, thus relaxing the chromatin and subsequently enabling the activation of gene transcription (Garmpis et al., 2017).

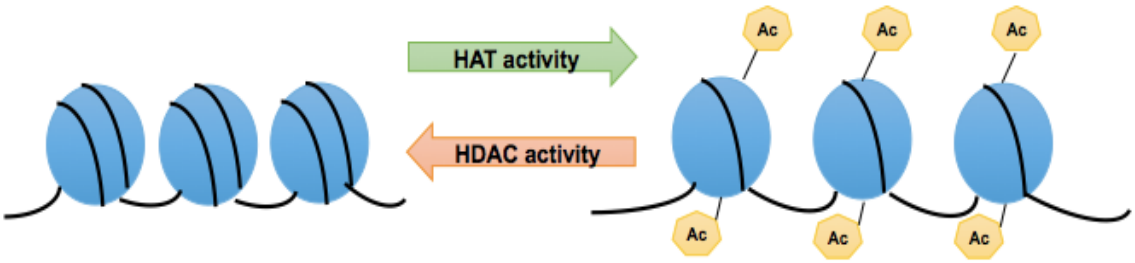


Figure 4. Transcription is modulated by the reversible effects of HAT and HDAC activities. Following HDAC activity the chromatin is compacted, and acetylation occurs after HAT activity thus loosening the chromatin.

This epigenetic approach targets the heritable patterns in gene expression not attributed to changes in DNA sequence (Ramachandran, Ient, Göttgen, Krieg, & Hammond, 2015), and epigenetic approaches are attracting more attention because of their amenability to pharmacological intervention. Epigenetic modifications are potentially reversible as demonstrated by the acetylation/deacetylation in Figure 4, unlike genetic mutations, which almost never can be reversed (Wagner, Hackanson, Lübbert, & Jung, 2010).

Anti-tumoral mechanisms of some HDAC inhibitor result from their acetylation of both histone and non-histone proteins. This can lead to arresting the eukaryotic cell cycle, by altering the expression of regulators within the cell cycle, inducing cell differentiation, or activating the cell death program known as apoptosis (Yoshida et al., 2001). HDAC inhibitors can increase expression of pro-apoptotic genes as well as decrease expression of anti-apoptotic genes, down-regulate other genes that promote angiogenesis and metastasis, or alter expression of DNA repair genes (Thomas, Miller, Thurn, & Munster, 2011).

HDAC inhibitors (HDACi) can be classified as either pan-HDAC inhibitors or class-selective HDAC inhibitors. A class-selective HDAC inhibitor targets a specific class of HDACs, whereas a pan-HDAC inhibitor targets all classes of HDACs (Eckschlager, Plch, Stiborova, & Hrabeta, 2017). There are currently four classes of HDACs and a total of eighteen HDACs identified in humans. However, Class III HDACs are usually considered separate from the classes of "classical" HDACs because they are structurally dissimilar and depend on a different co-substrate. Class I, Class IIa, and Class IIb, and Class IV HDACs all require Zn^{2+} as a cofactor and are considered the

classical HDAC classes, as illustrated in Figure 5. Class I HDACs are generally found in the nucleus, whereas class II HDACs are localized in both the nucleus and the cytoplasm (Dokmanovic et al 2007). What is not pictured in Figure 5 is Class III HDACs, often referred to as "the sirtuins." Rather than being dependent on Zn^{2+}, the sirtuins are NAD^{+} - dependent; in yeast they are referred to as silent information regulator-2 (SIR2) protein homologs (Eckschlager et al 2017, Tang et al 2013).

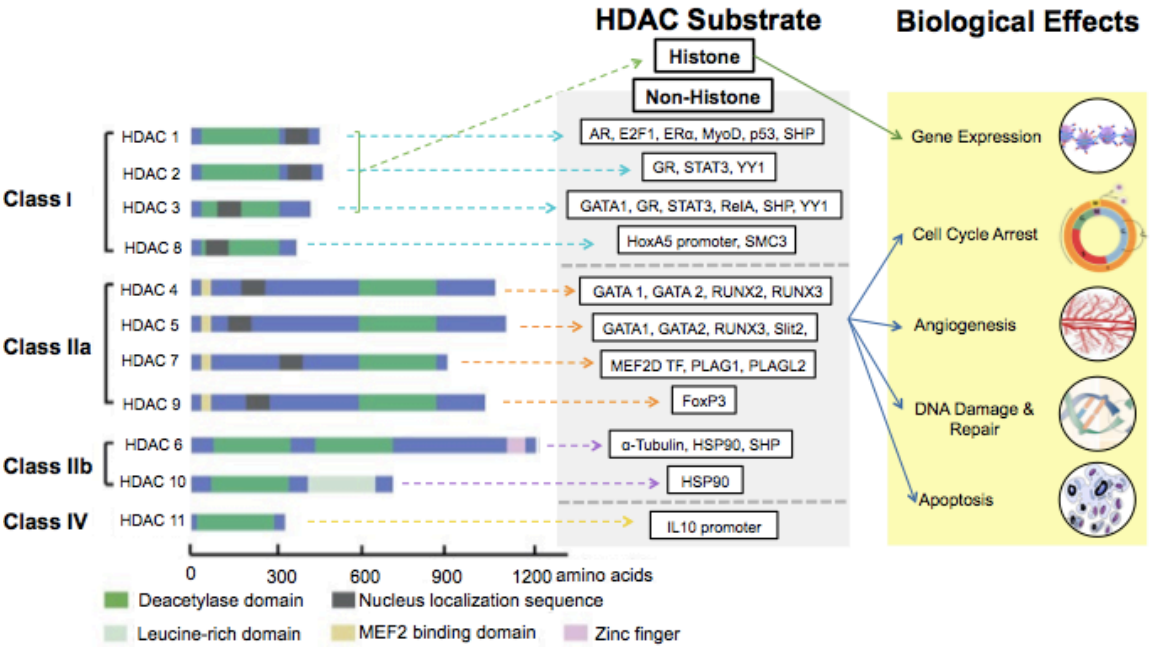


Figure 5. The classical HDACs are subdivided by their binding domains into 4 classes. Their various substrates ultimately yield different biological effects. (Adapted from Chun, 2015; Wang, Qin, & Yi, 2015).

HDAC inhibitor mechanism of action often involve the HDACi chelating the zinc cofactor and thereby blocking HDAC's catalytic role. HDAC inhibitors can be divided into four distinct chemical classes: hydroxamic acids or hydroxamates (e.g. vorinostat, Trichostatin-A, panobinostat), benzamides (e.g. entinostat, tacedinaline), cyclic tetrapeptides (e.g. depsipeptide/romidepsin), and short chain fatty acids or aliphatic acids

(sodium butyrate, valproic acid) (Eckschlager et al., 2017; Falahi, van Kruchten, Martinet, Hospers, & Rots, 2014; Garmpis et al., 2017). In addition to their chemical properties, HDACi can also be divided by their selectivity in targeting specific HDAC classes: pan-HDAC inhibitors versus class-selective HDAC inhibitors.

Notable differences between the pan-HDAC inhibitors and class-selective HDAC inhibitors are their isoform selectivity and efficacy against certain diseases, as well as their overall HDACi potency (Gryder, Sodji, & Oyelere, 2012). Even though pan-HDAC inhibitors are more potent, class-selective HDAC inhibitors have a more restricted scope of activity and are therefore less toxic, often yielding a higher therapeutic index. US Food and Drug Administration (FDA) approved and clinically used HDAC inhibitors currently include pan-HDAC inhibitors like sodium butyrate, valproate, vorinostat, and class-selective HDAC inhibitors like entinostat, and romidepsin. In contrast, the well-studied pan-HDACi, trichostatin-A, remains a prototypic drug used solely for investigational studies due to its poor bioavailability (Glozak & Seto, 2007). Earliest approvals by the FDA of HDACi against human malignancies focused on a pan-HDAC inhibitor with hydroxamic acid-like properties: suberanilohydroxamic acid (vorinostat), for the treatment indication of cutaneous T-cell lymphoma, as well as the class I-selective HDAC inhibitor, romidepsin, for the same clinical indication. Since then, other pan-HDACi such as panobinostat have become FDA approved for use against other types of hematopoietic malignancies such as multiple myeloma; but, apart from entinostat, no other HDACi has yet been approved for the treatment of other epithelial cancers (Barbarotta & Hurley, 2015; Garmpis et al., 2017; Libby et al., 2015; Mann et al., 2007).

Phase I and phase II clinical trials with many different kinds of HDACi have been and continue to be conducted in patients with many different kinds of malignancies, including breast cancer. These commonly reveal that HDAC inhibitors induce only minor adverse side effects in most patients, but as single agents, produce beneficial anticancer effects that are mostly limited to patients with specific hematopoietic malignancies like T-cell lymphomas and multiple myeloma (Barbarotta & Hurley, 2015; Mann et al., 2007; Woo, 2016). Preclinical studies suggest that HDACi may be most effective against solid tumors when administered in combination with standard cancer therapeutics. For example, the combination of vorinostat with the approved chemotherapeutic, oxaliplatin, synergistically induced cell cycle arrest during the G2 and M phase, induced apoptosis, and showed a decrease in hepatic tumorigenicity in both *in vitro* and *in vivo* settings (Liao, Zhang, Sun, & Jiang, 2018). In breast cancer preclinical studies, several different HDACi have been tested *in vivo* and *in vitro* and demonstrated single agent effectiveness at arresting cancer cell growth and development, inducing cellular differentiation and apoptosis (Falahi et al., 2014). Breast cancer studies have also shown that HDACi co-administered with radiotherapy or other cytotoxic agents may produce synergistic anticancer activity especially against ER+ breast cancer models. To date, however, the only HDACi that has received FDA approval for use in breast cancer patients is entinostat, although breast cancer clinical trials evaluating other HDACi, alone and in combination with hormonal agents and chemotherapeutics continue (Garmpis et al., 2017; Jones, Issa, & Baylin, 2016; Marks, Olson, & Fernandez-Zapico, 2016).

HDAC inhibitors and mechanisms mediating HER2 mRNA stability

While preclinical *in vitro* and *in vivo* studies indicate that HDAC inhibitors can induce significant rapid decay of oncogenic transcripts like HER2 mRNA, the exact mechanism underlying this effect remains unknown. Earlier observations in the Benz lab suggested that stability of HER2 mRNA depends largely on proteins and microRNAs (miRs) binding to the 3' untranslated region (UTR) of HER2 mRNA while the transcript is polysome-associated and being translated to protein. Highlights from those early findings include the following: 1). HDACi inducible miR-125a/b binds specifically within the 3'UTR of HER2 mRNA and induces its decay (Scott et al., 2008). 2). A small basic protein, HuR, also regulates HER2 mRNA stability by binding to the uracil-rich region of its 3'UTR (Scott et al., 2008; Yi et al., 2009). 3). A subclass of HDAC inhibitors can destabilize HER2 mRNA almost as effectively as pan-HDACi, suggesting the mechanistic involvement of isotype-selective HDAC in stabilizing HER2 mRNA and the need to employ isotype-selective HDACi (Scott et al., 2008). Figure 6 provides a summary schematic illustrating both known and proposed mechanisms by which HDACi are capable of inducing the rapid decay of HER2 mRNA.

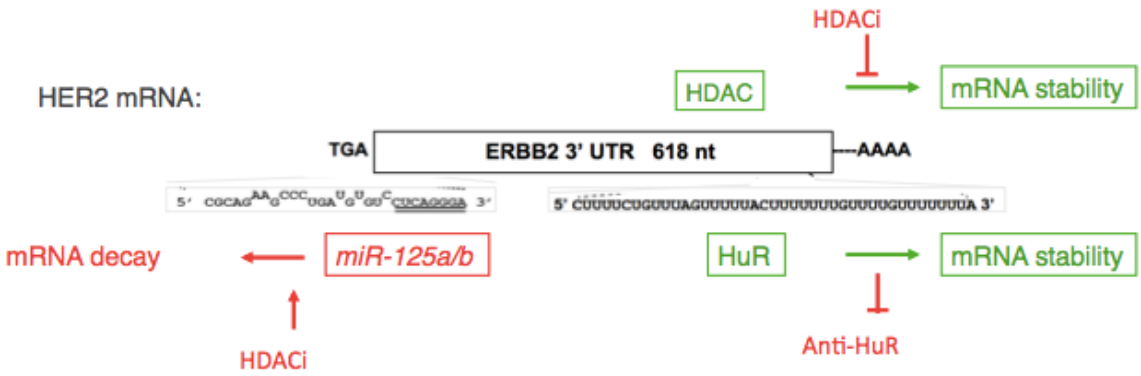
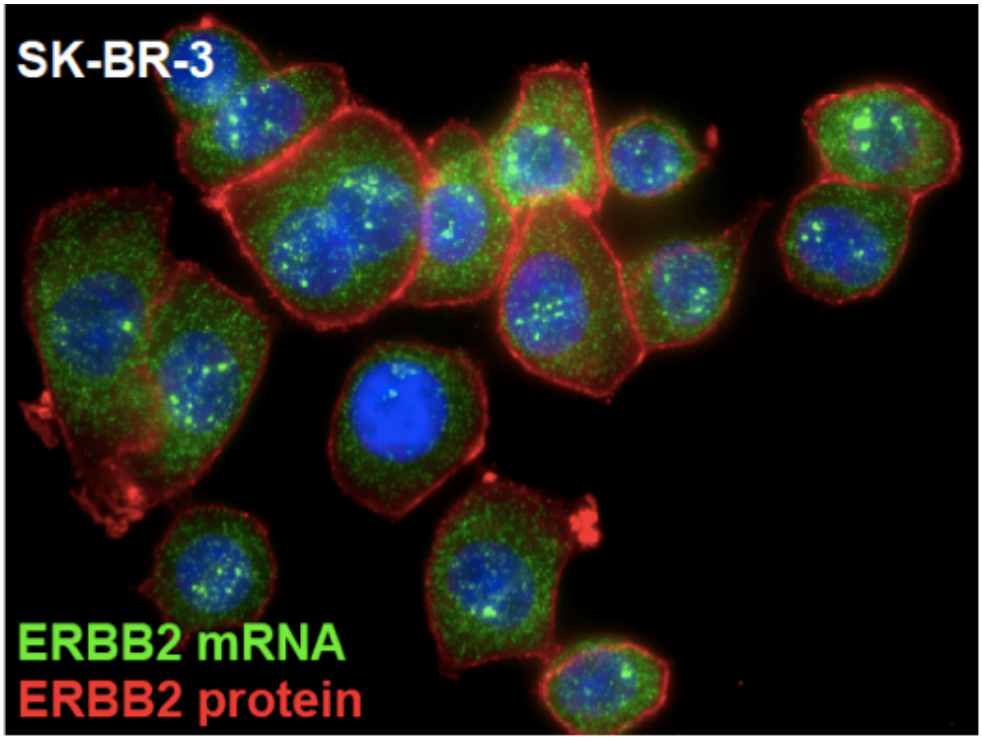


Figure 6. Three factors regulating HER2 mRNA stability include microRNA miR-125a/b which regulates HER2 mRNA stability upon treatment with HDACi as shown in the 5' direction of the HER2 mRNA. HDACi also targets a specific HDAC and regulates the overall mRNA stability as shown in the top right. The 3' end of the mRNA has a uracil-rich region. The protein HuR is located in that U-rich region and plays a role in regulation.

From past Benz Lab studies, we know that non-specific pan-HDACi treatment of HER2+ breast cancer cell line models, like SKBR3, rapidly induce HER2 mRNA decay and cause breast cancer growth arrest and apoptosis. Figure 7 shows that TSA can visibly reduce both nuclear and cytoplasmic HER2 mRNA within 6 h of administration to SKBR3 cells (Orjalo Jr., Johansson, & Ruth, 2011). What remains to be determined is whether this transcript stability mechanism requires a specific class or isotype of HDAC and if an isotype-selective HDACi can induce HER2 mRNA degradation as effectively as a pan-HDACi, like TSA.



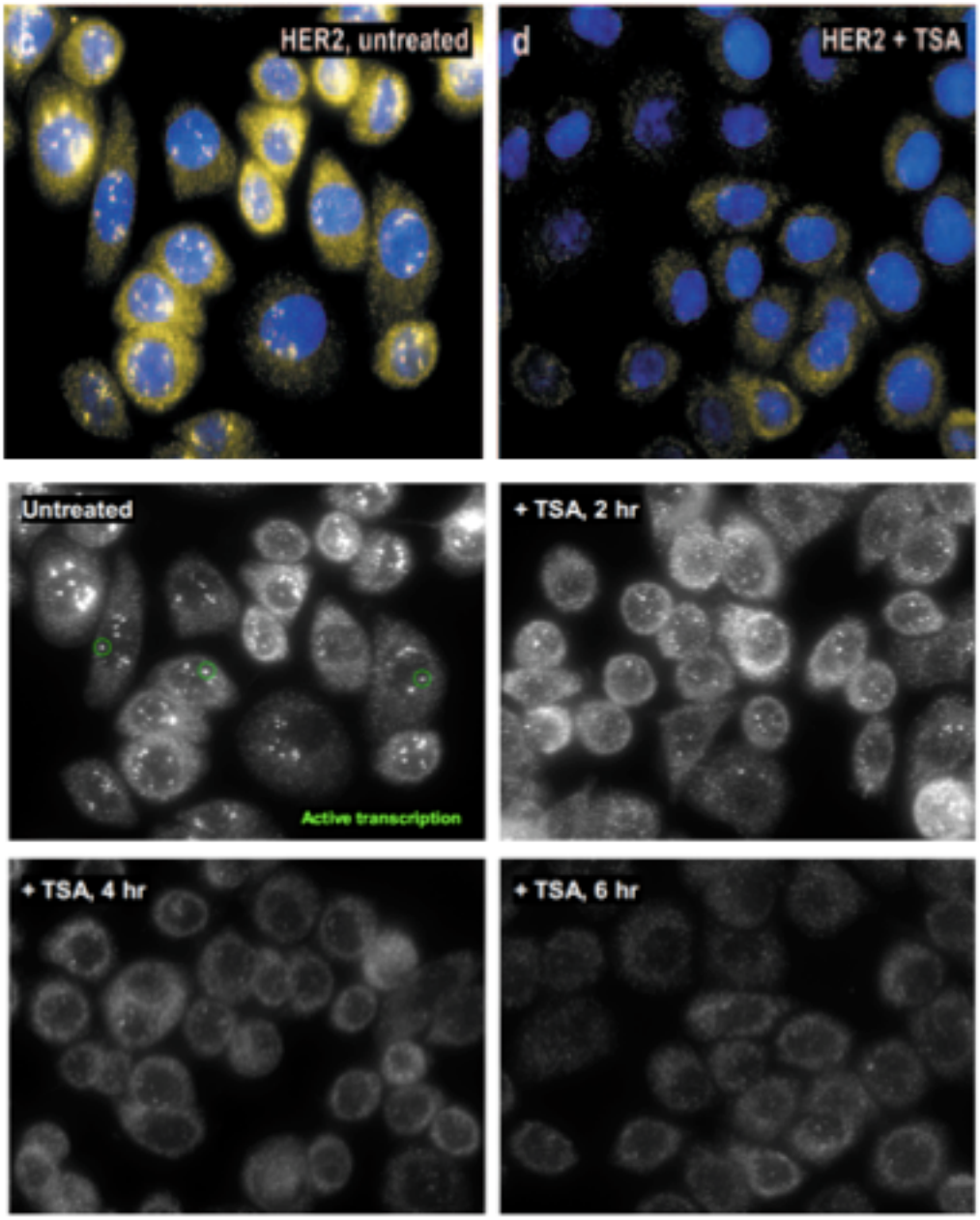


Figure 7. The images show HER2+ breast cancer cell line, SKBR3. HER2 (ERBB2) is being overexpressed in both the nucleus and the cytoplasm (upper panel). The panel on the bottom demonstrates SKBR3 cells that are untreated versus cells treated with 1 μ M TSA from 2-6 hours. In just 6 hours, the mRNA decay is clearly visible. (Orjalo Jr. et al., 2011).

Figure 8 outlines an experimental flow chart to accomplish my project's objective and to not only find a target HDAC and its selective inhibitor as effective as TSA in

causing HER2 mRNA decay but also to further understand how the HDAC acetylation substrate plays a role in HER2 mRNA stability during translation. We believe that the HDAC mediated and translation-associated HER2 mRNA stability mechanism is either polysome- or ribosome-associated; therefore, we will look for the HDAC(s) and their acetylated substrates of interest by polysome profiling, using cell fractions containing ribosomes and polysomes (ribopellet) before and shortly (2-6 h) after HDACi treatments. Polysome profiles provide a visual representation of where translational activity is taking place (Figure 9). For example, pre-ribosomal activity would precede the 40s ribosome peak, while anything between the 40-80s peaks would be ribosome associated and anything following the 80s peak would be polysome associated.

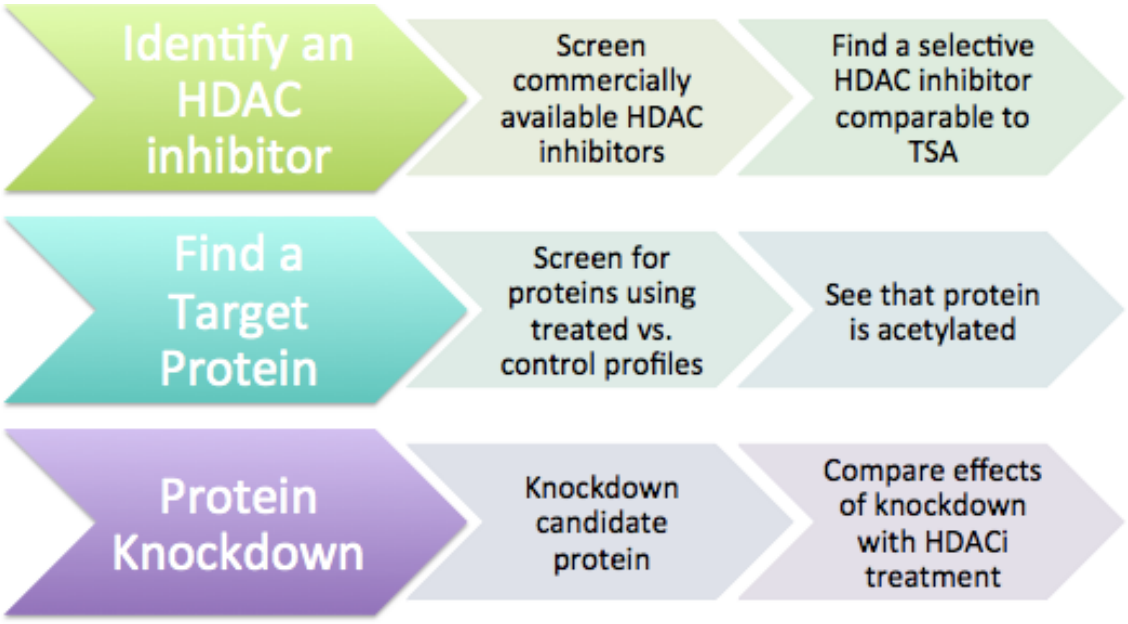


Figure 8. Outline of project objectives.

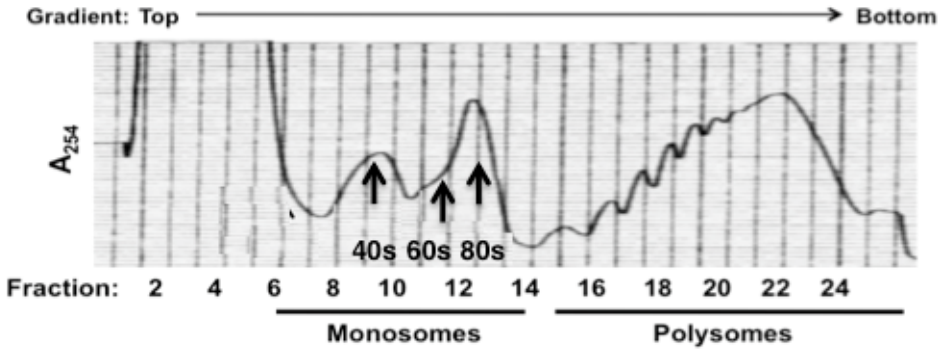


Figure 9. The UV visual graph shows a 40s peak, 60s peak, and the 80s peak (the sum of 40s and 60s peaks) in the ribosomal region and what the peaks would look like past the 80s into the polysome region. The fraction numbers would correlate to the fractions collected in the polysome profiling process.

HDACi treatment is expected to leave a candidate substrate of interest acetylated on one or more of its lysine (K) residues. Having identified acetylated substrate candidates of interest, that protein will be knocked down by siRNA in order to confirm that it is playing a mechanistic role in the HDACi induced decay of HER2 mRNA. If HDACi induced acetylation of a candidate protein is associated with its loss of function, then knock down of that protein by siRNA will simulate the HDACi induced decay of HER2 mRNA. If its acetylation is associated with any gain or function, or no change in function whatsoever, then knockdown of the protein in the absence of HDACi treatment will not affect HER2 mRNA stability. HER2 mRNA stability will be assessed by Northern blotting or RT-PCR measurement of HER2 mRNA levels relative to the housekeeping gene, GAPDH. By 24 hours, destabilization of HER2 mRNA will also lead to loss of HER2 protein expression and this will be measured by Western blotting.

Specific Aims

Based on the observation that the pan-HDAC inhibitor TSA promoted the accelerated decay of HER2 mRNA, it is hypothesized that there is a class-selective HDAC inhibitor that will mediate this HER2 mRNA decay. In addition, it is hypothesized that the class-selective HDAC inhibitor mediating the HER2 mRNA decay will be present in either pre- or post-polyribosomal fractions with acetylated protein(s) responsible for mediating HER2 mRNA decay. These hypotheses will be experimentally examined by completing the following specific aims:

Specific Aim 1: Examine the effect of a various class-selective HDAC inhibitors for their ability to promote HER2 mRNA decay as assayed by Northern blot analysis.

Specific Aim 2: Examine pre- and post-polyribosome fractions extracted from treated (vs. control) HER2-positive breast cancer cell lines (e.g. SKBR3) for acetylated protein resulting from HDAC inhibitor treatment that potentially mediate the decay of HER2 mRNA then use mass spectrometry (MS) to identify acetylated protein(s).

Specific Aim 3: Use siRNA technique to knockdown in breast cancer cells (e.g.SKBR3) expression of protein candidate(s) to evaluated their impact on HER2 mRNA decay using both Northern blot analysis and RT-PCR to assess HER2 mRNA levels.

As mentioned in the specific aims, a class-selective HDAC inhibitor comparable to TSA has to first be identified. Once there is a candidate HDAC inhibitor, its substrate and class selectivity will be evaluated using Western blot techniques and polysome profiling. Localization within the nucleus and in the cytosol will also be examined using

immunohistochemistry. A comprehensive list of the commercially available HDAC inhibitors that will be studied is available in Figure 10.

HDAC Inhibitor	Type of HDACi	Target
Acetylon 775 (ACY-775)	Class Selective (IIB)	HDAC 6
Acetylon 1035 (ACY-1035)	Class Selective (I)	HDAC 1 - HDAC 3
Acetylon 1215 (ACY-1215)	Class Selective (II B)	HDAC 6
Entinostat	Class Selective (I)	HDAC 1 - HDAC 3
Romidepsin (FK228)	Class Selective (I)	HDAC 1 & HDAC 2
Trichostatin A (TSA)	Pan (I & II)	
Tubacin	Class Selective (II B)	HDAC 6
Vorinostat	Pan (I & II)	

Table 1. This table lists the commercially available class-selective HDAC inhibitors targeting a spectrum of HDACs. TSA is a baseline reference pan-HDAC inhibitor that the other HDACs will be compared to. Vorinostat has no significance in this project but was examined simply because it was the first clinically available HDAC inhibitor.

Materials and Methods

Cell Culture. SKBR3 HER2-positive human breast cancer cell line was obtained from American Type Culture Collection for use in this study. The SKBR3 cells were cultured in McCoy's medium (Cellgro, Manassas, VA) containing 10% fetal bovine serum (VWR-international, Randor, PA) and 1% penicillin/streptomycin (Cellgro, Manasass, VA) under 5% CO_2 at 37°C.

Cell Treatment. SKBR3 cells were treated with commercially obtained trichostatin-A (TSA) (Sigma-Aldrich, St. Louis, MO) or commercially obtained romidepsin (FK228) (Sellekchem, Houston, TX) for 2 hours, 4 hours, or 6 hours. Concentrations of 40 nm to 1.5 μM were used to treat the cells. SKBR3 cells were also treated for 6 hours at 1μM using the following: ACY-1035, ACY-775 (2-((1-(3-fluorophenyl)cyclohexyl)amino)-*N*-hydroxypyrimidine-5-carboxamide), ACY -1215 [2-(diphenylamino)-N-(7-

(hydroxyamino)-7-oxoheptyl)pyrimidine-5- carboxamide] were synthesized by ChemPartner (Shanghai, China) and obtained from Acetylon Pharmaceuticals (Boston, MA), while Entinostat (MS-275) was obtained from Sellekchem (Houston, TX)

Cell Harvest. Control SKBR3 cells as well as drug-treated SKBR3 cells were harvested by douncing in a hypotonic buffer comprised of 0.05M HEPES (pH 7.4), 0. 1 M KCl, 0.03% Tergitol-type NP-40, 0.01 M $MgCl_2$, 100 units/mL of SUPERasein (Ambion, Grand Island, NY), and mini-complete protease inhibitors (Roche Diagnostics, Nutley, NJ). Cell lysates were centrifuged at 13,000 rpm for 5 minutes to remove nuclear and insoluble components with the remaining supernatant comprising the cytoplasmic lysate fraction.

Polysome Profiling. Polysome profiling was performed using the cytoplasmic lysate obtained as described above. The samples were layered on top of a sucrose gradient (10% sucrose, 20% sucrose, 30% sucrose, 40% sucrose and 50% sucrose) as illustrated by the schematic illustration of Figure 11. The samples were then centrifuged at 38,000 rpm for 2 hours at 4°C using the Beckman Optima XL-100K Ultracentrifuge (Fullerton, California). Fractionation was performed on the gradients using Biocomp Gradient Station (model 153, Wolflabs, Pocklington, England), and fractions were collected by the Gilson FC203B Fraction Collector in tandem with the BioRad Econo UV Monitor, creating UV graphs of the samples.

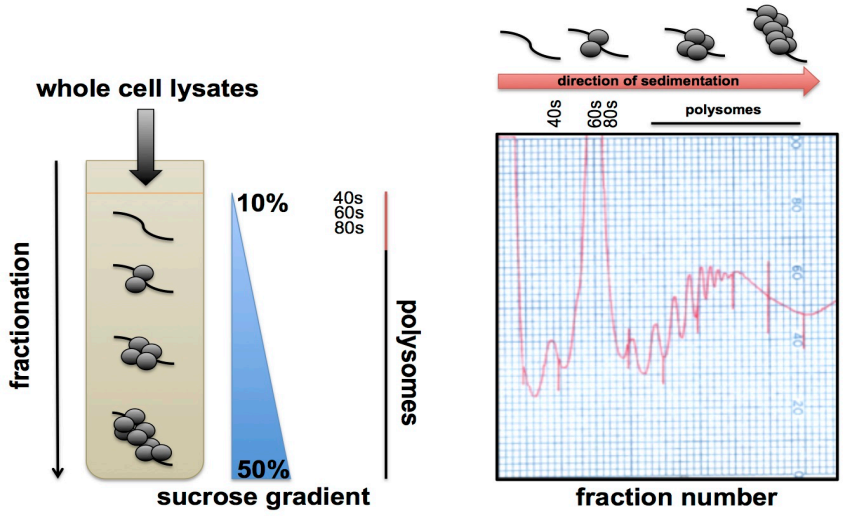


Figure 10. Schematic representation of polysome profiling utilizing a continuous sucrose gradient and the UV/Vis graph correlating to obtained fractions. (Adapted from Abdelmohsen, 2012).

Western Blot Analysis. Lysates (either from whole cell or cytoplasmic fractions) from control or treated SKBR3 cells were boiled in sample loading buffer (1% SDS, 20% glycerol, 100 mm DTT, and 50 mm Tris, pH 6.8) and electrophoresed using 4-12% Bis-tris precast polyacrylamide gels (Invitrogen) with lanes loaded with a constant 15 µg of protein. Proteins were transferred onto membranes (Immobilon-P, Millipore), and the protein-bound Immobilon-P membranes (Amersham Bioscience, Pittsburgh, PA) were hybridized with a primary antibody in TBS (50 mM Tris pH 7.5, 100 mM NaCl and 0.01% Tergitol-type NP-40 and supplemented with 4% non-fat milk powder) followed by a horseradish peroxidase-conjugated secondary goat anti-mouse affinity purified antibody (Sigma Chemical Co.). Primary antibodies used: anti-ERBB2 (Calbiochem, San Diego, CA), anti-β-actin (Abcam, Cambridge, MA), anti-α-tubulin (Sigma), anti-acetylated α-tubulin (Sigma), anti-K12-acetylated H4, anti-HDAC1, anti- HDAC2, and anti-RACK-1

(Cell Signaling Technology, Danvers, MA**)**. Final primary antibody concentrations were set at 0.2 ug/ml.

Immunohistochemistry (IHC). The SKBR3 cells were fixed for 10 minutes using 4% paraformaldehyde, blocked using 5% goat serum in PBS containing 0.3% Tergitol-type NP-40, and then incubated with mouse monoclonal anti-HDAC1 primary antibody (Santa Cruz Biotechnology), followed by anti-mouse Alexa 488 secondary antibody (Invitrogen). DAPI (Sigma –Aldrich) was used for nucleus staining. Cells were imaged using LSM 510 NLO Confocal Microscope Scanning System that was perched on Axiovert 200 Inverted Microscope (Carl Zeiss Limited, Cambridge, UK) and a dual-photon Chameleon laser (Coherent Inc., Santa Clara, CA). Confocal images were obtained using a 40x objective and a 4x digital zoom.

SYPRO Ruby Protein Gel Stain. Fixation was performed using 100 ml of 50% methanol and 100 ml of 7% acetic acid for 30 minutes. Staining was done in polypropylene containers using 60 ml of SYPRO Ruby gel stain (Sigma Aldrich, St. Louis, MO) overnight. The gel was washed with 100 ml of 10% methanol and 7% acetic acid for 30 minutes. All fixations, staining and washing was done gently on an orbital shaker kept at 50 rpm. The gel was imaged and then passed to the mass spectrometry core at the Buck Institute for Research and Aging (Novato, CA) for analysis.

RNA Isolation. RNA from SKBR3 cells was isolated using TRIzol (Invitrogen) using the manufacturer's standard protocol and quantified using NanoDrop2000 spectrophotometer (Thermo Scientific).

Northern Blot Analysis. 15 μg of total RNA per lane was electrophoresed on 1% agarose-formaldehyde gels and transferred onto Hybond Plus membranes (Amersham, Piscataway, NJ). The membranes were UV cross-linked, hybridized using ExpressHyb (Clontech) at 68°C using P^{32} labeled cDNA probes for HER2 or GAPDH and washed at 64°C in 0.2× SSC and 0.5% SDS. The hybridization bands were visualized by autoradiography, and the ratios of HER2 to GAPDH were calculated using the program ImageJ freely available from the NIH (https://imagej.nih.gov/ij/).

Reverse Transcriptase PCR (RT-PCR). RNA was reverse transcribed by oligo dT priming using SuperScript II reverse transcriptase (Invitrogen) in 20 μl volumes, according to manufacturer's instructions. 2 μl aliquots of the RT reaction were then used for PCR reactions to quantify HER2 and GAPDH levels using HER2 forward and reverse primers and GAPDH forward and reverse primers described below. The HER2 specific primer pair was designed to amplify a 137 nt sequence within the HER2 3′ UTR, upstream of the terminal U-rich sequence (465–505 nt). The primers used were: HER2 (137 bp amplimer) forward: 5′GGTACTGAAAGCCTTAGGGAAGC 3′ and reverse: 5′ACACCATTGCTGTTCCTTCCTC 3′; for GAPDH (234 bp amplimer) forward: 5′ CGAATTGGCTACAGCAACAGG 3′ and reverse: 5′ GTACATGACAAGGTGCGGCTC 3′. PCR reactions were performed with Phusion

(New England BioLabs, Ipswich, MA) following the manufacture's recommend guidelines. Denaturing was done at 95°C for 60 seconds. Annealing was performed at 70°C for 20 seconds. Extension was done at 72°C for 20 seconds. Final extension was performed at 72°C for 60 seconds for a total of 27 cycles and finally held constant at 4°C. PCR samples were electrophoresed using 8% polyacrylaminde TBE gels (Invitrogen) and then stained with ethidium bromide for UV visualization.

Small interfering RNA (siRNA). Validated siRNA reagents (and associated control siRNAs) specific for HDAC1, HDAC 2, and p300 (Dharmacon, Lafayette, CO) were commercially obtained. Equal number of cells were plated and cells were transfected with the various siRNAs at a final concentration of 50 nM using liptofectamine 2000 (Invitrogen) in complete serum medium for 7 hours. After transfection, cells were incubated for an additional 72 hours prior to RNA and/or protein extraction. Transfections were repeated three times to assure consistency of results.

Immunoprecipitation (IP). Immunoprecipitation using various polysome fractions were typically performed using 7 µg of the immunoprecipitating antibody (either acetylated lysine or p300) at 4°C for 6 hours where approximately 12 µl of protein G sepharose beads (GE Healthcare) were added to precipitate the antibody-antigen complexes. Spun down beads were extracted with 0.3 mls Trizol (ThermoFisher Waltham, MA) and the RNA isolated in 20 µl H2O according to manufacturer's protocol.

Results

Identifying class-selective HDAC inhibitor that induces HER2 mRNA decay

An initial screening was done to find a class-selective HDAC inhibitor with HER2 mRNA decaying effects comparable to TSA. SKBR3 cells were treated with pan-HDAC inhibitor Trichostatin A (TSA) and class-selective HDAC inhibitors: Acetylon 1215 (AC-1215), Entinostat (Ent), Entinostat mixed with Acetylon 775 (AC-775), and Romidepsin (FK228). All HDAC inhibitors were administered at 1 μM for six hours. Following the treatment period, RNA from the various treatment conditions was isolated, with RNA from untreated SKBR3 cells serving as control RNA. Northern blot analysis was then performed to determine the effects of these various commercially available HDAC inhibitors on HER2 mRNA levels (Figure 12). When the Northern blot was probed with a ^{32}P-labeled HER2 DNA probe, the effect of each HDAC inhibitor's ability to degrade mRNA message of HER2 was clearly determined. Normalizing HER2 mRNA expression levels to the housekeeping gene GAPDH, Figure 12 indicates that of the various HDAC inhibitors tested, only the class I selective inhibitor FK228 was able to induce HER2 mRNA decay to a degree comparable to TSA. Specifically, TSA, the most potent HDAC inhibitor, decreased HER2 message levels by 87% while FK228 produced a relatively comparable 82% decrease in HER2 mRNA levels (Figure 12). The combination of Entinostat/AC-775 had a HER2/GAPDH ratios of 45%. Combining class I and class II inhibitors did not yield significant HER2 mRNA degradation but was still more effective than a pure class II inhibitor. AC-1215 was representative of a class II inhibitor and produced only a 30% suppression of HER2 mRNA levels. Class I

Entinostat yielded a 50% drop in HER2 message levels, which was similar to the HER2 mRNA suppression induced by combining Entinostat with AC775.

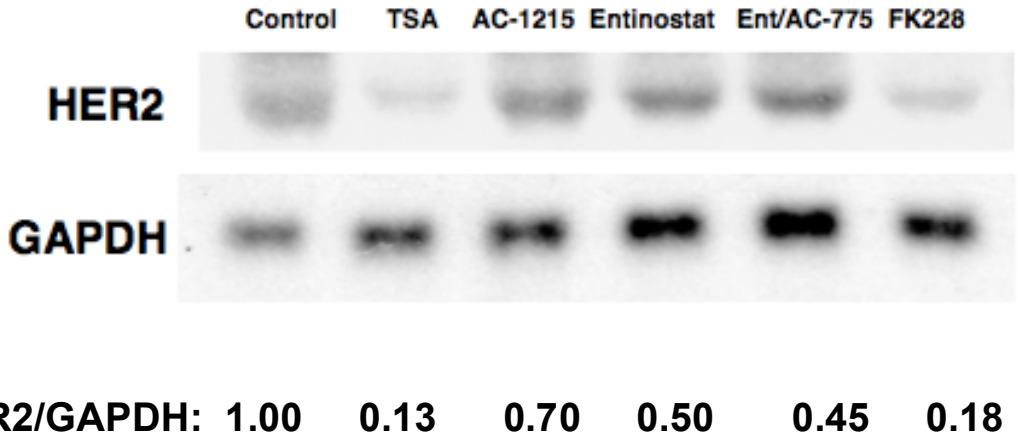


Figure 11. In this Northern blot analysis, untreated SKBR3 cells provided a baseline for HER2 message. TSA was included as a baseline for an effective inhibitor of HER2 mRNA expression. AC12-15 is a pure class II and Entinostat and FK228 are pure class I inhibitors. Entinostat/AC-775 examined the effects of combining class I and class II inhibitors. All HDAC inhibitors suppressed HER2 mRNA levels to some degree, but only FK228 matched the efficacy of TSA.

Although all HDAC inhibitors suppressed HER2 mRNA levels to some degree, only FK228 matched the efficacy of TSA. To examine FK228 as a potent class I HDAC inhibitor, experiments were performed using acetylated histone 2b (H2B) as a marker for class I HDAC inhibition, while class II HDAC inhibition was examined using acetylated tubulin as a marker. As the western blot analysis shown in Figure 13 illustrates, at higher treatment doses (> 0.5 µM x 6 hours), a potent class I inhibitor like FK228 (Avendaño & Menéndez, 2008; Chessum, Jones, Pasqua, & Tucker, 2015; Zahnow et al., 2016) can produce class IIB inhibitor effects such as acetylating tubulin. This is also evident with pan-HDAC (TSA) or class I/IIB (AC-1215) inhibitors, but not induced by other less potent class I inhibitors (AC-1035, Entinostat).

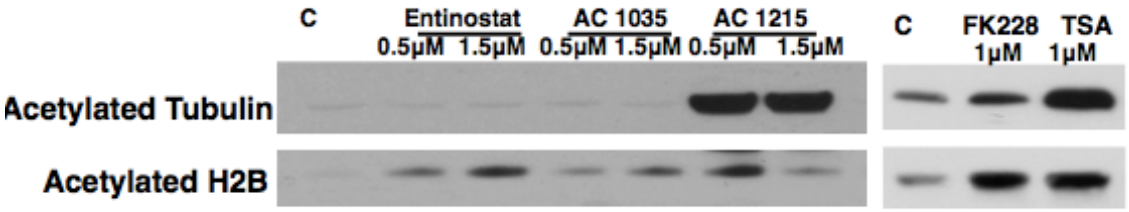


Figure 12. Using western blot analysis, Entinostat, AC-1035 and FK228 are class I inhibitors and appeared to acetylate histone 2b (H2B) at higher concentrations. AC-1215 is a class II inhibitor, acetylating tubulin, and pan-HDACi TSA targets both class I and class II HDACs, acetylating both histone 2b and tubulin.

Localization of HDAC1

As the literature reports that HDAC inhibitor activity of FK228 is directed primarily against HDAC1 and HDAC2 (Stubbs et al., 2015), experiments were conducted to see if HDAC1 could be detected in either pre- or post-polyribosome fractions (Figure 14). The western blot using polysome profile fractions from SKBR3 cells treated for 2 hours with 0.5 μM FK228 detected HDAC1 in fractions 3-11, meaning HDAC1 was found in pre-polysome fractions (fractions 3-7) as well as in initial polysome fractions (9-11) where the eukaryotic 40S ribosomal protein RACK1 (Receptor For Activated C Kinase 1) was used as a marker for polysome fractions. The expression of RACK1 suggests that HDAC1 is potentially loosely bound to the ribosomal region and also potentially associated with an unidentified acetylated protein of interest. The significance of this is that western analysis of polysome profiles probed with antibodies that detected acetylated lysine detected acetylated proteins marked by the arrows in figure 14 and later are the same fractions used in the SPYRO ruby gel in figure 17.

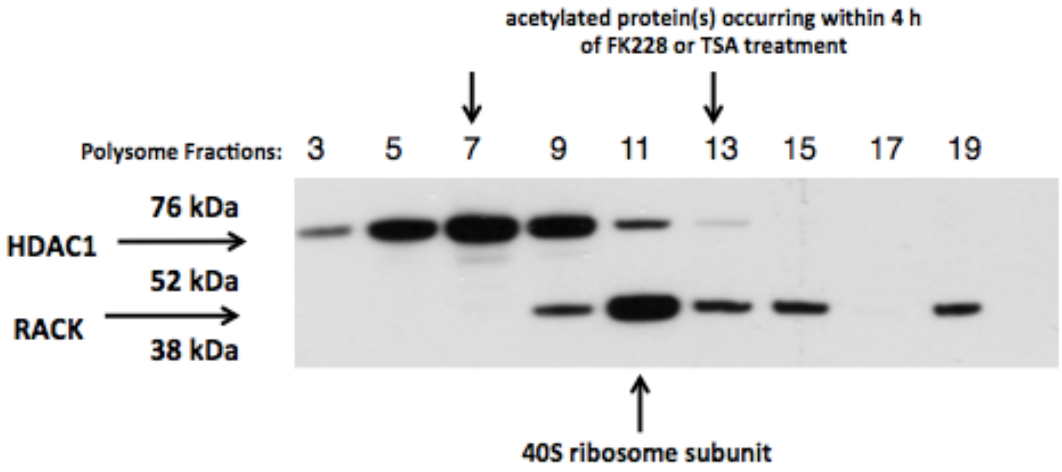


Figure 13. Western blot analysis of HDAC1 using fractions from a polysome profile of lysate from SKBR3 cells treated for 2 hours with 0.5 μM FK228. RACK1 is a marker for the eukaryotic 40S ribosome (Jannot et al., 2011). Arrows directed at fraction 13 and fraction 7 indication regions where acetylated proteins were detected.

Because western blot data demonstrated that HDAC1 migrated with the 40S ribosomal subunit, this suggested that in addition to its established nuclear localization, HDAC1 would demonstrate cytoplasmic localization. Therefore, HDAC1 was further investigated using immunohistochemistry. As shown in the Figure 15, the confocal images of SKBR3 cells stained with DAPI to see the nuclei (blue) and the HDAC1 staining (green) showed that HDAC1 was primarily nuclear in location. However, a punctate HDAC1 staining in the cytoplasm was also evident (Figure 15, middle panel), with the merged imagine (Figure 15, right panel) confirming a cytoplasmic HDAC1

localization.

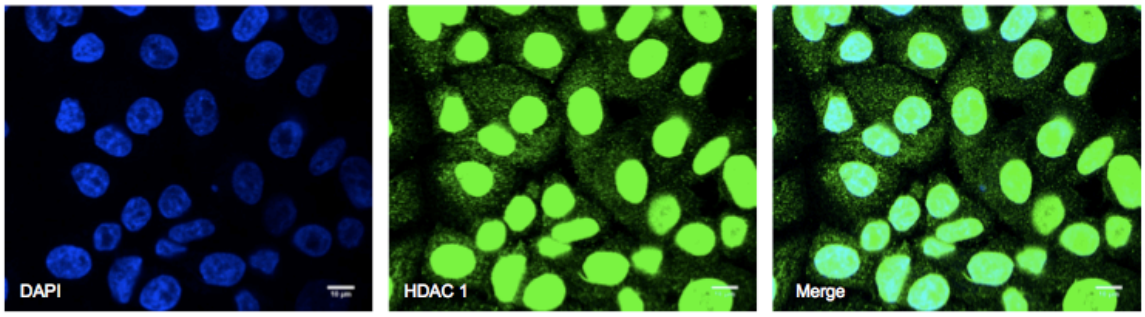


Figure 14. Confocal images of SKBR3 cells: left panel shows the blue DAPI channel staining for nuclei. The middle panel is stained for HDAC 1 localization. The right panel shows the merged images.

To better understand the influence of FK228 upon the polysome profile, polysome profiles comparing SKBR3 lysates from control and 2-hour treatment with 0.5 uM FK228 were examined (Figure 16; see Figure 11 for polysome profile details). Both control and FK228-treated SKBR3 profiles displayed relatively similar structure except for the significant loss of 80S peak intensity in the FK228-treated profile. This suggested that a subset of mRNAs in the treated cells did not reach ribosomal subunits, allowing the assembly of the 80S complex. Since we have previously demonstrated that FK228 treatment can induce mRNA decay of HER2 (see Figure 12), it is possible that FK228 mediates decay of additional mRNAS, impeding their association with the 80S ribosome, to account for the drop in 80S intensity in FK228-treated SKBR3 cells.

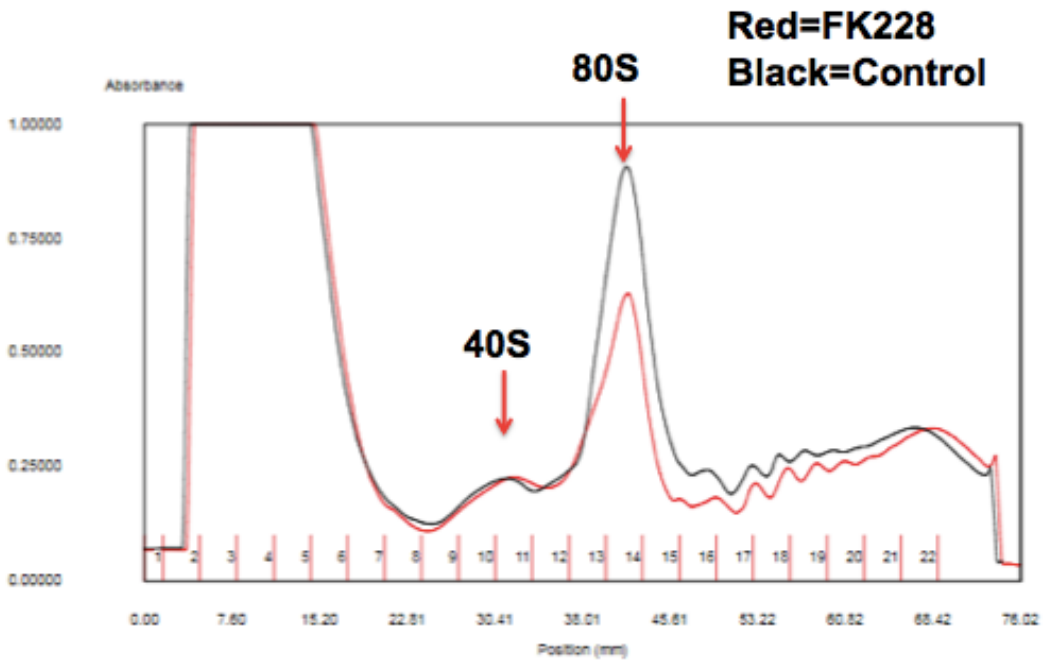


Figure 15. Polysome profiles comparing control (black) versus FK228-treated (red) SKBR3 cells. Profiles appear similar except for 80S intensity loss in the FK228-treated profile.

Proteins involved in FK228-induced decay of HER2 mRNA

Proceeding with trying to find out what proteins were involved with HER2 mRNA decay, further western blot examination of polysome profiles centering fractions 7-13 revealed there are acetylated protein bands in fractions 7 and 12-13 (figure 17).

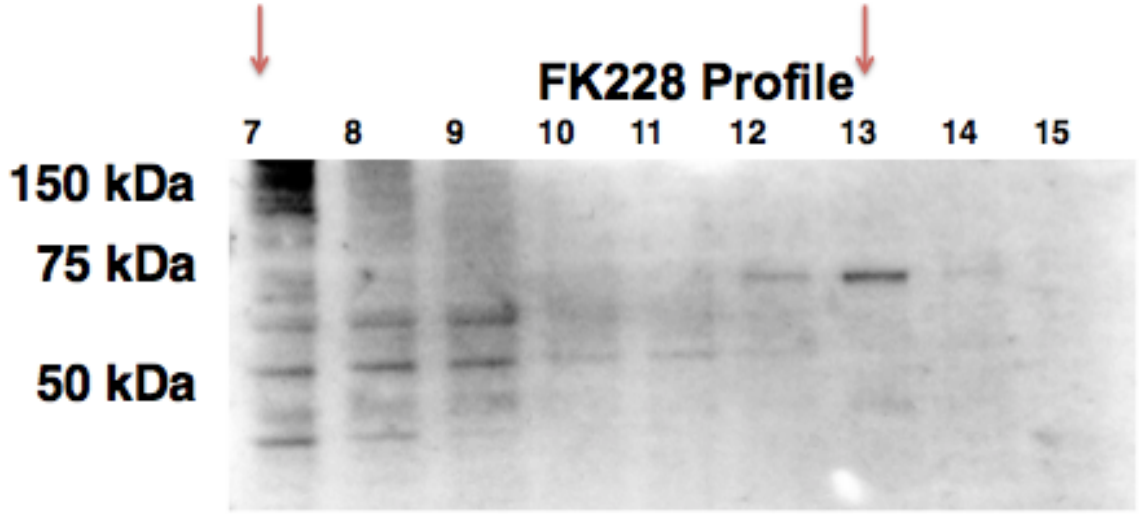


Figure 16. After probing with anti-acetylated lysine, there are bands for unidentified proteins at approximately 150 kDa and 68 kDa in fraction and fraction 13 respectively.

The acetylated protein detected in FK228 polysome profiles in fractions centered on fraction 7 and 13 were further examined by mass spectrometry. To this end, these fractions were first subjected to immunoprecipitation using an antibody against acetylated lysine to enhance the content of acetylated proteins as shown by the western blot in figure 18. Additionally, immunoprecipitation using the acetylated antibody was performed with a control lysate, to discriminate against non-specifically bound proteins. The immunoprecipitated proteins from FK228-treated and control cells were run on 4-12% polyacrylamide gels, and the proteins visualized by SYPRO Ruby staining. Bands in the gel, marked by the red boxes in figure 18, were excised for analysis by mass spectrometry (MS), which was performed by the MS core at the Buck Institute for Research and Aging.

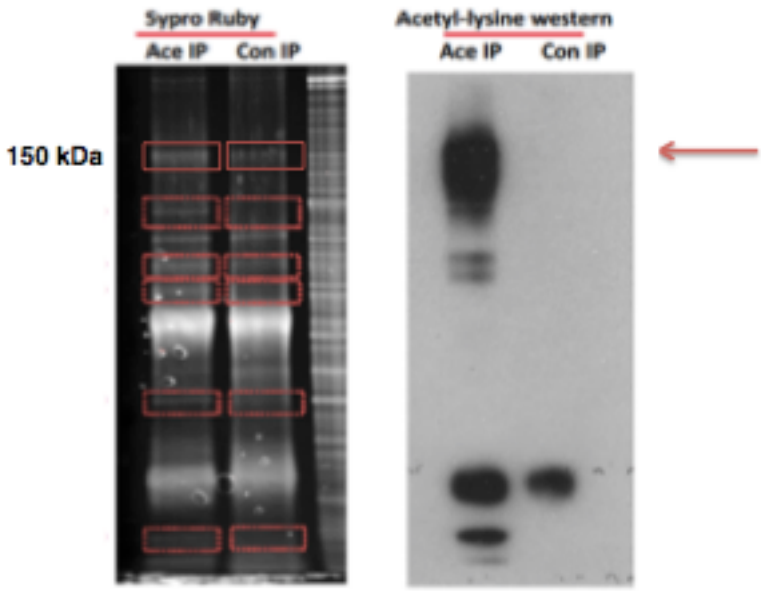


Figure 17. Proteins immunoprecipitated using an antibody against acetylated lysine from fraction 7 of FK228-treated (Ace-IP) and control (Con-IP) lysates. SKBR3 lysates were run on a 4-12% acrylamide gel and stained with SYPRO ruby. The bands that were excised are marked by red boxes (left panel). Immunoprecipitates from fraction 7 of FK228-treated and control lysates were analyzed by western blot using the anti-acetyl lysine antibody to confirm immunoprecipitation specificity (right panel).

MS data provided a list of proteins that were acetylated by FK228 treatment. Proteins with a higher percentage of lysine peptides acetylated were considered and examined (Appendix 1). Of those proteins detected by the mass spectrometry, the histone acetyltransferase (HAT) p300 was of particular interest, as it was found to be acetylated at twelve different lysine residues. These findings led to immunoprecipating p300 and acetylated lysine from polysome fraction 7 of FK228-treated SKBR3 lysates. Through western blot analysis, it was determined that the acetylated lysine contained multiple p300 isoforms (Figure 19). Additonally, immunoprecipitated p300 appeared as

a number of distinct molecular bands, a surprising result that was confirmed by p300 siRNA experiments (see Figure 21).

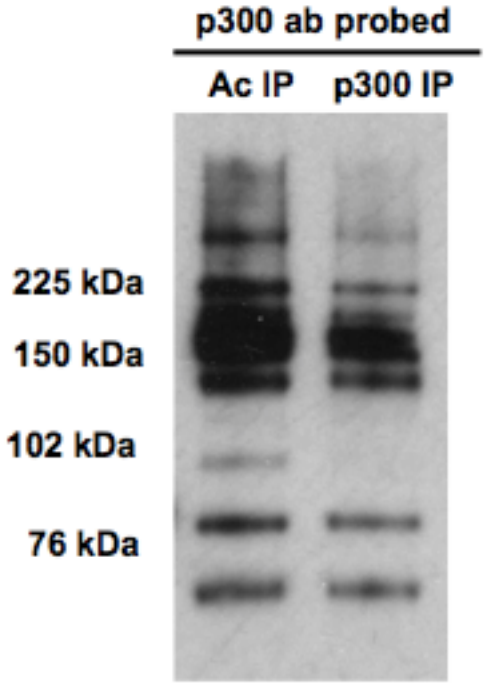


Figure 18. Fraction 7 lysates from FK228-treated SKBR3 cells were immunoprecipitated using either an anti-acetylated lysine antibody (Ac IP) or an anti-p300 antibody (p300 IP). Western blot analysis with the p300 antibody demonstrated that the Ac-IP contained multiple p300 isoforms**.**

In the interest of investigating p300 as a potential protein involved in the proposed mechanism of HER2 mRNA decay, the next step was to knock it down using p300 siRNA and monitor what effect the p300 knockdown had upon HER2 mRNA levels. Additionally, as FK228 inhibits HDAC1 and HDAC2, simultaneous HDAC1 and HDAC2 (H1/H2) siRNA was performed to examine to what extent this alone had the ability to induce HER2 mRNA decay. Northern blot analysis of RNA from SKBR3 cells treated for either 48 hours or 72 hours with p300 siRNA demonstrated a dramatic reduction in HER2 mRNA (0.34 and 0.35 respectively), when normalized against the

housekeeping gene GAPDH (Figure 20). On the other hand, combined HDAC1 and HDAC2 siRNA knockdown reduced HER2 mRNA levels by 30% at 48 hours and only by 10% at 72 hours (Figure 20).

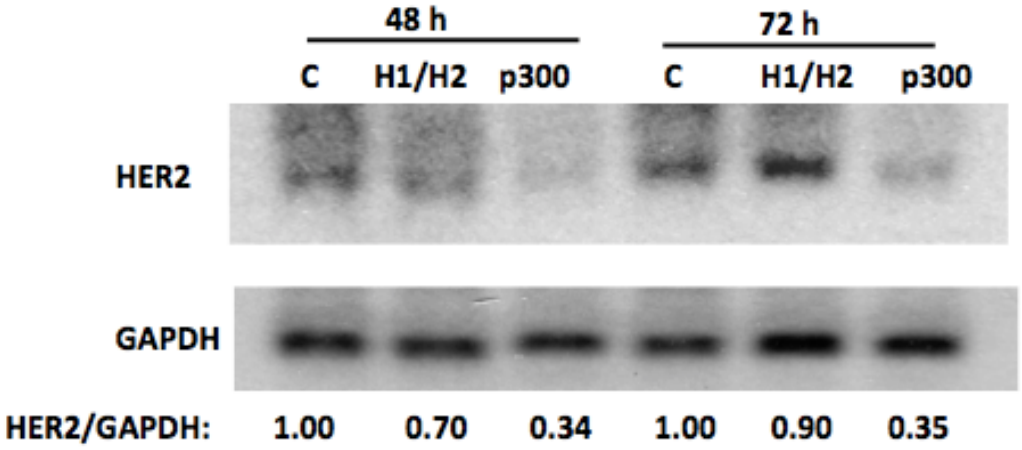


Figure 19. Using Northern blot analysis, siRNA knockdown of p300 after 48 and 72 hours in SKBR3 cells showed a marked decrease in HER2 mRNA levels, when normalized to GAPDH (red numbers). siRNA knockdown of HDAC1 and HDAC2 (H1/H2) produced a minimal to moderate reduction in HER2 levels as compared with siRNA control (C).

To validate the effectiveness of the siRNA knockdowns, western blot analysis of whole cell lysates from the various siRNA conditions was performed. The siRNA knockdown of HDAC1 was very effective, as essentially no HDAC1 was detected in either the HDAC1 (H1) siRNA cell lysate or in the combined HDAC1 and HDAC2 (H1/H2) siRNA cell lysate (Figure 21, left panel). Most interestingly, p300 siRNA resulted in the suppression of several p300 isoforms in the molecular weight range between 102 kDa and 225 kDa (Figure 21, right panel), thus validating the acetylated p300 isoforms previously seen in this molecular weight range (see Figure 18).

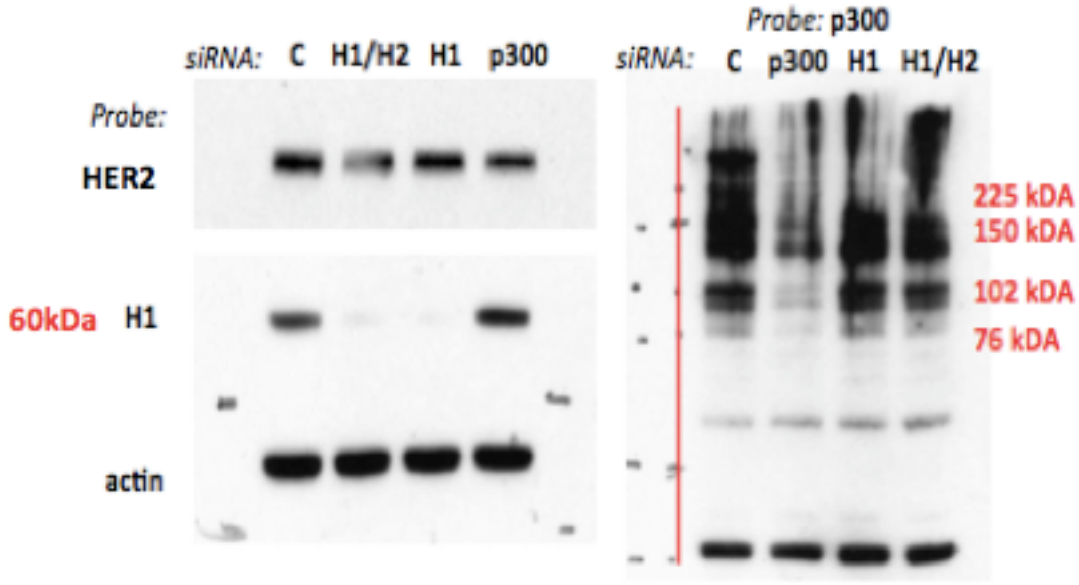


Figure 20. HER2 protein levels are reduced following p300 siRNA (top left panel). SKBR3 whole cells lysates from combined HDAC1 and HDAC2 siRNA (H1/H2), HDAC1 siRNA (H1) or p300 siRNA (p300) following 48 hours of siRNA treatment were examined by western blot for HDAC1 (left panel) or p300 (right panel).

To determine if HER2 mRNA was directly associated with p300, p300 was immunoprecipitated (IP) from control cytoplasmic lysate as well as from FK228-treated cytoplasmic lysate, with the resulting protein G sepharose beads containing immunoprecipitated p300 resuspended in Trizol for isolation of RNA. Following the reverse transcription of the RNA to cDNA, PCR was used to determine the HER2 and GAPDH mRNA levels contained in the p300 immunoprecipitates. Additionally, to validate previous Northern analysis, RT-PCR was performed on RNA from control cells and p300 siRNA-treated SKBR3 cells, which had by Northern analysis demonstrated reduced HER2 mRNA levels relative to control levels (see Figure 20). RT-PCR results for HER2 mRNA levels using RNA from p300 siRNA-treated cells versus control cells, while not showing the same degree of suppression seen in the Northern analysis,

confirmed the general robustness of the RT-PCR procedure by producing a 40% suppression in HER2 mRNA levels (Figure 22). Significantly, levels of GAPDH mRNA appeared relatively low and displayed little variation across the different IP conditions. HER2 mRNA associating with p300 under control (untreated) conditions was enhanced almost 3-fold relative to its matched control IP (4.88 vs. 1.79). However, HER2 mRNA associating with p300 under FK228 treatment displayed only a 1.5-fold enhancement relative to its matched control IP (2.60 vs. 1.64), suggesting that FK228 treatment promoted a decrease of HER2 mRNA from p300.

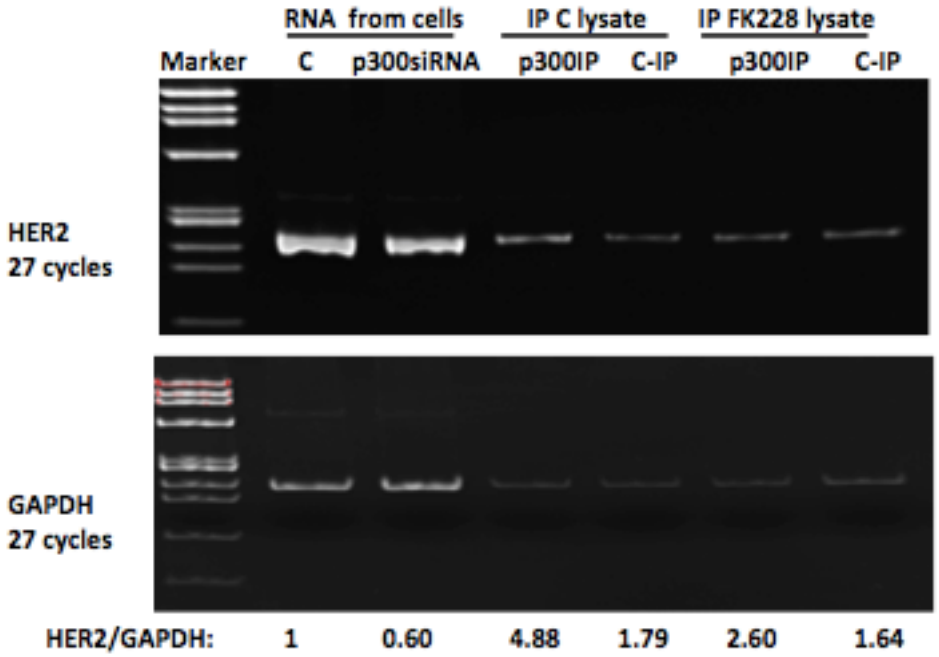


Figure 21. Top Panel: Ethidium bromide stained gels of RT-PCR products for measuring HER2 mRNA from control cells (C) versus p300 siRNA-treated cells (p300 siRNA), IP from control lysates (IP Ct lysate) using p300 antibody (p300IP) or control antibody (C-IP) and IP from FK228-treated lysates (IP FK228 lysate) using p300 antibody (p300 IP) or control antibody (C-IP). Bottom Panel: Identical to the top panel except using PCR primers for GAPDH.

Discussion

Building upon earlier studies with the pan-HDACi, TSA, this project's first aim was to examine the effect of various class-selective HDAC inhibitors for their ability to promote HER2 mRNA decay, as assayed by Northern blotting. Having previously demonstrated the TSA comparable effectiveness of other pan-HDACi like Vorinostat and LAQ824 in inducing HER2 mRNA decay in SKBR3 cells, even when such agents were also administered *in vivo* against several other HER2+ breast cancer models (Drummond et al., 2005), this project first acquired and then evaluated a number of more selective inhibitors of specific HDAC classes. Notably, this effort demonstrated that a potent and FDA-approved class-1 HDACi, specifically the HDAC1/2 inhibitor Romidepsin (also known as depsipeptide or FK228), was able to rapidly induce the degradation of HER2 mRNA just as effectively as TSA (Figure 12). While class-II selective HDACi (including those specific to HDAC6 like ACY-775 and Tubacin) proved essentially ineffective at inducing HER2 mRNA decay, other class-I HDACi (e.g. ACY-1215/Ricolinostat and Entinostat/ACY-1035) exhibited significant ability to induce HER2 mRNA decay but not to the same degree as TSA or FK228. Consistent with these observations, western blotting of polysome profile fractions demonstrated the presence of HDAC1/2 in the pre-ribosome (pre-40S) fraction of control and HDACi-treated SKBR3 cells (Figure 14), and subsequent immunohistochemical imaging of HDAC 1 confirmed the detectable presence of HDAC1 proteins in the cytosol compartment of SKBR3 cells (Figure 15).

This project's second aim was to evaluate pre- and post-polyribosome fractions from FK228-treated (vs. control) SKBR3 cells, looking for evidence of any acetylated proteins resulting from HDACi treatment, as these proteins would then be candidate

mediators of the HER2 mRNA decay mechanism. After identifying potentially acetylated protein candidates using our immunoprecipitation-immunoblotting (IP-IB) approach (using an antibody specific for acetylated lysine residues; see Figure 18), we collaborated with Buck Institute mass spectrometry (MS) experts to identify those acetylated protein candidates. Specific pre-polyribosome fractions evaluated first by immunoprecipitation/immunoblotting and detected acetylated protein bands with different molecular weights that were not present in control cells but were detectable within 2 hours of treatment with either TSA or FK228. MS analysis of those cut-out pre-polysome bands identified many different proteins that were present only in the FK228-treated IP-IB extracts, but only one of these proteins showed clear MS evidence of newly formed acetylated lysine (K) residues: the histone acetyl transferase (HAT) protein, p300. In particular, FK228-induced acetylation of p300 was found to be one of the most abundant proteins detected by MS, and this finding was independently confirmed using a specific anti-p300 antibody to immunoblot the same anti-acetylated protein immunoprecipitates used for the MS analyses (Figure 19). These IP-IB confirmation assays also demonstrated multiple isoforms of p300 each with different molecular sizes, possibly due to selective proteolytic cleavage (although alternative splicing cannot be ruled out). Moreover, the MS studies demonstrated that FK228 induced acetylation of p300 on 12 different lysine residues, largely confined to its HAT domain.

While p300 is thought to be largely localized within the nuclear compartment, a very recent study demonstrated its key involvement, along with HDAC1/2, in regulating mRNA stability via poly(A) length (Sharma *et al* 2017). As well, in recent immunohistochemical studies of human breast cancers, p300 was overexpressed and

predominantly localized in the cytoplasm, as compared to its nuclear localization in normal breast tissue samples (Fermento *et al*., 2014). Therefore, the above two studies provided additional mechanistic credence to our current hypothesis that p300, possibly in co-association with another yet-to-be-identified acetylated substrate of its HAT domain, acts in concert with HDAC1/2 to stabilize HER2 transcripts; and shortly after treatment with the HDAC1/2 inhibitor FK228, p300 becomes extensively acetylated and enzymatically activated to trigger the rapid decay of all p300-associated HER2 mRNA. Supporting this mechanistic hypothesis, RT-PCR analysis of p300 immunoprecipitates showed selective binding of p300 to HER2 transcripts that significantly declined almost immediately after FK228 treatment when p300 also became acetylated (figure 22).

In order to test the above mechanistic hypothesis, we undertook the project's third specific aim, using siRNA to knockdown expression of this candidate protein in SKBR3 cells and thereby determine if its intracellular loss produced any impact on HER2 mRNA stability (Figure 20). We also compared the impact of siRNA knockdown of p300 with that of HDAC1/2 knockdown; furthermore, the effectiveness of our respective siRNA knockdown procedures was documented by western blot analysis, which also served to confirm that the multiple sizes of p300 isoforms were comparably reduced by the p300 knockdown (Figure 21). As expected and convincingly demonstrated by both Northern blot analysis and RT-PCR assays, siRNA knockdown of p300 induced a rapid decay in HER2 transcript levels yielding a ERBB2(HER2)/GAPDH mRNA ratio = 0.3 at 48 hours, comparable to that observed with FK228 treatment (see Figure 12). This p300 knockdown impact in reducing HER2 mRNA levels clearly exceeded that produced by our partial knockdown of HDAC1/2 proteins, which yielded a ERBB2(HER2)/GAPDH

mRNA ratio = 0.7 at 48 hours. With these findings, we successfully confirmed the hypothesis generated from our specific aims results, demonstrating the critical role of p300 in maintaining HER2 mRNA stability and making the therapeutically relevant observation that HER2 mRNA decay comparable to that produced by FK228 treatment can be achieved without targeting HDAC1/2 but simply by functionally knocking down p300.

Conclusions and Future Perspectives

Based on the above findings and completion of this project's three specific aims, future studies will evaluate the potential impact on HER2 mRNA stability of commercially available inhibitors of the p300 bromo and HAT domains, as well as perform intracellular enzymatic assays of p300 to compare its HAT activity under control and FK228 treatment conditions. As well, future MS efforts will be aimed at identifying potential p300-associated substrates that may also be mechanistically involved with the FK228-induced destabilization of HER2 mRNA. In summary, this project's findings shed new light on the relatively unrecognized subcellular mechanism by which HDACs regulate specific aspects of the cell's translational machinery, in particular those ribosome-/polyribosome-associated components that can dysregulate cell growth during tumorigenesis by rapidly altering the stability of cancer-promoting transcripts like HER2 mRNA. Non-selective inhibitors of all classes of histone deacetylases (pan-HDACi) represent a broad class of drugs that have been used for almost two decades to treat hematologic malignancies, yet only recently have found limited utility in the treatment of solid malignancies like breast cancer. This limitation to their use is primarily due to two

facts: i) pan-HDACi also produce many unwanted clinical side effects, and ii) they impact many different targets as well as largely unexplored subcellular systems beyond their earliest documented effects on chromatin structure and gene expression. These limitations have prevented their more widespread clinical deployment and evaluation, especially in an era when new FDA-approved anticancer indications require biomarker-specified targeting of credentialed cancer-driving mechanism, such as amplified and overexpressed HER2.

With ongoing therapeutic development of more class-selective HDACi as well as Benz Lab progress including this project's most recent discovery revealing that the stability of oncogenic transcripts like HER2 mRNA depends not only on a cytosolic and ribosome/polysome-associated subset of class-1 HDACs (HDAC1/2) but also their cytosolic substrate p300 that binds specifically to HER2 mRNA, It is now possible to consider the use of an investigational or already FDA-approved class-selective HDACi like FK228 as a new treatment strategy for patients suffering with clinically-resistant HER2+ breast cancer. Moreover, this project's findings implicating the critical role of p300 in regulating HER2 mRNA stability provides new rationale for pharmaceutical development of a new class of p300-targeted agents that can fully simulate the HER2 mRNA degrading effects we observed with siRNA knockdown of p300, hopefully yielding an equipotent but better tolerated therapeutic than FK228. As new therapeutic strategies are increasingly needed to counter clinical resistance to currently available HER2-targeted agents, the repurposing of selective HDAC1/2 inhibitors as well as the emergence of new p300 inhibitors may find appropriate clinical utility against treatment refractory HER2-positive breast cancers. First generation investigational anti-p300 agents

are currently under pharmaceutical development as potential anti-cancer therapeutics, but even fully optimized versions of this new cancer drug class will require cancer-specific biomarkers to guide their clinical indications (Lasko *et al.*, 2017). While future preclinical studies are also needed to fully delineate the translational machinery and complete set of HDAC1/2 substrates beyond p300, as well as the essential functional domains of p300 critical to the regulation of HER2 mRNA stability, we now have reason to anticipate that life-threatening HER2+ breast cancers with clinical resistant to Trastuzumab/Pertuzumab/taxane treatment may become a future indication for the need for agents like FK228 or a newly developed and optimized p300-targeted therapeutic.

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APPENDIX 1

Table 2: Complete list of acetylated proteins detected from SPYRO ruby gel during mass spectrometry analysis.

Protein	Gene	Gel Cut #	Checked
60 kDa heat shock protein, mitochondrial	HSPD1 (synonym: HSP60)	3	
60S ribosomal export protein NMD3	NMD3 (orf: CGI-07)	4	x
60S ribosomal protein L4	RPL4 (synonym: RPL1)	5	
78 kDa glucose-regulated protein	HSPA5 (synonym: GRP78)	1	
Annexin A6	ANXA6 (synonym: ANX6)	1 & 2	
Arginine--tRNA ligase, cytoplasmic	RARS	2	
Aspartate--tRNA ligase, cytoplasmic	DARS (orf: PIG40)	4	
ATP synthase subunit alpha	ATP5A1 (synonym: ATP5A, ATP5AL2, ATPM)	4 & 5	
ATP synthase subunit beta, mitochondrial	ATP5B (synonym: ATPMB, ATPSB)	5	
Basigin	BSG (orf: UNQ6505/PRO21383)	4	x
Bifunctional glutamate/proline--tRNA ligase	EPRS (synonym: GLNS, PARS, QARS, QPRS; orf: PIG32)	3	x
Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A (synonym: CPT1)	1	x
CREB-binding protein	CBP (synonym: CREB BP)	2 & 3	x
Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	5	
Dihydrolipoyl dehydrogenase, mitochondrial	DLD (synonym: GCSL, LAD,	4 & 5	

	PHE3)		
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	DLAT (synonym: DLTA)	2 & 3	x
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	DLST (synonym: DLTS)	5	
DNA-directed RNA polymerase I subunit RPA49	POLR1E (synonym: PAF53, PRAF1)	5	
DNA repair protein XRCC1 OS	XRCC1	1	x
DnaJ homolog subfamily C member 11	DNAJC11	3	
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	RPN1	2	x
E3 ubiquitin-protein ligase synoviolin	SYVN1 (synonym: HRD1, KIAA1810)	2	
Elongation factor 1-alpha 2	EEF1A2 (synonym: EEF1AL, STN)	5	
Eukaryotic translation initiation factor 3 subunit E	EIF3E (synonym: EIF3S6, INT6)	5	
Eukaryotic translation initiation factor 3 subunit L	EIF3L (synonym: EIF3EIP, EIF3S6IP; orf: HSPC021, HSPC025, MSTP005)	3	
Galectin-3-binding protein	LGALS3BP (synonym: M2BP)	1	x
Glutamine--tRNA ligase	QARS	1	
Golgi membrane protein 1	GOLM1 (synonym: C9orf155, GOLPH2; orf: PSEC0242, UNQ686/PRO1326)	1	
Heat shock 70 kDa protein 1B	HSPA1B	2	
Heat shock cognate 71 kDa protein	HSPA8 (synonym: HSC70, HSP73, HSPA10)	1	

Heterogeneous nuclear ribonucleoprotein K	HNRNPK (synonym: HNRPK)	4	x
Heterogeneous nuclear ribonucleoprotein L	HNRNPL (synonym: HNRPL; orf: P/OKcl.14)	3	
Heterogeneous nuclear ribonucleoprotein R	HNRNPR (synonym: HNRPR)	1	
Histone acetyltransferase p300	p300 (synonym: p300 HAT; E1A-associated protein p300; EP300)	2 & 3	x
Lamina-associated polypeptide 2, isoform	TMPO (synonym: LAP2)	1	
Large subunit GTPase 1 homolog	LSG1	1	
Ly6/PLAUR domain-containing protein 3	LYPD3 (synonym: C4.4A; orf: UNQ491/PRO1007)	1	
Lysine--tRNA ligase	KARS (synonym: KIAA0070)	2	x
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	NDUFS1	1	
NADPH--cytochrome P450 reductase	POR (synonym: CYPOR)	1	
Neutral amino acid transporter B(0)	SLC1A5 (synonym: ASCT2, M7V1, RDR, RDRC)	1	
Nucleolin	NCL	1, 2, 3, & 4	
Pentatricopeptide repeat domain-containing protein 3, mitochondrial	PTCD3 (synonym: MRPS39; orf: TRG15)	1	
Phenylalanine--tRNA ligase alpha subunit	FARSA (synonym: FARS, FARSL, FARSLA)	4	
Phenylalanine--tRNA ligase beta subunit	FARSB (synonym: FARSLB, FRSB; orf: HSPC173)	2 & 3	
Plasminogen activator inhibitor 1 RNA-binding	SERBP1 (synonym:	5	

protein	PAIRBP1; orf: CGI-55)		
Prelamin-A/C	LMNA (synonym: LMN1)	2	
Pre-mRNA-processing factor 19	PRPF19 (synonym: NMP200, PRP19, SNEV)	4	x
Proliferation-associated protein 2G4	PA2G4 (synonym: EBP1)	5	
Pyruvate dehydrogenase protein X component, mitochondrial	PDHX (synonym: PDX1)	4 & 5	
Ras GTPase-activating protein-binding protein 1	G3BP1 (synonym: G3BP)	3	
Ras GTPase-activating protein-binding protein 2	G3BP2 (synonym: KIAA0660)	4	x
Signal recognition particle subunit SRP72	SRP72	1	
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	1	
SRSF protein kinase	SRPK1	1	
Stress-70 protein, mitochondrial	HSPA9 (synonym: GRP75, HSPA9B, mt-HSP70)	1	x
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	SDHA (synonym: SDH2, SDHF)	2	
T-complex protein 1 subunit beta	CCT2 (synonym: 99D8.1, CCTB)	4	x
T-complex protein 1 subunit delta	CCT4 (synonym: CCTD, SRB)	4	x
T-complex protein 1 subunit epsilon	CCT5 (synonym: CCTE, KIAA0098)	3	
T-complex protein 1 subunit theta	CCT8 (synonym: C21orf112, CCTQ, KIAA0002)	4	
T-complex protein 1 subunit gamma	CCT3 (synonym: CCTG, TRIC5)	3	
T-complex protein 1 subunit zeta	CCT6A (synonym: CCT6, CCTZ)	4	
Translation initiation factor eIF-2B subunit epsilon	EIF2B5 (synonym: EIF2BE)	1	x
Translocation protein SEC62	SEC62 (synonym: TLOC1)	5	
Trifunctional enzyme subunit	HADHA	1	x

alpha, mitochondrial	(synonym: HADH)		
Trifunctional enzyme subunit beta, mitochondrial	HADHB (orf: MSTP029)	5	
Tripeptidyl-peptidase 2	TPP2	5	
tRNA-splicing ligase RtcB homolog	RTCB (synonym: C22orf28; orf: HSPC117)	4	
Ubiquitin-60S ribosomal protein L40	UBA52 (synonym: UBCEP2)	3	x
V-type proton ATPase catalytic subunit A	ATP6V1A (synonym: ATP6A1, ATP6V1A1, VPP2)	2	
V-type proton ATPase subunit B, brain isoform	ATP6V1B2 (synonym: ATP6B2, VPP3)	4	
X-ray repair cross-complementing protein 6	XRCC6 (synonym: G22P1)	2	
Zinc finger protein 622	ZNF622 (synonym: ZPR9)	3	