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Defining YAP/TAZ-dependency in human breast cancer cells

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DEFINING YAP/TAZ-DEPENDENCY IN
HUMAN BREAST CANCER CELLS

by

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ABSTRACT

**Overview:** Hyperactivation and amplification of the oncogenic transcriptional co-factors YAP and TAZ are common in breast cancer. However, it is unknown if breast cancer cells are dependent on YAP/TAZ for growth and survival. In addition, key transcriptional targets of YAP/TAZ that enable breast cancer growth have yet to be defined. To address these unresolved questions, we will define YAP/TAZ-dependencies across a large cohort of breast cancer cells and generate gene expression signatures for both YAP/TAZ-dependent and YAP/TAZ-independent lines. We aim to identify YAP/TAZ-target genes that are essential for the growth and survival of YAP/TAZ-dependent breast cancer cells. This approach may reveal genetic dependencies in breast cancer that can then be therapeutically exploited.

**Methods:** A comprehensive cohort of breast cancer cells (45 cell lines) was obtained from the American Type Culture Collection (ATCC). The majority of time allotted for this thesis was spent culturing and expanding the cell lines. Five complete breast cancer cell line libraries were successfully generated and annotated. These libraries will be a useful resource for the Boston University School of Medicine Cancer Research Community. Protein and RNA extracts were collected from all cell lines. RNA extraction was performed in all cell lines with the Qiagen RNase Kit as per the manufacturer’s instructions. Protein extracts were collected from the cell lines with RIPA lysis buffer.
Protein lysates were then run on an acrylamide gel and the relative abundance of YAP and TAZ was quantified. RNA extracts were sent for microarray analysis to obtain gene expression profiles. Cell lines were also fixed and stained for YAP and TAZ at subconfluence (50%) and confluence (90%) and visualized through immunofluorescence to assess the relative subcellular localization of YAP and TAZ.

**Results:** Our results indicate that YAP/TAZ levels and activity are highly variable across breast cancer cell lines. Seven cell lines were found to overexpress only YAP, nineteen cell lines were found to overexpress only TAZ, and two cell lines (BT-474 and HCC 1599) were found to overexpress both YAP and TAZ. Two cell lines (MDA-MB-134-VI and DU4475) had negligible protein expression levels of YAP/TAZ. We were also able to identify a subset of cells as being resistant to Hippo pathway activation, as seen in MCF 10A, MCF 10F, and MCF-12A cells, which maintained nuclear YAP and TAZ even under confluent conditions, and with MDA-MB-231 cells, which maintained only nuclear YAP under confluence. Given the importance of YAP and TAZ in cellular proliferation and survival, these results suggest that these Hippo pathway inactive cell lines may be dependent on YAP and TAZ for survival, which will be assessed at a future time point. We plan to complete our analysis of the subcellular localization of YAP and TAZ for all 45 breast cancer cell lines. Microarray profiling and gene expression signature analysis of all 45 cell lines are also ongoing.

**Discussion:** We surmise that increased levels/activity of YAP/TAZ will predict increased dependency on these oncogenes for growth and survival. This prediction will be directly tested by assessing cell viability following YAP/TAZ knockdown experiments. We also
hypothesize that YAP/TAZ-dependent cells will be dependent on the transcription of specific YAP/TAZ target genes for survival. Current work detailed in this thesis will form the foundation for future work focusing on therapy-relevant YAP/TAZ target genes that are critical to breast cancer pathogenesis and disease progression. Our long-term aim is to identify pharmacologically-tractable YAP/TAZ target genes with the ultimate goal of finding novel chemotherapeutics that will improve prognosis for breast cancer patients.
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LIST OF ABBREVIATIONS

ABCP ........................................................................................................... Apicobasal Cell Polarity
AIP4/Itch .............................................................. Atrophin-1-Interacting Protein
AMOT ........................................................................................................ Angiomotin
AMPK .......................................................... 5’Adenosine Monophosphate-Activated Protein Kinase
ATCC ........................................................................................................ American Type Culture Collection
ATM ........................................................................................................ Ataxia-Telangiectasia Mutated
BRCA 1/2 .......................................................... Breast Cancer Genes 1/2
BSA ........................................................................................................ Bovine Serum Albumin
BUMC .................................................................................................. Boston University Medical Campus
BUSM .................................................................................................. Boston University School of Medicine
CO2 ........................................................................................................ Carbon Dioxide
DAPI ......................................................................................... 4’,6-Diamidino-2-Phenyindole
DMSO .................................................................................................. Dimethylsulfoxide
DNA ..................................................................................................... Deoxyribonucleic Acid
EGF ..................................................................................................... Epidermal Growth Factor
EMT ...................................................................................................... Epithelial-to-Mesenchymal Transition
f-actin ................................................................................................. Filamentous Actin
FBS ..................................................................................................... Fetal Bovine Serum
FITC ................................................................................................. Fluorescein Isothiocyanate
g ........................................................................................................ Gravitational Force
shRNA ............................................................. Short Hairpin Ribonucleic Acid
T ................................................................................................ Threonine
TAZ ................................................... Transcriptional Co-Activator with PDZ-binding motif
TBS ................................................................................................ Tris-Buffered Saline
TEAD .................................................................. Transcriptional Enhancer Activator DNA-Binding
TGF-β ............................................................ Transforming Growth Factor-Beta
TNF-induced apoptosis ..................................... Tumor Necrosis Factor Induced Apoptosis
TRITC .............................................................................. Tetramethylrhodamine
WW domain ............................................................... Tryptophan-Rich Domain
WWTR1 .................................................. WW Domain Containing Transcription Regulator 1
YAP ................................................................................. Yes-Associated Protein
INTRODUCTION

Breast cancer is the most common cancer in the world and the second leading cause of cancer-related deaths among women in developed regions around the world, trailing slightly behind lung cancer (IARC, 2012; Patel et al., 2011). Figure 1 shows the distribution of age-adjusted incidences per 100,000 people of breast cancer cases in the world in 2012 (IARC, 2012). Developed countries have higher rates of breast cancer when compared with developing regions, pointing to the importance of environmental factors and lifestyle habits in addition to commonly known genetic factors (e.g. common mutations in the DNA repair \textit{BRCA} genes) that can contribute to the development of cancer (Patel et al., 2011).

The United States, as a developed country, has one of the highest incidences of breast cancer in the world, with a rate greater than 64.8 cases per 100,000 females (Figure 1). In 2012 alone, 226,272 new cases of breast cancer were diagnosed in the U.S., and 41,555 female patients died from breast cancer (“CDC - Breast Cancer Statistics,” 2015). The rate of breast cancer diagnosis among females has increased since 1981 from around 100 new cases per 100,000 females to 120 new cases per 100,000 females in 2005 due to increased screening (Figure 2, Jemal et al., 2012). However, the death rate from breast cancer has significantly decreased from 30 cases per 100,000 females in 1990 to 20 cases per 100,000 females in 2005 (Figure 3) due in part to the ability to detect and treat breast cancer at earlier stages than previously possible. Nevertheless, breast cancer still remains the second leading cause of death in women, highlighting the urgency to define new innovations or therapeutic strategies to combat breast cancer.
Figure 1: Age-Adjusted Incidence of Breast Cancer per 100,000 People Worldwide. This map published by the World Health Organization depicts the incidence of breast cancer around the world in 2012. Dark blue represents higher incidences, and lighter colors show lower incidences of breast cancer. The developed countries tend to have a higher rate of breast cancer. Adapted from IARC (2012).
Figure 2: Age-Adjusted Incidence Rates of Cancer Among Females in the United States between 1975-2005. The number of breast cancer diagnoses has increased significantly since 1975, and is still the most common cancer diagnosis among females over 40 years of age. Adapted from Jemal et al. (2009).
Figure 3: Age-Adjusted Breast Cancer Death Rates Among Females in the United States, 1930-2005. The number of deaths due to breast cancer among females has decreased slightly since 1990, while the number of lung and bronchus cancer-related deaths has risen since 1965. Breast cancer still remains the second most common cause of death among female cancer patients over 40 years of age. Adapted from Jemal et al. (2009).
Sub-Classifications of Breast Cancer and Current Chemotherapeutics

Once a diagnosis for breast cancer has been made, histological and pathological examinations are performed in order to characterize the subtype of breast cancer for each patient. These analyses provide critical information regarding the most effective mode of therapy. Breast cancer is typically characterized into subtypes according to the type of growth factor/hormone receptors the cancer cells contain and/or lack. Some of the most common subtypes include Estrogen Receptor positive or negative (ER+ or ER-), Progesterone Receptor positive or negative (PR+ or PR-), Human Epidermal Growth Factor Receptor 2 positive or negative (HER2+ or HER2-), or triple negative if the breast cancer cells lack all three of these receptors ("Your Breast Cancer Diagnosis | Breastcancer.org," 2016).

The subtype of breast cancer is useful for deciding which chemotherapeutic the patient’s breast cancer cells will be responsive to. For example, ER+ breast cancers rely on estrogen to fuel their growth and are commonly responsive to estrogen hormonal therapy that targets estrogen (such as tamoxifen, an estrogen antagonist), whereas an ER-subtype that does not rely on estrogen for growth would not be responsive to hormone therapy (Patel et al., 2011). Aromatase inhibitors are also commonly used for ER+ subtypes and act by inhibiting production of estrogen in estrogen-dependent cells (Sausville & Longo, 2015; Patel et al., 2011).

Trastuzumab is another common chemotherapeutic, specific for breast cancers overexpressing the growth factor receptor HER2 (Patel et al., 2011). HER2 is a protein in...
the cell membrane that relays critical extracellular cell proliferation signals into the nucleus (Gajira & Chandarlapaty, 2011; Ménard et al., 2000; Moasser, 2007). Overexpression of HER2 provides sufficient pro-growth factor signaling to allow cells to overcome the G1/S checkpoint that would normally arrest the cell via cyclin D1 and p27 (Moasser, 2007; Timms et al., 2002). Trastuzumab acts by binding to and inactivating the HER2 receptor, thus arresting the cell in G1 (Gajira & Chandarlapaty, 2011).

In addition to hormone therapy, polychemotherapy is the most common adjuvant treatment regimen for breast cancer. According to The MD Anderson Manual of Medical Oncology, the current standard of care is a combination of three drugs: doxorubicin, cyclophosphamide and 5-flourouracil (Patel et al., 2011). Doxorubicin intercalates with the DNA, which inhibits its two strands from reannealing (“Definition of doxorubicin hydrochloride - NCI Drug Dictionary - National Cancer Institute,” n.d.). This action halts transcription and promotes double-strand breaks in the DNA by stabilizing the Topoisomerase II complex (“Definition of doxorubicin hydrochloride - NCI Drug Dictionary - National Cancer Institute,” n.d.). Cyclophosphamide activates apoptotic pathways by creating unusual linkages between guanine base pairs at the N7 position in the DNA that the cell machinery recognizes as errors (“Definition of cyclophosphamide - NCI Drug Dictionary - National Cancer Institute,” n.d.; Sausville & Longo, 2015). 5-flourouracil inhibits the production of thymidine, causing failures in S-phase, and ultimately cell death (Sausville & Longo, 2015). Therefore, these three drugs halt DNA synthesis, activate apoptosis, and destroy cancer cells.
Another commonly used class of chemotherapeutics includes the taxanes (paclitaxel, taxol), which act by stabilizing microtubules during mitosis (Weaver, 2014). Stabilization of microtubules prevents normal mitotic completion, thus promoting cell death after a period of prolonged mitotic arrest.

Drug resistance from breast cancer chemotherapeutics has become a topic of increasing concern. Even with ample access to these various chemotherapeutics and treatments, prognosis is often poor among certain types of breast cancer. Some breast cancers do not respond to the currently existing armament of treatment options. Cancers can also acquire resistance to chemotherapeutic agents and are able to proliferate despite the presence of the various drugs.

Cells develop resistance to chemotherapeutics for several reasons. One theory is that cancer cells gain a survival advantage through natural selection processes (Harvey et al., 2013; Lai et al., 2011). Drug-resistant cells are less fit to survive normally, and comprise a small portion of the population of cancerous cells. However, these cells are able to proliferate during chemotherapy when the cells that are not drug resistant die, thus giving them a selective advantage (Harvey et al., 2013; Lai et al., 2011). Another theory is that chemotherapy drugs are excluded from the cells through increased expression of multi-drug resistant efflux pumps, such as ATP-dependent p-glycoproteins, which then remove the drugs from cells (Gottesman et al., 2002).

Even with the various treatment options, breast cancer continues to result in a significant number of deaths per year. Breast cancer is caused by a wide array of genetic mutations, and such genetic heterogeneity poses a significant challenge in terms of
implementing therapeutics. The scientific community as well as the government has devoted a significant amount of time and money into trying to gain a better understanding of cancer, moving towards personalized medicine and pharmacogenetics in order to improve patient treatment and survival. Research is now focused on identifying novel, effective and more targeted chemotherapeutics to improve breast cancer prognosis.

**Common Drivers of Breast Cancer**

There are a host of different factors that can promote the development of breast cancer. Loss-of-function mutations in tumor suppressor genes, or gain-of-function mutations in proto-oncogenes can lead to uncontrolled cellular proliferation and are common hallmarks of cancer (Meric-Bernstam & Pollock, 2014).

Tumor suppressors are involved in a multitude of different regulatory processes, including cell cycle regulation, apoptosis, and DNA repair (“Tumor suppressor gene | Broad Institute of MIT and Harvard,” n.d.). Functional tumor suppressor genes are essential to prevent abnormal cells from proliferating, and mutations in these genes can lead to deregulation of the cell cycle, uncontrolled cell growth in the presence of damaged DNA, and ultimately cancer (Meric-Bernstam & Pollock, 2014). The most commonly known loss-of-function mutations of tumor suppressor genes in breast cancer include *TP53*, which codes for p53, a critical cell cycle regulator, and *BRCA1/BRCA2*, which are both DNA repair genes (Lee and Muller, 2010). Forty percent of breast cancer cases contain a mutation in *TP53* while *BRCA1* and *BRCA2* mutations account for 25% of hereditary breast cancers (Lippman, 2015).
Oncogenes arise from proto-oncogenes through changes in the genetic code, such as chromosome rearrangement, gene duplications, or mutations in base pairs in coding sequences (Morin et al., 2015). Oncogenes promote cell proliferation, growth, differentiation, and survival and include growth factors/mitogens, receptor tyrosine kinases, serine/threonine kinases, regulatory GTPases, and transcription factors (Morin et al., 2015). Commonly mutated oncogenes in cancer include: CCND1, which codes for Cyclin D1, an important cell cycle regulator; PI3KCA which codes for a PI3K, a kinase that promotes growth, proliferation and survival; ERBB2, which codes for a receptor tyrosine kinase; and MYC, a transcription factor promoting proliferation (Lee and Muller, 2010).

Recent studies have also shown that aberrant Hippo pathway functioning can drive tumor formation and metastasis due to its critical role in regulating cellular proliferation and organ size (Aragona et al., 2013; Low et al., 2014). In recent years, inactivation of the Hippo tumor suppressor pathway (see below) has come to the forefront as a recognized occurrence in human breast cancers.

The Hippo Tumor Suppressor Pathway

The Hippo pathway plays a critical role in controlling the vital biological process of cellular proliferation, growth and survival (Morioishi et al., 2015; Ganem et al., 2014) Although originally discovered through genetic screening of tumor suppressor genes in Drosophila melanogaster, the Hippo pathway is important in human biology and has broad implications in disease etiology, gaining recognition particularly for its role in cancer (Ganem et al., 2014; Morioshi et al., 2015; Pan, 2010).
The Hippo pathway exists to negatively regulate YAP and TAZ activity. Central to the Hippo pathway are protein kinases known as LATS1/2, which negatively regulate the two transcriptional co-activators, YAP and TAZ (Ganem et al., 2014; Morioishi et al., 2015). YAP and TAZ are functional paralogues containing a WW domain, a 14-3-3 binding domain, a coiled-coil motif and a PDZ-binding motif (Liu et al., 2011). LATS1 and LATS2 are tumor suppressor proteins that are members of the NDR family of kinases, which are proteins involved in regulating mitosis, as well as cell growth and development (Hergovich et al., 2006; Hergovich et al., 2013). These kinases require phosphorylation of serine or threonine residues within their activation domain to become functional (Hergovich et al., 2006).

When the Hippo pathway is turned on, LATS1/2 is activated by several different mechanisms. One commonly studied mechanism is through MST1/2. The protein kinase MST1/2 dimerizes and binds to its adapter protein, SAV1, which enhances its kinase activity (Ambrose 1996; Hong & Guan, 2012; Ni et al., 2015; Yu & Guan, 2013). MST1/2-SAV1 then phosphorylates MOB, a kinase-binding protein, at two threonine residues at positions 12 and 35, allowing it to form a complex with LATS1/2 (Hong & Guan, 2012; Ni et al., 2015; Yu & Guan, 2013). The MST1/2-SAV complex then phosphorylates a threonine residue in the hydrophobic motif of LATS1/2 (T1079 in LATS1; T1041 in LATS2) in the LATS/MOB complex (Hong & Guan, 2012; Ni et al., 2015; Yu & Guan, 2013). After threonine-1079 is phosphorylated, LATS 1 is able to then autophosphorylate a serine residue (S909) in the T-loop of its activation domain (Hong & Guan, 2012; Ni et al., 2015; Yu & Guan, 2013). LATS1/2 must be phosphorylated at
both sites to become active (Ni et al., 2015; Yu & Guan, 2013). Once activated, LATS 1/2 binds via a PPxY motif to the WW domains of YAP/TAZ (Hong & Guan, 2012; “YAP1 Gene - GeneCards | YAP1 Protein | YAP1 Antibody,” n.d.). LATS1/2, a kinase, phosphorylates the transcriptional co-activators YAP and TAZ at up to 5 (YAP) or 4 (TAZ) sites (Hong & Guan, 2012; Yu & Guan, 2013). Phosphorylated YAP/TAZ binds to the 14-3-3 adaptor proteins that sequester YAP/TAZ in the cytoplasm, which can be subsequently ubiquitinated and degraded by proteasomes (Ganem et al., 2014; Lamar et al., 2012; Morioishi et al., 2015; Yu & Guan, 2013).

The degree of phosphorylation of YAP and TAZ also dictates the cytoplasmic to nuclear ratios. When all five sites are phosphorylated, YAP will likely be sequestered almost exclusively in the cytoplasm, while YAP with only one site phosphorylated will have less protein sequestered in the cytoplasm and more protein in the nucleus. Phosphorylation of YAP/TAZ is critical to its sequestration in the cytoplasm, as serine to alanine mutations in the critical phosphorylation sites (YAP5S4A or TAZ4S4A) result in constitutively active YAP and TAZ located almost exclusively in the nucleus (Ganem et al., 2014).

While LATS1/2 activation through MST1/2 seems to be essential in Drosophila melanogaster, newer studies discovered that Hippo pathway and LATS1/2 activation in humans are MST1/2-independent and can be activated by other kinases, such as MAP4K family members (Meng et al., 2015; Zheng et al., 2015). Regardless of how it is activated, activated LATS1/2 results in phosphorylation of YAP and TAZ and leads to cytoplasmic sequestration and decreased transcription of YAP/TAZ target genes.
When the Hippo pathway is off, LATS1/2 is inactive and thus unable to phosphorylate YAP and TAZ (Morioishi et al., 2015). Without the added phosphate groups, YAP and TAZ can then move into the nucleus. YAP and TAZ lack the ability to bind DNA, so instead, they must bind to a family of DNA-binding transcription factors (Yu & Guan, 2013). Transcriptional enhancer activator DNA-Binding (TEAD) proteins, members of the transcriptional enhancer family of proteins, are the most common target transcription factor of the canonical Hippo pathway (Yu & Guan, 2013). Other target transcription factors include SMAD, p73, Paired Box Gene 3 (PAX3), and Runt-Related Transcription Factor 2 (RUNX2) (Mauviel et al., 2012; Nallet-Staub et al., 2014). Binding of YAP and TAZ to TEAD leads to the transcription of pro-survival, pro-growth, and anti-apoptotic genes, such as connective tissue growth factor (CTGF), epidermal growth factor proteins such as amphiregulin, and cysteine-rich angiogenic inducer 61 (CYR61) (Aqeilan, 2013; Miroishi et al., 2015; Plouffe et al., 2015; Zhao et al., 2010). A host of other YAP and TAZ target genes have yet to be defined, and are part of the focus of this project. Figure 4 is a pictorial representation of the on and off states of the Hippo pathway.
When the Hippo pathway is on, LATS1/2 is activated via phosphorylation and forms a complex with MOB. LATS1/2 then phosphorylates the two transcriptional co-activators YAP and TAZ, which sequesters them in the cytoplasm. When sequestered in the cytoplasm, YAP and TAZ are then bound to 14-3-3 adaptor proteins, and ultimately degraded via an E3 ubiquitination dependent mechanism. When the Hippo pathway is off, LATS1/2 is inactive, and YAP and TAZ are not phosphorylated, so they are able to move into the nucleus where they bind to TEAD and turn on transcription of pro-growth and survival genes. Adapted from Johnson and Halder (2014).
Upstream Regulators of the Hippo Pathway

The Hippo pathway with its homologous transcriptional co-activators YAP and TAZ are part of an intricate network that can be influenced by a host of other factors. While many of these factors are still unknown, increased evidence shows that the Hippo pathway plays a role in a number of cellular processes.

As shown in Figure 5, activators of the Hippo pathway inhibit cell growth and promote apoptosis and include metabolic stressors (oxidative or energetic), cytoskeletal defects caused by detachment, contact inhibition, or growth factor deprivations, and cytokinesis failure (Ganem et al., 2014; Hansen et al., 2015; Kim et al., 2015; Wennmann et al., 2014). Inhibitors of the Hippo pathway promote cellular proliferation and include osmotic, endoplasmic reticular and mechanical stressors as well as DNA damage (Hansen et al., 2015). The precise mechanism detailing how each process regulates the Hippo pathway has yet to be elucidated.

Nevertheless, upon examining upstream regulators of the Hippo pathway from a broader perspective, it becomes apparent that Hippo pathway regulation can be categorized into one general concept: changes in the actin cytoskeleton. The Hippo cascade appears to consistently respond to changes in its cellular cytoskeletal network. When a cell expands or stretches, the Hippo pathway is inactivated, promoting nuclear accumulation of YAP and TAZ (Dupont et al., 2011; Yu & Guan, 2013). When a cell is compressed, the Hippo pathway is on and prevents YAP/TAZ accumulation in the nucleus and cell growth (Dupont et al., 2011; Yu & Guan, 2013). Complete disruption of
the cytoskeleton, such as through treatment with Latrunculin A, an actin polymerization inhibitor, results in Hippo pathway activation, which then sequesters YAP in the cytoplasm (Thomasy et al., 2013). Hippo pathway activity can also be modulated through changes to the actin cytoskeleton induced by tetraploidy, glucose deprivation, serum starvation, contact inhibition and loss of cell adhesion, which are also, not coincidentally, key players in cancer progression (Ganem et al., 2014; Hansen et al., 2015). Each of these different upstream Hippo regulators will be discussed briefly in the next sections.
Figure 5: Examples of the Upstream Regulators of the Hippo Pathway.
YAP and TAZ serve as the effectors of the Hippo pathway. These two transcriptional co-activators bind to TEAD and are responsible for promoting growth and survival. The Hippo pathway is regulated by a plethora of different factors, many of which have yet to be defined. Some of the possible upstream regulators are shown in this figure. Adapted from Hansen et al. (2015).
Cellular Polarity

Cellular polarity, defined as having a defined apical and basal side, is important to maintaining the structure and function of epithelial cells. ABCP (apicobasal cell polarity) proteins define the polarity of the cell.

Proteins that establish cell polarity in epithelial cells at cell-cell junctions can activate the core Hippo cascade and thus play an important regulatory role (Hansen et al., 2015; Yu & Guan, 2013). For example, in *Drosophila melanogaster*, the formation of an apical trimeric complex between NF2, Expanded and Kibra is known to activate the Hippo pathway (Yu & Guan, 2013; Yu et al., 2010). Specifically, NF2 and Expanded (Ex) associate with Salvador (Sav) and Hippo (Hpo), the homologues of SAV1 and MST1/2, respectively, and Kibra associates with Warts (Wts), the homologue of LATS1/2 (Yu & Guan, 2013). The association of these Hippo kinases with the trimeric complex at the apical domain triggers activation of the Hippo kinase cascade (Genevet et al., 2011; Hansen et al., 2015; Yu & Guan, 2013; Yu et al., 2010).

Scribble (SCRIB), a basolateral protein, is also known to have a dual role in regulating cellular polarity and Hippo signaling. SCRIB responds to changes in cell density by promoting the assembly of the Crumbs complex at the apical border of the cell at tight junctions that promotes YAP/TAZ phosphorylation by LATS1/2 and subsequent cytoplasmic retention (Varelas et al., 2010).

Alpha-catenin, located at the adherens junction, is another known ABCP that influences Hippo pathway activation. Alpha-catenin links the actin cytoskeleton to other
cadherins (Yu & Guan, 2013). When the Hippo pathway is on, alpha-catenin binds with p-YAP and sequesters it at the adherens junction, thereby preventing it from moving into the nucleus (Wang et al., 2012; Yu & Guan, 2013). Mutations in E-cadherin, another protein located at the adherens junction, can lead to the loss of proper cell-cell contact, and promote EMT (Hansen et al., 2015; Harvey et al., 2013). Other adherens junction proteins such as Protein Tyrosine Phosphatase 14 are also able to bind to and sequester p-YAP in the cytoplasm (Wang et al., 2012; Yu & Guan, 2013). The ability of these proteins to sequester p-YAP in the cytoplasm prevents transcription of YAP/TAZ target genes, and thus are important regulators of the Hippo pathway (Wang et al., 2012; Yu & Guan, 2013).

AMOT proteins maintain cellular polarity and are able to sequester YAP/TAZ in the cytoplasm by recruiting them to tight junctions or the actin cytoskeleton (Wells et al., 2006; Yu & Guan, 2013; Zhao et al., 2011). AMOTL1, a tight junction protein, is one isoform of angiomotin, and responds to low energy states by sequestering YAP in the cytoplasm either by physically binding directly to YAP, or by activating LATS1/2 which then phosphorylates YAP, or by decreasing the stability of YAP by promoting its ubiquitination via AMOT130 and AIP4/Itch E3 ligase (Alder et al. 2013 (a); DeRan et al., 2014).

Proper maintenance of cellular polarity is critical to maintaining Hippo pathway functioning and tissue homeostasis. Changes to the proteins involved in regulating cellular polarity at the adherens junctions or tight junctions can lead to dysregulation and
inactivation of the Hippo pathway, resulting in increased and uncontrolled transcription of YAP/TAZ target genes (Varelas et al., 2010).

Cell Adhesion

Cellular adhesion exists in two general forms: cell-cell adhesion and cell-matrix adhesion. Proper adhesion is important in maintaining structure and function, and loss of proper connectivity in either type can cause cellular distress and promote tumorigenesis.

Epithelial cells are connected with each other through cell-cell adhesion molecules at tight junctions and adherens junctions. Proteins that promote cell-cell adhesion include SCRIB, AMOT, PALS, E-cadherin and alpha-catenin activate the Hippo pathway by binding with YAP and TAZ, localizing them to the cell-cell junctions, and preventing YAP and TAZ from entering the nucleus (Hansen et al., 2015; Harvey et al., 2013).

Anoikis, or loss of adhesion between the cell and the extracellular matrix and movement into lumen, is tightly controlled by the Hippo pathway and normally induces programmed cell death (Paoli et al., 2013). Cell detachment due to cytoskeletal changes such as actin or microtubule reorganization triggers activation of the Hippo pathway, turning on LATS1/2 and excluding p-YAP from the nucleus (Zhao et al., 2012). Thus, appropriate functioning of the Hippo kinase cascade is critical in promoting nuclear exclusion of YAP/TAZ and inducing anoikis; dysregulation of the Hippo pathway results in uncontrolled proliferation via upregulation of YAP/TAZ as well as anoikis resistance (Paoli et al., 2013; Zhao et al., 2012). Resistance to anoikis allows cells to survive that would have otherwise died following detachment from the extracellular matrix, and
promotes changes in cellular morphology such as EMT and invasiveness (Hansen et al., 2015; Zhao et al., 2012). Such resistance then transforms relatively benign cells into malignant cells that can grow anchorage-independently, promoting EMT, invasion of distant sites and tumor progression (Paoli et al., 2013; Zhao et al., 2012).

**Contact Inhibition**

Contact inhibition is vital in controlling tissue growth and homeostasis. When cells come into contact with neighboring cells, normal proliferating cells typically undergo contact inhibition, and stop proliferating in order to avoid overgrowth (Meric-Bernstam & Pollock, 2014). Contact inhibition is of particular interest in cancer research as one common characteristic of cancer cells is loss of contact inhibition (Meric-Bernstam & Pollock, 2014). While the evidence points to the importance of the Hippo pathway in regulating this process, the details to understanding the underlying mechanism of how it occurs is currently being investigated.

The Hippo pathway plays a role in contact inhibition due to its importance in controlling growth and proliferation as well as organ size. Studies have found that the Hippo pathway modulates cellular responses to changes in cell density, where high density cell cultures had more cell-cell contact including increased adherens and tight junctions with increased signaling that lead to Hippo pathway activation and YAP inactivation (Meng et al., 2016; Zhao et al., 2007). High cell density has also been shown to activate the Hippo pathway through TGF-β and other mechanisms (Varelas et al. 2010). Cells with an inactivated Hippo pathway, through either knockdown of NF2 (an upstream activator of the Hippo pathway) or overexpression of YAP, are not contact
inhibited and can continue to divide indefinitely (Harvey et al., 2015; Lallemand et al., 2003).

Mitogenic signaling and soluble growth factors can also mediate contact inhibition through Hippo pathway inactivation. For example, EGF was found to stimulate PI3-kinase (PI3K) and prevent PDK1 binding to the SAV1/LATS complex, thus inactivating the Hippo pathway and stimulating growth (Fan et al., 2013; Gumbiner and Kim, 2014). An interesting result discussed by Gumbiner and Kim (2014) from the Kim et al. (2009) study is the dose-dependent nature of contact inhibition in cells. Within a cluster of cells, the Hippo pathway tends to be inactivated towards the edges rather than at the center of the cluster, and contains higher levels of nuclear YAP on the edges (Gumbiner & Kim, 2014; Kim et al., 2009). Selective activation of the Hippo pathway would allow cells to proliferate in less populated areas rather than in more populated areas at the center of its cluster (Gumbiner & Kim, 2014; Kim et al., 2009).

Marked changes in cell density can also alter cell morphology from cytoskeletal rearrangements. Such changes are intricately linked to Hippo pathway activity (Wada et al., 2011). At low densities, the Hippo pathway is inactive, and cells adapt a more flattened and spread morphology, with visible f-actin stress fibers to promote cell proliferation (Wada et al., 2011). At higher densities, the Hippo pathway becomes activated, and cells adapt a more rounded and less spread out morphology, with less f-actin stress fibers (Wada et al., 2011).
Glucose Deprivation

Glucose or energy starvation has been shown to activate the Hippo pathway and sequester YAP in the cytoplasm. Glucose deprivation activates an AMPK-mediated signaling cascade, leading to cytoplasmic retention of YAP via the formation of an AMOT-YAP complex (DeRan et al., 2014).

Serum Starvation

Serum contains hormones and growth factors that allow cells to grow and proliferate in culture. Serum starvation inhibits cell growth and movement and rearranges the cytoskeleton into a more flattened morphology (Giuliano & Taylor, 1990). Depriving cells of serum also activates the Hippo pathway in a LATS1/2 and AMOT 130-dependent fashion, ultimately binding and sequestering YAP in the cytoplasm in order to prevent cell growth in the absence of growth factors (Adler et al., 2013b).

Tetraploidy

Whole genome duplication has been documented in around 37% of human cancers (Dewhurst et al., 2014; Ganem et al., 2014; Zack et al. 2013). Genomic duplication arises through genetically unstable tetraploid cells that can then become oncogenic if proliferative (Ganem et al., 2014).

Tetraploidy, or cells with twice the number of normal chromosomes, is a common characteristic of many types of cancers, including breast cancer (Davoli & de Lange, 2011). Tetraploid cells can result from several different processes, including endoreduplication, oncogene activation, viral-induced cell fusion, chronic inflammation,
telomere erosion and most commonly errors in mitosis and cytokinesis (Davoli & de Lange, 2011; Ganem et al., 2014; Ganem et al., 2012; Ganem et al., 2007). As a cell becomes tetraploid, it can not only accumulate more copies of the same gene, but also random rearrangements and mutations, which can then confer a selective advantage to abnormal tetraploid cells over normal diploid cells (Davoli & de Lange, 2011). This phenomenon is advantageous to the tetraploid cells for two reasons. First, tetraploid cells contain extra copies of the same gene and thus are more able to survive mutations in the genome due to this buffering effect (Davoli & de Lange, 2011; Thompson et al., 2006). Secondly, increases in the amount of centrosomes can disrupt proper spindle assembly during mitosis and lead to abnormal segregation of chromosomes, increases in the number of tetraploid cells, and disruption of proper cellular functioning (Bolgioni & Ganem, 2016; Ganem et al., 2009).

Normal cells possess checkpoints to avoid proliferation of tetraploid cells. The two checkpoints are at the transition between G\(_1\)-phase (Gap1) and S-phase (synthesis) and G\(_2\)-phase (Gap2) and M-phase (Mitosis) (Meric-Bernstam & Pollock, 2014). Rb serves as the main gatekeeper at the G\(_1\)/S checkpoint (Clark and Longo, 2015; Meric-Bernstam & Pollock, 2014). Once the cell is prepared to synthesize its DNA in S-phase, Rb is phosphorylated by cyclin-dependent kinases, and inactivated, allowing the cell to progress into S-phase (Clark and Longo, 2015; Meric-Bernstam & Pollock, 2014). Prior to mitosis and after completion of DNA replication, the cell must go through another checkpoint to ensure the fidelity of the replication (Clark and Longo, 2015). If DNA damage is detected, the ATM pathway is activated to repair DNA. ATM activates p53,
which either halts cell cycle progression or repairs damaged DNA or initiates apoptosis for irreparable DNA damage (Clark and Longo, 2015).

In contrast to normal cells, tetraploid cancer cells do not possess proper checkpoints. This lack of regulation allows cells to bypass critical checkpoints and proliferate in instances that it would not have otherwise progressed through the cell cycle (Ganem et al., 2014). The ability of tetraploid cells to bypass these critical checkpoints to proliferate is thought to be a direct result of low levels of the p53 tumor suppressor. Depletion of p53 enables tetraploid cells to progress through both the G1/S checkpoint and the G2/M checkpoint (Ganem et al., 2014). Deletion or mutation of the p53 gene can thus allow cancer cells to grow and proliferate rapidly, and is found in almost 40% of cancers (Lippman, 2015). Interestingly, cells can also evade p53-dependent tetraploid arrest through sustained growth factor signaling mechanisms (Ganem et al., 2014).

Tetraploidy is intimately linked to the core Hippo kinase cassette, and has gained recognition for its role in cancer. Proliferating tetraploid cells are chromosomally and genetically unstable (Ganem et al., 2007). Additionally, Ganem et al. (2014) discovered that the level of p53 was directly correlated with Hippo pathway activity in tetraploid cells. When the Hippo pathway was active, the amount of p53 increased in the cell; when the Hippo pathway was inactive, p53 levels were low (Ganem et al., 2014). LATS2, a core Hippo pathway kinase, is a negative regulator of p53. When the Hippo pathway is active, LATS2 binds to MDM2, an E3 ubiquitin ligase, and prevents it from interacting with and degrading p53 (Aylon et al., 2006; Ganem et al., 2014). Thus, an activated Hippo pathway stabilizes and increases p53 levels in the cell, decreasing proliferation
two-fold in tetraploid cells (Ganem et al., 2014). When the Hippo pathway is inactive, MDM2 is free to interact with p53, and repress its tumor suppressor functions.

**The Role of Hippo Pathway Inactivation in Breast Cancer**

Hyperactivation of the proto-oncogenic transcriptional co-activators YAP and TAZ, due to either inactivation of the Hippo pathway or copy number amplification of \( YAP1 \) and \( WWTR1 \), the genes that code for YAP and TAZ on the 11q22 and 3q25 ampiclons, respectively, has been implicated in several types of cancers, including breast cancer, pancreatic ductal metaplasia, hepatocellular carcinomas, squamous cell carcinomas, gastrointestinal dysplasia, and medulloblastomas (Bao et al., 2011; Barron & Kagey, 2014; Cordenonsi et al., 2012; Lamar et al., 2012; Muramatsu et al., 2011; Overholtzer et al., 2006). Nuclear accumulation of hyperactivated YAP/TAZ stimulates increased transcription of pro-growth and survival genes, leading to uncontrolled proliferation and inhibition of apoptosis.

The oncogenic properties of YAP/TAZ are due to their role in promoting transcription of pro-growth and survival genes. Mutant forms of YAP/TAZ fail to transcribe these target genes, even when overexpressed, highlighting their role as transcriptional activators in inducing oncogenic transformation (Zhao et al., 2012). Overexpression of YAP itself is not sufficient to develop cancer, as the transcriptional co-activator lacks a DNA-binding domain. YAP must bind to its transcriptional factor, TEAD in the nucleus in order for its target genes to be transcribed. Disruption of the interaction between YAP and TEAD via verteporfin inhibits YAP’s oncogenic activity (Lamar et al., 2012; Liu-Chittenden et al., 2012). Thus, YAP/TAZ’s role as an oncogene
is highly dependent on its ability to resist changes in the TEAD binding site that promotes its transcriptional activity.

In addition to an increasing proliferative potential, overactivation of the oncogenic transcriptional co-activators YAP/TAZ can promote cancer progression and metastasis through epithelial-to-mesenchymal transition (EMT), loss of contact inhibition, loss of cell-cell adhesion, resistance to anoikis, and uncontrolled proliferation and inhibition of apoptosis through TNF-induced and CD95 (FAS) induced apoptosis (Lamar et al., 2012; Overholtzer et al., 2006; Paoli et al., 2013).

Breast cancer cells are especially interesting to study with regards to the Hippo pathway, as they typically are characterized as being tetraploid, containing twice the normal number of chromosomes (Ganem et al., 2007). In a normal cell, tetraploidy activates the Hippo pathway and halts cell cycle progression to prevent proliferation of tetraploid cells, and the cells are quiescent and enter into the resting G0 phase. However, in breast cancer cells, hyperactivated YAP/TAZ allows cells to bypass cellular checkpoints and continue to grow and proliferate abnormally as well as progress through the cell cycle more rapidly (Harvey et al., 2013; Huang et al., 2005; Tapon et al., 2002). Thus, breast cancer cells serve as a useful model to better understand the implications of Hippo pathway inactivation and as a potential route to exploit in the development of chemotherapeutics.

While the Hippo pathway is often functionally inactivated in cancer cells, the mechanism of what causes Hippo pathway inactivation and subsequent YAP/TAZ hyperactivation in breast cancer cells has yet to be elucidated. Somatic and germline
mutations are rare, yet Hippo pathway inactivation is common among cancer cells, begging an explanation as to how these cells can all functionally inactivate the Hippo pathway (Johnson & Halder, 2014). Additionally, little is known about whether or not hyperactivation of YAP/TAZ makes the cancer cells become more dependent on the YAP/TAZ target genes for their survival.

**Specific Aims**

Hyperactivation of YAP/TAZ in breast cancer cells, due to Hippo pathway inactivation or from copy number amplification of the YAP/TAZ gene, is common in human breast cancers. However, the mechanism by which YAP/TAZ hyperactivation leads to tumor formation has not yet been clearly defined. It is unknown whether or not breast cancer cells are dependent on YAP/TAZ for survival and growth. Additionally, the downstream target genes of YAP/TAZ important for promoting cell growth and survival have not yet been defined.

In order to better understand the role of YAP/TAZ hyperactivation in breast cancer cells, the goals of this study are two-fold:

1. **To determine YAP/TAZ-dependency in a comprehensive panel of breast cancer cells:**
   a. We will use shRNA to knockdown YAP and TAZ activity, and assess cell viability using previously established threshold criteria.
   b. We will then compare basal state YAP/TAZ expression levels with our results from the YAP/TAZ-dependency experiments by using orthogonal and unbiased approaches.
2. To generate gene expression profiles for YAP/TAZ-dependent and YAP/TAZ-independent cell lines:

   a. We will derive gene expression profiles for all of our breast cancer cell lines using RNA sequencing techniques.

   b. We will then compare the transcriptome of the YAP/TAZ-independent cells with that of the YAP/TAZ-dependent cells, identifying any target genes that display increased expression in YAP/TAZ-dependent cells.

The first aim will allow us to characterize each cell line as either YAP/TAZ-independent or YAP/TAZ-dependent, and provide critical information regarding the dependence of breast cancer cells on YAP/TAZ for cell growth and survival. We will also be able to determine if there is an association between YAP/TAZ transcriptional activity and YAP/TAZ-dependency status, and the nature of that association. The second aim will allow us to characterize important YAP/TAZ target genes, such as those important in driving tumor cell growth, proliferation, and survival, which can generate novel ideas for developing effective chemotherapeutics in patients diagnosed with YAP/TAZ-dependent breast cancers.

We believe that a better understanding of the Hippo pathway can be a powerful tool in developing chemotherapeutics. Our hypothesis is that certain breast cancer cells are dependent on YAP/TAZ for their survival, explaining why YAP/TAZ is commonly hyperactivated. This discovery would lend insight into the development of novel chemotherapeutics for patients with YAP/TAZ-dependent subtypes of breast cancer.
Since YAP and TAZ themselves have no enzymatic activity and thus are not “druggable”, identifying and targeting YAP and TAZ target genes would provide the means of effectively targeting the YAP/TAZ-dependent subtypes of breast cancer. With our current study, we hope to gain insight into better understanding the role of the Hippo pathway in breast cancer cells.
METHODS

Cell Culture:

Our panel of breast cancer cells consisted of 45 verified breast cancer cell lines obtained from American Type Culture Collection (ATCC). All cell lines were cultured in a sterile tissue culture hood according to the manufacturer’s instructions (ATCC). All cell lines in this panel were certified to be mycoplasma-free and were authenticated by ATCC using short tandem repeat (STR) profiling. STR profiling is used to identify cross-contaminated, mislabeled, or genetically drifted cells. This cell line authentication is critical, as many funding agencies and scientific journals are now making this practice mandatory. Cell lines were immediately stored in our liquid nitrogen tank.

To thaw cell lines, one ml of aliquoted cells was placed into a 15 cm conical vial with 9 ml of the appropriate media, and then spun down in the centrifuge at 1250 rpm for 5 minutes. The supernatant was aspirated, and the pellet was re-suspended in 10 ml of media plus Invivogen plasmocin (25 µg/ml), plated on a 10 cm dish and then placed in an incubator at 37°C, 5% CO₂.

Twenty-four hours later, the media was replaced with fresh and warm media with plasmocin (25 µg/ml). After one week, the concentration of plasmocin was lowered to 2.5 µg/ml. All cells were grown in an incubator at 5% CO₂ (with the exception of cells growing in Leibovitz’s L-15 media, which required 0% CO₂), 37°C, until reaching around 70% confluence. Cells were monitored daily, and media was replenished every 2-3 days, with a PBS wash prior to media replacement. Media was stored in a dark and cold place.
All cell lines required serum in their media. In order to minimize variability due to batch-to-batch variations in serum, the same lot number serum was used for all cells. For cells with fetal bovine serum in their media, we used ATCC Lot # 62818223, Product #30-2020. For cells with horse serum, we used ATCC Lot # 61373986 Product #30-2040. All cell lines were treated with media containing 1% penicillin/streptomycin.

Cells were split once they reached 70-80% confluence. For cell lines that used trypsin, the media was aspirated, the cells were then washed with 1 ml of PBS and aspirated off, and 2.5 ml of 0.25% trypsin-EDTA was added directly to the cells. Once the trypsin was added, the 10 cm dish was placed at 37°C to facilitate detachment. Cells were closely monitored under the microscope, and after about 3 to 5 minutes (or however long it took the cells to detach), seven ml of media was added to inactivate the trypsin. MCF10A required the use of 3 ml of 0.1% soybean trypsin inhibitor instead of media to neutralize the trypsin. Two adherent cell lines, MDA-MB-415 and MDA-MB-436, required scraping using a cell scraper instead of trypsin to detach cells for splitting. Cells were collected into a 15 ml vial, and spun down at 1250 rpm for 5 minutes. The supernatant was aspirated, and 10 ml of media was added directly to the pellet.

Ten microliters from the 10 ml vial of cells were counted using a hemocytometer. After counting cells, we calculated the appropriate amount of cells to add to each well in order to get 50,000 cells per well for a total of six wells per cell line. For each cell line, three of the six wells were grown to subconfluence (50%), and three were grown to confluence (90%). The remainder of the cells was divided among two 10 cm culture
dishes and four 15 cm culture dishes. The 10 cm and 15 cm culture dishes contained a total volume of 10 ml and 20 ml, respectively.

Six cell lines were suspension cells. For the suspension cell lines, we added 1 ml of the aliquot of cells to 9 ml media, spun them down, and then placed them into a flask. Each time we split these cells, we took half of the media plus cells and placed that into a new flask, and then added more fresh media. We did not fix and stain these cell lines.

Once the cell lines in either the 10 cm dishes (adherent cells) or the flasks (suspension cells) reached 70% confluence, we extracted protein and RNA (see below). When the adherent cells in 15 cm culture dishes and the suspension cells in the flasks reached 70-80% confluence, these cells were trypsinized, neutralized with media, spun at 1250 rpm for five minutes, and resuspended into 18 ml of media containing 5% DMSO to be frozen down into 18X 1 ml aliquots. We placed all 18 of the 1 ml aliquots into a Nalgene Cryo 1°C freezing containers, which we then put into the -80°C freezer for 24 hours. The Nalgene Cryo containers allowed the cells to freeze at a constant rate of -1°C every hour. After 24 hours, we stored the aliquots in the liquid nitrogen tank. Ten vials were stored in the Ganem Lab’s library, and 2 vials were stored in each of the other four libraries to be distributed to other labs at Boston University. Each library’s contents was recorded.

**RNA Extraction:**

RNA extraction was performed to obtain gene expression profiles for all cell lines. Cells plated in 10 cm culture dishes were cultured until they reached 70% confluence. RNA was extracted from all cell lines using the Qiagen RNeasy Mini Kit and
protocol, and performed in a chemical hood. The chemical hood, all reagents and supplies used, and gloves were thoroughly sprayed and wiped using Ambion’s RNAse Zap Wipes and spray to remove any contamination. For the adherent cell lines, old media was aspirated, and 600 µl of the RLT lysis buffer was added directly to the 10 cm dish, and then collected after cell scraping into a 1.5 ml conical tube. The suspension cell lines were spun down in the centrifuge at 1250 rpm, and the RLT lysis buffer was added directly to the cell pellet. Lysed cells with RLT buffer were passed through a 20 gauge needle five times to homogenize the sample. The remaining steps were followed exactly as outlined in the Qiagen RNeasy kit instructional manual, including performing all optional steps and DNA digestion. Upon completion of the protocol, we used the Nanodrop 2000 spectrophotometer to measure the RNA concentration of each sample and ensure purity. The samples were stored at -80°C until we collected RNA from all cell lines. The RNA samples were sent as one batch to the BUSM Microarray Core, where they were assessed with Human Gene 2.0 ST microarrays.

**Protein Extraction and Western Blot:**

Stock solutions of Radio Immune Precipitation Assay (RIPA) buffer for use in protein extraction were made by adding 1 Roche Diagnostics GmbH Complete Mini EDTA-Free protease inhibitor tablet and 100 µl of ThermoFisher Scientific’s phosphatase inhibitor cocktail to 10 ml of Boston BioProduct’s RIPA buffer (50 mM Tris-hydrochloric acid, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS and mixed by vortexing. Five hundred microliter stocks were stored at -20°C for future use.
Once the cell lines reached 70% confluence, we extracted protein from either the 10 cm dish for adherent cells or the 50 ml flasks for suspension lines.

For adherent cell lines, the old media was aspirated, and the cells were washed twice with 1-2 ml of cold PBS. After the second wash of PBS was aspirated, 400 microliters of RIPA buffer with protease and phosphatase inhibitors (thawed and stored on ice) was added in a drop-wise fashion to the 10 cm culture dish and swirled for 30 seconds. A cell scraper was used to collect all of the extract into a 1.5 ml conical tube. For suspension cell lines, the cells plus media were first centrifuged at 1250 rpm for 5 minutes, the supernatant was aspirated, and 400 µl of RIPA buffer with protease and phosphatase inhibitors was added directly to the pellet.

Next, the extract containing RIPA buffer with protease and phosphatase inhibitors with cells (either suspension or adherent) was rotated in the cold room for 10 minutes. After 10 minutes, the extract was centrifuged for 10 min at 4°C at 17.0Xg. Avoiding the pellet, 400 µl of the sample’s supernatant was collected, and 133 µl of 4X Laemmli buffer was added to obtain a final concentration of 1X. The sample was then vortexed, touch spun in the microcentrifuge for 10 seconds, boiled at 95°C for 5 minutes, and then touch spun again in the microcentrifuge for 10 seconds. The extracted protein was labeled and stored in the -20°C freezer to be run on a gel once all proteins were extracted.

Equal amounts of total protein were resolved by SDS–PAGE and transferred onto PVDF (polyvinylidene fluoride) membranes. Twenty microliters of each lysate in RIPA buffer was loaded into a 15-well, 7.5% polyacrylamide gel, run for 15 minutes at 130 Volts and then run again for 30 minutes at 230 Volts. Proteins resolved in gels were
transferred to PVDF membrane using a Bio-Rad semi-dry transfer system at 25 Volts and 0.4 Amps for 60 minutes. Blots were blocked in 5% non-fat milk in TBS-Tween. Blots were probed overnight at 4 °C in 1% milk in TBS-Tween with anti-YAP (Cell Signaling, D8H1X Rabbit Monoclonal Antibody, 1:1000 dilution factor), anti-TAZ (GE Healthcare, Mouse Monoclonal antibody, 1:500 dilution factor), and anti-Vinculin (BD Biosciences anti-mouse monoclonal antibody, 1:2000 dilution factor). PVDF membranes were washed 3 times with 0.1% tris-buffered saline and Tween 20 (0.1% TBS-Tween) for 10 minutes each. The samples were then incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies, anti-mouse (GE Healthcare, anti-mouse IgG HRP-linked antibody, 1:8000 dilution factor) or rabbit (Cell Signaling, anti-rabbit IgG HRP-linked antibody, 1:5000 dilution factor). The samples were then washed three more times for 10 minutes with 0.1% TBS-Tween. Proteins were detected via chemiluminescence using ECL prime (GE Healthcare) for YAP/TAZ exposure, and ECL (ThermoFisher Scientific) for Vinculin. Images were captured using the Bio-Rad ChemiDoc XRS with ImageLab software. Levels of protein were normalized using Vinculin, and the protein level expression was compared to that of HMEC. Quantitative analysis of the immunoblots was performed using the ImageLab software with Excel to perform statistical analysis following raw data collection.

**Fixing and Staining Cells:**

Cells from each cell line were grown on glass cover slips to either subconfluence (50%) or confluence (90%) in order to fix and stain the cells for YAP and TAZ. For both subconfluent and confluent cells, the fixing and staining protocol was the same. Due to
time constraints and bad Triton, only 8 cell lines for YAP and six for TAZ are presented in this paper.

Using tweezers, glass cover slips were washed in 70% ethanol for one minute, dabbed with a Kim Wipe, and then placed into the well. The appropriate amount of cells (50,000 cells/well, as mentioned above) was added to each well in a 12-well plate, and then media was added to get a final volume of 2 ml per well. Cells were placed in the incubator to grow to the appropriate density.

Once cells reached the appropriate density on the glass cover slips, the cover slips were removed from the well using tweezers and placed into a weigh boat containing PBS for one minute, with the cell side face up. Then, each cover slip was dabbed with a Kim Wipe, transferred into weight boats containing 4% paraformaldehyde in PBS for 15 minutes, with cell side facing up.

After this, one of the three cover slips was dapped with a Kim Wipe, and placed in one well of a 12-well plate filled with 2 ml of PBS and labeled appropriately. The plate was wrapped in parafilm to avoid evaporation and stored in a cold place. This served as an extra cover slip for future use.

The remaining two cover slips per cell group were then placed in a weigh boat containing PBS with 0.5% Triton for 5 minutes, cell side facing up. This step is critical in making the membrane permeable to the antibodies added in the next step. The cover slips were then dabbed with a Kim Wipe, and placed into weigh boats containing TBS-5% BSA for 30 minutes.
After blocking, the cover slips were transferred into a humidified chamber, cell side facing up. Diluted primary antibody (200 µl) was added to the cover slips for one hour: one cover slip was stained with anti-YAP antibody (Santa Cruz 63.7) at a 1:250 dilution factor in TBS with 5% BSA, and the other cover slip was stained with anti-TAZ antibody (BD Biosciences) at a 1:250 ratio in TBS with 5% BSA. Two hundred microliters of the diluted antibody was added to the appropriate cover slip and the humidified chamber was sealed.

After one hour, cover slips were dabbed off with a Kim Wipe, and placed into a weigh boat containing TBS with 5% BSA to be washed, cell side facing up. After five minutes, the cover slips were placed in an additional wash with PBS for five minutes.

Two hundred microliters of secondary antibody was then added to the cover slips for 30 minutes. All cover slips received the same secondary antibodies: Alexa Fluor anti-Mouse 488 green and Life Technology’s Rhodamine Phalloidin, each diluted with TBS-5% BSA with Hoechst (1:2500) at a dilution ratio of 1:500 and 1:250, respectively.

Cover slips were then transferred into a TBS with 5% BSA weigh boat (as above), cell side face up, for five minutes, and then into a weigh boat with PBS also for five minutes.

Following the PBS wash, cover slips were mounted onto glass microscope slides, cell side facing down, using Molecular Probes by Life Technology Prolong Diamond Anti-Fade Mounting reagent. Slides were left in the dark for at least 24 hours before imaging.
**Microscopy:**

Prior to imaging, cover slips were rinsed with de-ionized water to remove any residual PBS and BSA.

Cells were imaged using a Nikon TiE2000 microscope with the NIS Elements software, with a 20X objective (200X total magnification). We utilized the DAPI, TRITC and FITC channels. Subconfluent and confluent cells of the same cell line were imaged together, using the same exposure. Eight images were taken, and the best five images were used for analysis.

Localization of YAP and TAZ was analyzed using the ROI method. In this method, one quadrant per image was picked at random to analyze using the DAPI stain, and odd number boxes were placed in nucleus, and even number boxes were placed in cytoplasm of a cell for a total of five cells per image. The data was then exported into an Excel file, and the difference between nuclear and cytoplasmic signal was calculated. We then averaged all of the differences between nuclear and cytoplasmic signals and calculated the standard error of the mean for each cell line. The Excel Data Package was used for all analysis.
RESULTS

We aimed to characterize YAP/TAZ-dependency in a large panel of breast cancer cell lines. To do this, we individually cultured 45 distinct cell lines collected from ATCC. Cell lines and culture conditions are shown in Table 1.

Growing an entire panel of breast cancer cell lines presented many challenges. Each cell line grew at a different rate and required different media, so it was of utmost importance to be organized, keep a detailed record of the cell lines, check cell lines daily under the microscope and label the media to ensure the correct ingredients were added and that it was used on the proper cell line.

In the suspension cell lines, it was difficult to estimate confluence to split the cells. Some of them grew in clumps, which sometimes looked like cellular debris, so we had to be very careful in maintaining them. We spun them down, re-suspended the pellet and then re-plated the suspension lines in a new flask if the media had a lot of clumps. Additionally, some of the suspension cell lines did not grow well in large flasks, so we used smaller flasks and stood them up inside the incubator instead of laying them flat to allow them to grow better. Estimating confluence was also challenging in suspension cell lines for protein and RNA extraction. The cell lines had more media in them (sometimes with 80 ml of media) since they grew in flasks compared with the adherent cells in 10 cm culture dishes. We did our best to ensure that there was a similar amount of cells by counting cells and looking at confluence.
Within the adherent cell lines, there was a wide variability in growth rate. The HCC 70 cell line grew in high density clumps of cells, instead of spreading out on the dishes, so it was difficult at times to predict confluence. Some grew more slowly on the cover slips compared with normal 10 cm culture dishes. The HCC 1937 and HCC 1500 cell lines took more than two months to grow to confluence and did not grow well when plated at a low density. MCF-12A needed media replacement more often than the other cell lines, thus the media color was monitored and changed often, every 1-2 days. MDA-MB-134-VI and MDA-MB-361 seemed to die after they reached a certain confluence (around 75%). HCC 1937 also contained brown spots that did not go away even after adding antibiotics so we thought it might be cellular debris.
Table 1: Breast Cancer Cohort and Culture Method. Breast cancer cell lines were obtained from ATCC and cultured according to their specific media requirements.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ATCC Code</th>
<th>Media</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU-565</td>
<td>CRL-2351</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>DU4475</td>
<td>HTB-123</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC38</td>
<td>CRL-2314</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC70</td>
<td>CRL-2315</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC202</td>
<td>CRL-2316</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC1187</td>
<td>CRL-2322</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC1395</td>
<td>CRL-2324</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC1419</td>
<td>CRL-2326</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
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<td>HCC1428</td>
<td>CRL-2327</td>
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<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
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<td>HCC1569</td>
<td>CRL-2330</td>
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<td>10% FBS</td>
</tr>
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<td>HCC1599</td>
<td>CRL-2331</td>
<td>RPMI 1640</td>
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<td>CRL-2335</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC1937</td>
<td>CRL-2336</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC2218</td>
<td>CRL-2343</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>HCC1954</td>
<td>CRL-2338</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>CRL-1500</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Subculture</td>
<td>Medium</td>
<td>Serum (%)</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ZR-75-30</td>
<td>CRL-1504</td>
<td>RPMI 1640</td>
<td>10% FBS</td>
</tr>
<tr>
<td>BT-483</td>
<td>HTB-121</td>
<td>RPMI 1640</td>
<td>20% FBS, 0.01mg/ml bovine insulin</td>
</tr>
<tr>
<td>HCC2157</td>
<td>CRL-2340</td>
<td>RPMI 1640</td>
<td>5% FBS</td>
</tr>
<tr>
<td>BT-549</td>
<td>HTB-122</td>
<td>RPMI 1640</td>
<td>10% FBS, 0.023 IU/ml insulin</td>
</tr>
<tr>
<td>T-47D</td>
<td>HTB-133</td>
<td>RPMI 1640</td>
<td>10% FBS, 0.2 Units/ml bovine insulin</td>
</tr>
<tr>
<td>MDA kb2</td>
<td>CRL-2713</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>HTB-24</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-175-VII</td>
<td>HTB-25</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>HTB-26</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>HTB-131</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>HTB-132</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>UACC-893</td>
<td>CRL-1902</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS</td>
</tr>
<tr>
<td>MDA-MB-134-VI</td>
<td>HTB-23</td>
<td>Leibovitz’s L-15</td>
<td>20% FBS</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>HTB-27</td>
<td>Leibovitz’s L-15</td>
<td>20% FBS</td>
</tr>
<tr>
<td>UACC-812</td>
<td>CRL-1897</td>
<td>Leibovitz’s L-15</td>
<td>20% FBS, 2 mM L-glutamine, 20ng/ml human EGF</td>
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<td>MDA-MB-415</td>
<td>HTB-128</td>
<td>Leibovitz’s L-15</td>
<td>15% FBS, 2mM L-glutamine, 10 mcg/ml insulin, 10 mcg/ml glutathione</td>
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<td>MDA-MB-436</td>
<td>HTB-130</td>
<td>Leibovitz’s L-15</td>
<td>10% FBS, 16 mcg/ml glutathione (90%), 10mcg/ml insulin</td>
</tr>
<tr>
<td>MCF 10A</td>
<td>CRL-10317</td>
<td>MEBM (Lonza/Clonetics CC-3150 - don't use the GA-1000 mix)</td>
<td>100 ng/ml cholera toxin -do not filter complete medium</td>
</tr>
<tr>
<td>Cell Line</td>
<td>PCC</td>
<td>Media</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-----</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>184B5</strong></td>
<td>CRL-8799</td>
<td>MEBM (Lonza/Clonetics CC-3150 - don't use the GA-1000 mix)</td>
<td>1ng/ml cholera toxin</td>
</tr>
<tr>
<td><strong>SK-BR-3</strong></td>
<td>HTB-30</td>
<td>McCoy's 5a</td>
<td>10% FBS</td>
</tr>
<tr>
<td><strong>BT-20</strong></td>
<td>HTB-19</td>
<td>Eagles MEM</td>
<td>10% FBS</td>
</tr>
<tr>
<td><strong>CAMA-1</strong></td>
<td>HTB-21</td>
<td>Eagles MEM</td>
<td>10% FBS</td>
</tr>
<tr>
<td><strong>MCF7</strong></td>
<td>HTB-22</td>
<td>Eagles MEM</td>
<td>10% FBS, 0.01 mg/ml human recombinant insulin</td>
</tr>
<tr>
<td><strong>Hs 578Bst</strong></td>
<td>HTB-125</td>
<td>46X</td>
<td>10% FBS, 1.5g/L Sodium Bicarb, 30ng/ml mouse EGF</td>
</tr>
<tr>
<td><strong>BT-474</strong></td>
<td>HTB-20</td>
<td>46X</td>
<td>10% FBS, 1.5g/L Sodium Bicarb</td>
</tr>
<tr>
<td><strong>Hs 578T</strong></td>
<td>HTB-126</td>
<td>DMEM</td>
<td>10% FBS, 0.01 mg/ml bovine insulin</td>
</tr>
<tr>
<td><strong>MCF 10F</strong></td>
<td>CRL-10318</td>
<td>Base medium: Combine 14.8g/L Dulbecco's modified Eagle's medium and Ham's F12 base (Sigma, D-9785), 1.2g NaHCO3 (Sigma, S-5761), 0.365g L-glutamine (Sigma, G-3126), 0.059g L-leucine (Sigma, L-8912), 0.0912g L-lysine (Sigma, L-8662), 0.017g L-methionine (Sigma, M-5308), 0.0612g MgCl2.6H2O (Sigma, M-1028), 0.0488g MgSO4.7H2O (Sigma, M-3409), 0.006g CaCl2.2H2O (Sigma, C-8106), and 0.0086g Phenol Red (Sigma, P-3532). Fill to 1L with Ultrapure Cell Grade water (ATCC® 30-2205). Stir to dissolve. Adjust pH to 7.1 – 7.3. Filter-sterilize using a 0.22 μm filter. Complete growth medium: Combine base medium with 20 ng/mL epidermal growth factor (Sigma, E-9644), 100 ng/mL cholera toxin (Sigma, C-8052), 0.01 mg/mL human insulin (Sigma, I-2643), 500 ng/mL hydrocortisone (Sigma, H-0888), and 5% Chelex-treated horse serum.</td>
<td></td>
</tr>
<tr>
<td><strong>MCF-12A</strong></td>
<td>CRL-10782</td>
<td>1:1 DMEM and Ham's F12</td>
<td>5% Horse Serum, 500 ng/ml hydrocortisone (95%), 0.01mg/ml bovine insulin, 100 ng/ml cholera toxin, 20 ng/ml human epidermal growth factor</td>
</tr>
</tbody>
</table>
Three different methods were used to characterize YAP/TAZ activity in our breast cancer cell lines: Western Blotting to assess total YAP/TAZ protein levels, RNA extraction to identify gene expression signatures indicative of YAP/TAZ activation, and fixing and staining cells for the relative subcellular localization of YAP and TAZ to infer activity (nuclear YAP/TAZ are active while cytoplasmic YAP/TAZ are inactive).

We harvested protein from each cell line when they grew to 70% confluence. In line with current literature, we determined that normalized levels of YAP and TAZ would allow us to rapidly observe a cell line’s Hippo signaling status. As such, we performed protein immunoblotting against YAP and TAZ as described in the methods section above. Vinculin served as our loading control for our analysis, as it is a protein that is ubiquitously expressed at similar levels among the varieties of cell lines used in this assay. Images of the blots obtained can be found in Figure 6. Relative intensities of the YAP and TAZ bands were normalized using the corresponding intensity of the Vinculin band, and then total levels were compared to HMEC cells, which are a non-transformed human mammary epithelial cell line. The results for YAP are shown in Figure 7 and the results for TAZ are shown in Figure 8. Upon performing this analysis, we found that YAP protein levels were found to be greater than a readout of one (using HMEC as our normalizing control) in the following cell lines: MDA-MB-453 (1.40), HCC 2157 (1.14), HCC 1599 (1.14), HCC 1569 (2.88), T-47D (1.17), BT-474 (1.59), 184B5 (1.20) (Figure 7, Table 2). We found that YAP levels were particularly high in HCC 1569 (2.88). TAZ protein levels were greater than one in MDA-MB-157 (4.87), MDA-MB-436 (2.94), HCC 38 (5.16), HCC 70 (9.57), HCC 202 (4.45), HCC 1395 (1.58), HCC 1500 (1.24),
HCC 1599 (5.50), HCC 1806 (2.73), HCC 1954 (2.52), CAMA-1 (1.05), AU565 (1.83), ZR-75-1 (3.14), MCF7 (5.30), MCF 10F (1.17), BT-474 (2.95), BT-483 (8.74), UACC 812 (1.35), and SK-BR-3 (1.72) (Figure 8, Table 3). The HCC 1599 and BT-474 cell lines contained high protein levels of both YAP and TAZ. Two cell lines, MDA-MB-134-VI and DU4475, had almost no observable YAP/TAZ expression. This protein expression analysis will be more useful in the future to determine if higher YAP/TAZ protein level correlates with YAP/TAZ-dependency.
Figure 6: Western Blot for YAP and TAZ Protein levels in our Panel of Breast Cancer Cells. YAP and TAZ protein levels for our 45 breast cancer cell lines. Vinculin was used as a control to normalize protein levels of each cell line for comparison.
Figure 7: YAP Protein Levels in our Breast Cancer Panel. Protein levels of YAP in all 45 breast cancer cell lines normalized to HMEC. Protein levels were normalized in each cell line to Vinculin, and then each protein level was compared with the HMEC, which served as a control and was set equal to 1. A ratio greater than 1 means that the protein levels are higher than that of HMECs, while less than 1 means that the YAP protein levels are lower than HMECs.
Figure 8: TAZ Protein Levels in our Breast Cancer Panel. Protein levels of TAZ in all 45 breast cancer cell lines. Protein levels were normalized in each cell line to Vinculin, and then each protein level was compared with TAZ protein expression of HMEC, which served as an internal control and was set equal to 1. A ratio greater than 1 means that the protein levels are higher than that of HMEC, while less than 1 means that the TAZ protein levels are lower than HMEC.
Table 2: Cell lines with YAP Protein Level Ratio Greater than One. These seven breast cancer cell lines had high protein levels of YAP compared with HMEC cells. A ratio greater than one implies Hippo inactivation.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>YAP Protein Level Ratio (Relative to HMEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-453</td>
<td>1.40</td>
</tr>
<tr>
<td>HCC 2157</td>
<td>1.14</td>
</tr>
<tr>
<td>HCC 1599</td>
<td>1.14</td>
</tr>
<tr>
<td>HCC 1569</td>
<td>2.88</td>
</tr>
<tr>
<td>T-47-D</td>
<td>1.17</td>
</tr>
<tr>
<td>BT-474</td>
<td>1.59</td>
</tr>
<tr>
<td>184B5</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Table 3: Cell lines with TAZ Protein Level Ratio Greater than One. Nineteen breast cancer cell lines had high protein levels of TAZ compared with HMEC cells. A ratio greater than 1 is significant as it implies Hippo pathway inactivation.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>TAZ Protein Level Ratio (Relative to HMEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-157</td>
<td>4.87</td>
</tr>
<tr>
<td>CAMA-1</td>
<td>1.05</td>
</tr>
<tr>
<td>HCC 1500</td>
<td>1.24</td>
</tr>
<tr>
<td>HCC 1599</td>
<td>5.50</td>
</tr>
<tr>
<td>AU 565</td>
<td>1.83</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>3.14</td>
</tr>
<tr>
<td>HCC 70</td>
<td>9.57</td>
</tr>
<tr>
<td>HCC 38</td>
<td>5.16</td>
</tr>
<tr>
<td>HCC 1395</td>
<td>1.58</td>
</tr>
<tr>
<td>MCF 10F</td>
<td>1.17</td>
</tr>
<tr>
<td>HCC 1806</td>
<td>2.73</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>2.94</td>
</tr>
<tr>
<td>BT 474</td>
<td>2.95</td>
</tr>
<tr>
<td>BT-483</td>
<td>8.74</td>
</tr>
<tr>
<td>HCC 1954</td>
<td>2.52</td>
</tr>
<tr>
<td>UACC 812</td>
<td>1.35</td>
</tr>
<tr>
<td>HCC 202</td>
<td>4.45</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>1.72</td>
</tr>
<tr>
<td>MCF7</td>
<td>5.30</td>
</tr>
</tbody>
</table>
In order to characterize gene expression profiles for our panel of breast cancer cells, we individually cultured 45 cell lines (see Table 1) and grew the cells to 70% confluence prior to extracting RNA. We are currently in the process of collecting this data so the results are not presented in this thesis; however, they will be used in future studies to define gene expression signatures in both YAP/TAZ-dependent and YAP/TAZ-independent cell lines.

Our next project was to determine if cell density changed subcellular localization of YAP and TAZ as a surrogate for normal Hippo activity among these breast cancer cell lines. To do so, we grew cells on cover slips to either a low density (subconfluent, 50%) or high density (confluent, 90%) for each cell line and then fixed and stained each cover slip for either YAP or TAZ. We then analyzed nuclear localization of both proteins in our panel of breast cancer cells using immunofluorescence to evaluate YAP/TAZ activity levels.

Snapshots of the immunofluorescence images from fixing and staining for YAP at subconfluent (50%) and confluent (90%) densities for eight cell lines (HCC 202, HCC 1187, HCC 1599, MDA-MB-231, MCF 10A, MCF 10F, MCF-12A, and UACC 812) are shown in Figures 9 and 10. These images allowed us to visualize where YAP is located in each cell line. We then quantified these images, using subconfluent as the control (the ratio was set to 1), since we were testing how an increase in cell density impacted the Hippo pathway and YAP/TAZ activity (Figure 11). When grown to confluence, four cell lines (HCC 202, HCC 1187, HCC 1599 and UACC 812) had a notable change in the subcellular localization of YAP from nuclear (active) to cytoplasmic (inactive) YAP,
shown as a negative ratio. The amount of nuclear (active) YAP decreased significantly and the amount of cytoplasmic (inactive) YAP increased significantly between subconfluence and confluence in HCC 202 (-0.84), HCC 1187 (-0.29), HCC 1599 (-1.25) and UACC 812 (-0.09), with HCC 1599 showing the most significant change between the two states, represented as the most negative ratio (-1.25). In the MCF 10A, MCF 10F, and MCF-12A cell lines, YAP remained nuclear at a confluent density. However, nuclear YAP decreases (or becomes less active) by 30% and almost 50% when grown to confluence in MCF 10A and MCF-12A respectively. Nuclear YAP does not change significantly between subconfluent and confluent states in the MCF 10F cell line. MDA-MB-231 cells had a 50% reduction in nuclear YAP levels when grown from subconfluence to confluence.
Figure 9: Immunofluorescent staining for YAP at Subconfluent Densities. Eight cell lines (HCC 202, HCC 1187, HCC 1599, MCF 10A, MCF 10F, MCF-12A, MDA-MB-231, and UACC-812) were grown to subconfluence (50%) and subsequently fixed and stained for YAP. The three channels shown in the merged image are YAP (green, FITC), the nucleus (blue, DAPI), and the actin cytoskeleton (red, TRITC).
Figure 10: Immunofluorescent Staining for YAP at Confluent Densities. Eight cell lines (HCC 202, HCC 1187, HCC 1599, MCF 10A, MCF 10F, MCF-12A, MDA-MB-231, and UACC-812) were grown to confluence (90%) and subsequently fixed and stained for YAP. The three channels shown in the merged image are YAP (green, FITC), the nucleus (blue, DAPI), and the actin cytoskeleton (red, TRITC).
Figure 11: Quantification of the Nuclear to Cytoplasmic Ratio for YAP. Nuclear to Cytoplasmic ratio of YAP in MCF 10A, MCF 10F, MCF-12A, MDA-MB-231, HCC 1187, HCC 1569, HCC 202 and UACC 812 are shown above. Positive and negative ratios represent nuclear or cytoplasmic YAP, respectively. Subconfluence was used as the control for each cell line. Error bars represent standard error of the mean. N-C: nuclear minus cytoplasmic YAP.
We also imaged and analyzed four cell lines (HCC 202, MCF 10A, MCF 10F, and MCF-12A) to determine if cell density influenced subcellular localization of TAZ. Figures 12 and 13 show the immunofluorescence TAZ-labeled images at subconfluent and confluent densities for HCC 202, MCF 10A, MCF 10F, and MCF-12A. These images allowed us to visualize whether TAZ was nuclear or cytoplasmic in each cell line, which would tell us if TAZ were active or inactive, respectively. We then analyzed our images to quantify differences in TAZ nuclear localization between subconfluent and confluent densities. The amount of nuclear (active) TAZ decreased when cells were grown to confluence by about 21%, 25% and 80% in the MCF 10A, MCF 10F, and MCF-12A cell lines, respectively (Figure 14). The HCC 202 cells grown to confluence had a drastic change in subcellular localization of TAZ when compared with subconfluence, shifting from nuclear to cytoplasmic, or active to inactive, TAZ (-0.46).

We expected the changes in the levels of nuclear TAZ to be comparable to that of YAP within the same cell line. Interestingly, MCF 10F and MCF-12A seemed to have a more significant decrease when grown to confluence in nuclear TAZ levels (25% in MCF 10F and 80% in MCF-12A) than in nuclear YAP levels (no significant decrease in MCF 10F and 50% in MCF-12A).
Figure 12: Immunofluorescence staining for TAZ at Subconfluent Densities. Four cell lines (HCC 202, MCF 10A, MCF 10F, MCF-12A) were grown to subconfluence (50%) and subsequently fixed and stained for TAZ. The three channels shown in the merged image are TAZ (green, FITC), the nucleus (blue, DAPI), and the actin cytoskeleton (red, TRITC).
Figure 13: Immunofluorescence staining for TAZ at Confluent Densities. Four cell lines (HCC 202, MCF 10A, MCF 10F, MCF-12A) were grown to confluence (90%) and subsequently fixed and stained for TAZ. The three channels shown in the merged image are TAZ (green, FITC), the nucleus (blue, DAPI), and the actin cytoskeleton (red, TRITC).
Figure 14: Quantification of the Nuclear to Cytoplasmic Ratio for TAZ. Quantification of Nuclear to Cytoplasmic Levels of TAZ for MCF 10A, MCF 10F, MCF-12A, and HCC 202 are shown above. Positive and negative ratios represent nuclear or cytoplasmic TAZ, respectively. Subconfluence was used as the control for each cell line. Error bars represent standard error of the mean. N-C: Nuclear minus cytoplasmic.
DISCUSSION

The canonical Hippo tumor suppressor pathway plays a critical role in regulating cellular growth, proliferation and survival. YAP and TAZ are two transcriptional co-activators that serve as effectors of the Hippo pathway. Activation of the Hippo pathway leads to nuclear exclusion and degradation of phosphorylated YAP and TAZ, and is mediated by the protein kinases LATS1 and LATS2 and subsequent degradation by an E3 ubiquitination dependent mechanism. Hippo pathway inactivation allows YAP and TAZ to move into the nucleus to bind to TEAD, leading to the transcription of target genes that promote survival, growth and proliferation.

Disruption of the Hippo pathway has been implicated in several cancers, including breast cancer. Hyperactivation of YAP/TAZ is common in breast cancer, and we believe that this results in functional inactivation of the Hippo pathway. While it is currently known that amplification of YAP/TAZ is common to many breast cancers, the dependency of these cells on YAP/TAZ activity remains unknown. We believe that many of these cell lines are dependent on YAP/TAZ activity for survival, so this project’s goal was to define YAP/TAZ-dependency in a large cohort of breast cancer cells.

We obtained a comprehensive cohort of breast cancer cells from ATCC in order to test our hypothesis. These cell lines were chosen because they were certified as mycoplasma-free and authenticated by genetic profiling using short tandem repeat profiling by ATCC. Thus, we could be certain of the quality, identity and integrity of our cell panel lines. A large part of this project involved amplifying the cell lines and creating
libraries of breast cancer cells for the entire university. This aspect of the project was a large undertaking, as it took around six months to complete. We grew around 12 cell lines at a time, and amplified cells to obtain enough cells for 18 vials. Each cell line grew at a different rate, and some cells grew better than others. We have finally completed this project and look forward to using the breast cancer library to further our understanding the mechanisms of the Hippo pathway in breast cancer. We are also excited to share the libraries with other labs to help enhance breast cancer research at BUMC.

Gene expression data from these cell lines currently exists. However, we decided to obtain our own gene expression profiles for all 45 cell lines in order to have all cell lines grown to the same confluence. A consistent 70% confluence was important for our analysis, as differences in cell density impact Hippo pathway activation. Gene expression signatures obtained from our microarray analyses will be correlated with the results from our YAP/TAZ-dependency assays in the future to screen for a YAP/TAZ-dependency signature. We hope to find specific genes that are present in YAP/TAZ-dependent cells and not present in YAP/TAZ-independent cell lines, and will use this information to generate a gene expression signature for YAP/TAZ-dependent cancer cells. Given the importance of Hippo signaling in promoting chemoresistance and cellular survival, it is likely that a YAP/TAZ dependency signature will uncover genes critical to such processes in the context of breast cancer. Future efforts will focus on molecular deconvolution of such genes and their mechanisms of action.

The goal of our project was to determine YAP/TAZ-dependency in a large cohort of human breast cancer cells. While we have not completed our comprehensive analysis,
our preliminary data strongly support our hypothesis that some breast cancer cell lines are dependent on YAP/TAZ for survival. In four cell lines (MCF 10A, MCF 10F, MCF-12A, and MDA-MB-231), we observed that YAP was nuclear, and thus constitutively active, at both subconfluent and confluent densities. This finding indicates that YAP does not respond to changes in cell density that would normally lead to contact inhibition, and we believe that this will correlate with YAP-dependency.

We expected the subcellular changes in YAP to correlate with that of TAZ, and this trend is exactly what we saw. The three cell lines MCF 10A, MCF 10F, and MCF-12A that had nuclear, active YAP regardless of cell density also contained nuclear, active TAZ. The HCC 202 cell line that did change the subcellular localization of YAP from nuclear to cytoplasmic in response to an increase in cell density had the same result with TAZ.

Since YAP and TAZ have similar functions and negatively regulate each other, we expected to see high protein levels of either YAP or TAZ in each cell line. As anticipated, we detected high protein levels of YAP and TAZ in seven and nineteen breast cancer cell lines, respectively. High expression of both YAP and TAZ were noted in two cell lines, BT-474 and HCC 1599, and we predict that these cell lines have a functionally inactivated Hippo pathway, which will be verified in our future knockdown experiments. We believe that these cell lines with high YAP and/or TAZ expression will be dependent on YAP/TAZ for survival, and a clear answer to this will be evidence after completing our knockout studies. YAP/TAZ protein expression was essentially negligible in MDA-MB-134-VI and DU4475. With a lack of YAP/TAZ and presumably no Hippo
activity, these two breast cancer cell lines must have diverged significantly from mammary epithelial cells and reprogrammed their cellular machinery to use a different growth factor signaling pathway to promote tumor growth completely independent of Hippo signaling.

**Future Directions**

Our current hypothesis is that hyperactivation of YAP/TAZ must upregulate specific genes that promote the growth and survival of the cells within the specific context of YAP/TAZ-dependent cancers that are not upregulated in YAP/TAZ-independent cancers. Due to time constraints for this thesis project, we were unable to perform all analyses to test this hypothesis. However, we will continue to research until we can answer this question.

Our next step is to perform knockdown experiments of YAP and TAZ by lentiviral delivery of shRNA, and then test cell viability in each of the individual cell lines after 6 days using CellTiter-Glo luminescent assays. Once we knockdown YAP and TAZ, we will determine which cell lines rely on YAP/TAZ for survival, and then use this information along with our gene expression results to create a gene expression profile for YAP/TAZ-dependent breast cancer cells. Additionally, we will compare YAP/TAZ-dependency with protein level expression, and predict that the breast cancer cell lines with higher YAP/TAZ protein expression will be more dependent on YAP/TAZ than those cell lines with lower YAP/TAZ protein levels. We will also look more into the YAP/TAZ target genes to test which genes are important for the survival of the YAP/TAZ dependent cells. The results would allow us to also identify potential
biomarkers that could detect YAP/TAZ-dependent breast cancers. Our future experiments will allow us to either refute or support our hypothesis.

**Conclusion**

Preliminary data suggest that YAP/TAZ expression is upregulated in some breast cancer cell lines, but not others. Additionally, we found that YAP/TAZ subcellular localization in several breast cancer cell lines did not change, remaining nuclear even at a high density. This finding suggests that these cell lines are not contact inhibited, and may be dependent on YAP/TAZ for survival. However, these analyses will become more useful in the future when we perform our knockdown experiments and gain more information about these breast cancer cell lines.

By broadening our understanding of Hippo-pathway driven cancers, we hope to explore untapped opportunities to unveil novel, viable and effective targets for therapeutic manipulation. Identifying a distinct gene transcriptional program for YAP/TAZ-dependent cancers will help ascertain the mechanisms by which YAP/TAZ hyperactivation leads to tumorigenesis. Pinpointing target genes would also uncover an innovative way to combat YAP/TAZ-dependent cancers.

The ultimate goal is to provide valuable and novel ways of developing chemotherapeutics to lower the death rate of cancer in our society. Thus, we hope our findings will translate from benchside to bedside and be useful to physicians for diagnosing and treating breast cancer patients, thereby improve prognosis and ultimately clinical outcomes in the future.

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REFERENCES


CURRICULUM VITAE

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EDUCATION

BOSTON UNIVERSITY, Boston, MA
Master of Science in Medical Sciences, May 2016

BOSTON UNIVERSITY, Boston, MA
Master of Public Health, May 2013
Concentration: Environmental Health, Exposure and Risk Assessment Emphasis
Honors: Delta Omega Honors Society, Best Overall Practicum Poster (Summer 2012)

BOSTON UNIVERSITY, Boston, MA
Bachelor of Arts in Biology, May 2011
Honors: Cum Laude, College Honors Program, Dean’s List

LEADERSHIP EXPERIENCE

BOSTON UNIVERSITY, Boston, MA
Tutor, September 2015 - May 2016
• Tutored physiology and biochemistry for students in the Medical Sciences program and School of Dental Medicine.

GRADUATE MEDICAL SCIENCES STUDENT ORGANIZATION, BOSTON UNIVERSITY, Boston, MA
MAMS Sub-Committee, February 2015 - June 2016
• Coordinated and provided prospective student tours
• Answer questions for incoming students
• Met with program director monthly to plan events

BOSTON PUBLIC HEALTH COMMISSION, Boston, MA
Project Coordinator, City-Wide Water Access Initiative, October 2012-October 2013
• Developed and lead a multimedia awareness campaign to promote water consumption and reduce sugar-sweetened beverage consumption
• Planned, managed and executed project deliverables within the constraints of time, budget and scope
• Built relationships with external partners to include community-based participation in the campaign
• Created lead sampling protocol to test tap water in off-line Boston Public Schools; positive results could ultimately increase water access city-wide

BROOKLINE HEALTH DEPARTMENT, Brookline, MA
Environmental Health Intern, August 2012-May 2013
• Designed and implemented web-based restaurant inspection report for public use
• Performed restaurant menu checks to ensure compliance with local by-laws
• Provided guidance to food and retail establishments at town meetings regarding new town-by-laws for banning plastic bags and Styrofoam

GLOBAL WATER BRIGADES, Tegucigalpa, Honduras
Volunteer, September 2008-May 2011
Intern, June 2012-August 2012
• Conducted initial screening assessments, prioritized community needs and assessed feasibility of new infrastructure projects for two communities lacking safe water systems
• Assisted technician in training community water council on effective management and maintenance of water systems
• Completed follow-up assessments to check status of completed water systems
• Collaborated with staff technicians to design proposal for new water system including budget projection
• Created and executed my own research study to evaluate recontamination of drinking water in a community with a completed water system. Collected and processed data, and presented findings and recommendations to organization in English and also to community members in Spanish
• As volunteer, built new water systems for two communities (May 2010, January 2011)

ACTON HEALTH DEPARTMENT, Acton, MA
Health Intern, May 2010-September 2010
• Designed an educational campaign that increased awareness of Lyme disease among children under the age of 18 in towns of Acton, Boxborough, Stow, and Concord
• Developed stakeholder and assets report, strategic community-marketing model, and corresponding sustainability and budgeting report for educational campaign
• Liaised with the local health department and Boston University School of Public Health to develop Lyme Disease curriculum used to train local public health officials

BOSTON UNIVERSITY PEER ADVISOR, Boston, MA
Student Advisor, March 2008 – May 2011
• Supported new freshmen and transfer students during their transition into the Boston University community
**MEDICAL-RELATED COMMUNITY SERVICE**

**RESCUE MISSION CROSSROADS CLINIC**, Syracuse, NY  
Volunteer, January 2014-September 2014

**BRIGHAM AND WOMEN’S HOSPITAL**, Boston, MA  
Medical Careers Exploration Program, Volunteer, March 2012-December 2013

**PAID EMPLOYMENT**

**TUFTS MEDICAL CENTER**, Boston, MA  
Cardiology Technician (Per Diem), May 2009-April 2014

**RESEARCH EXPERIENCE**

**BOSTON UNIVERSITY SCHOOL OF PUBLIC HEALTH**, Boston, MA  
Research Assistant, Exposure Biology Research Group, October 2011-May 2012  
PI: Michael McClean

- This lab studies the relationship between chronic kidney disease and exposure to various pesticides in Nicaraguan sugarcane workers.

**PUBLICATIONS**


Summer 2013. This blog series consisted of bi-monthly blog posts that provided information about Boston’s tap water and discussed the health benefits of drinking water over sweetened-sugary beverages.