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Defining mechanisms directing YAP/TAZ-mediated tumorigenesis

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DEFINING MECHANISMS DIRECTING YAP/TAZ-MEDIATED

TUMORIGENESIS

by

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Is a hippopotamus really a hippopotamus or just a really cool opotamus?

-Mitch Hedberg

<u>DEDICATION</u>

To the Hiemer Family - Bob, Cathy, Chelsea, Taylor, Izzy, Charlie & Leon

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DEFINING MECHANISMS DIRECTING YAP/TAZ-MEDIATED

TUMORIGENESIS

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<u>ABSTRACT</u>

Dysregulated Hippo pathway signaling promotes the onset of aggressive cancers through the induced nuclear activity of yes-associated protein (YAP) and transcriptional co-activator with PDZ binding motif (TAZ) (YAP/TAZ). Uncontrolled nuclear YAP/TAZ activity evokes tumor-initiating properties in a range of epithelial-derived cancers, including oral and breast cancers, but their downstream targets and mechanisms of action are unclear. Recent studies have suggested that the pro-tumorigenic roles for YAP/TAZ relate to their convergence with growth factor signaling pathways. Based on these previous studies, I hypothesized that YAP/TAZ driven transcription contributes to carcinoma progression, and that cooperation with transforming growth factor β (TGFβ)-induced signals promotes aggressive oncogenic traits. In this thesis I show that dysregulated YAP localization precedes oral squamous cell carcinoma (OSCC) development, and that nuclear YAP/TAZ activity drives cell proliferation, survival, and migration *in vitro*, and is required for tumor growth and metastasis *in vivo*. Global gene expression studies in OSCC cells revealed that YAP/TAZ-mediated gene expression correlates with expression changes that occur in human OSCCs

identified by "The Cancer Genome Atlas" (TCGA), many of which encode cell cycle and survival regulators. By exploring the relationship with growth factor signaling, I found that YAP/TAZ induce pro-tumorigenic events by converging with TGFβ-induced signals, particularly in breast cancer cells where TGFβ is known to promote metastatic properties. My observations indicated that YAP/TAZ are necessary for maintaining and promoting TGFβ-induced tumorigenic phenotypes in breast cancer cells, and that these phenotypes result from the cooperative activity of YAP/TAZ, the TEA domain family of transcription factors (TEADs), and TGFβ-activated SMAD2/3 in the nucleus. Genome-wide expression analyses indicated that YAP/TAZ, TEADs, and TGFβ-induced signals coordinate a specific pro-tumorigenic transcriptional program. Importantly, genes cooperatively regulated by these complexes, such as the novel targets neuronal growth regulator 1 (*NEGR1*) and urothelial cancer associated 1 (*UCA1*), are necessary to maintain tumorigenic activity in metastatic breast cancer cells. Nuclear YAP/TAZ also cooperate with TGFβ signaling to promote phenotypic and transcriptional changes in non-tumorigenic cells to overcome TGFβ-mediated growth inhibition. This work thus defines novel roles for YAP/TAZ in cancer, offering molecular mechanisms that may be useful for identifying and targeting YAP/TAZ-driven cancers.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALDH .... aldehyde dehydrogenase

AMP .... adenosine monophosphate

AMPK .... AMP-mediated protein kinase

ANOVA .... analysis of variance

ASSIGN .... adaptive signature selection and integration

bp .... base pairs

BRCA .... breast cancer gene

BSA .... bovine serum albumin

BT20 .... breast tumor 20

CAF .... cancer-associated fibroblast

CCN .... cyclin

CD24 .... cluster of differentiation 24

CD44 .... cluster of differentiation 44

CDK .... cyclin dependent kinase

cDNA .... complementary DNA

ChIP .... chromatin immunoprecipitation

CK1ε/δ .... casein kinase 1 ε/δ

Co-IP .... co-immunoprecipitation

CSC .... cancer stem cell

CT .... cycle threshold

CTGF .... connective tissue growth factor

CHAPTER I - INTRODUCTION

Disclaimer: Sections of this chapter are adapted from Hiemer, S. E., and Varelas, X. (2012) Stem cell regulation by the Hippo pathway. *Biochimica et Biophysica Acta* (1) license number #3651551200333 from Elsevier Limited.

Epithelial cancers

Hallmarks of cancer and the tumor microenvironment

Cancers are the second leading cause of death in the United States (2). A vast majority of tumors (90%) arise from the epithelium and are classified as carcinomas (3). Epithelial tissue, one of the four main tissue types, is comprised of the cells lining the inside and outside of the body and can be further categorized based on cell shape (e.g. cuboidal, squamous) and tissue structure (e.g. simple, stratified) (**Figure 1.1**). Tumorigenesis is considered a multistep disease (**Figure 1.1**) and understanding the molecular pathways responsible for initiation and progression is important in developing and applying effective therapeutics (4). The acquisition of specific "hallmarks" is widely accepted to be fundamental in cancer development (5). These characteristics illustrate the complexity of the disease and highlight both intra- and extra-cellular traits of the tumor. For instance, the cell becomes immortal and continually proliferates, resists cell death and growth suppressive cues, and evades the immune system. These attributes are supported through increased nutrient availability by angiogenesis and deregulation of cellular metabolism. Aggressive properties are also acquired such as the ability to invade surrounding tissue and metastasize to distant sites. Genomic instability and the

Stratified squamous Oral mucosa

Simple cuboidal Breast tissue

Normal epithelium **Precancerous lesion** **Carcinoma *in situ*** **Invasive/metastatic carcinoma**
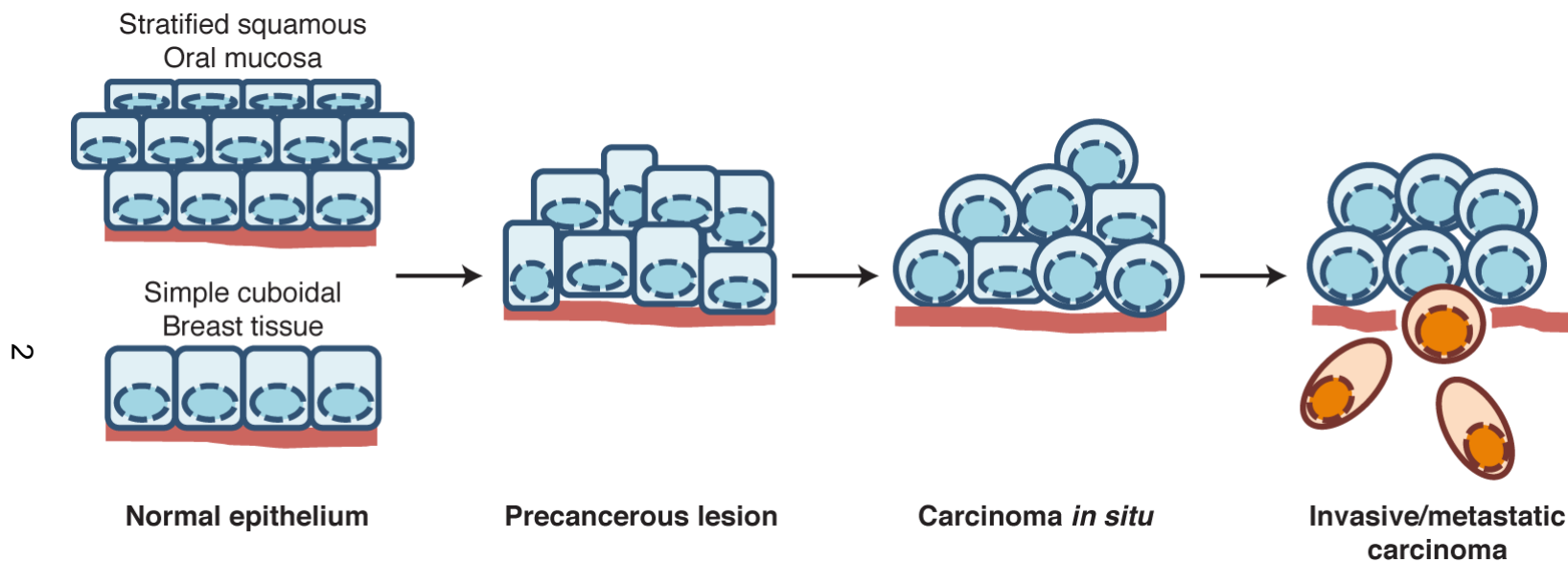
Figure 1.1 - Epithelial cancer progression. Tumor initiation and progression occur in a stepwise fashion, through the accumulation of mutations that manifest as "hallmarks of cancer." Carcinomas can arise from a range of normal epithelial tissue, like stratified squamous tissue found in the oral cavity to simple cuboidal tissue found in the breast.

accumulation of mutations underlie many of these characteristics. In addition, a tumor is no longer recognized as simply a homogenous mass of cancer cells but instead as a complex organ comprised of several different cell types. Cancer cells, cancer stem cells, cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, and immune cells, including tumor-associated macrophages, are all considered to communicate with one another and perpetuate the tumor microenvironment (**Figure 1.2**) (6).

Cancer stem cells

Cancer stem cells (CSCs) are a small subset of cells within a tumor that have the distinct ability to self renew and seed the heterogeneous populations which comprise the majority of the tumor (7). CSCs are hypothesized to promote tumor initiation and progression and have been implicated in chemoresistance and tumor relapse suggesting they drive a more aggressive disease state (6,8). CSCs do not necessarily originate from stem cell populations in normal tissue and can come from differentiated cancer cells populations that have acquired plasticity (9). Although the origins of CSC populations are not fully understood, the activation of an epithelial-to-mesenchymal transition (EMT) has been shown to confer stem-like properties on cancer cells (10-13). EMT occurs when a cell loses its epithelial characteristics, in particular cell-cell contacts and polarity, and gains a mesenchymal phenotype, allowing the cell to escape the epithelial layer, acquire motility, evade apoptotic cues, and invade the basement membrane (14,15). Overexpression of factors that promote EMT, such as transcription factors Twist
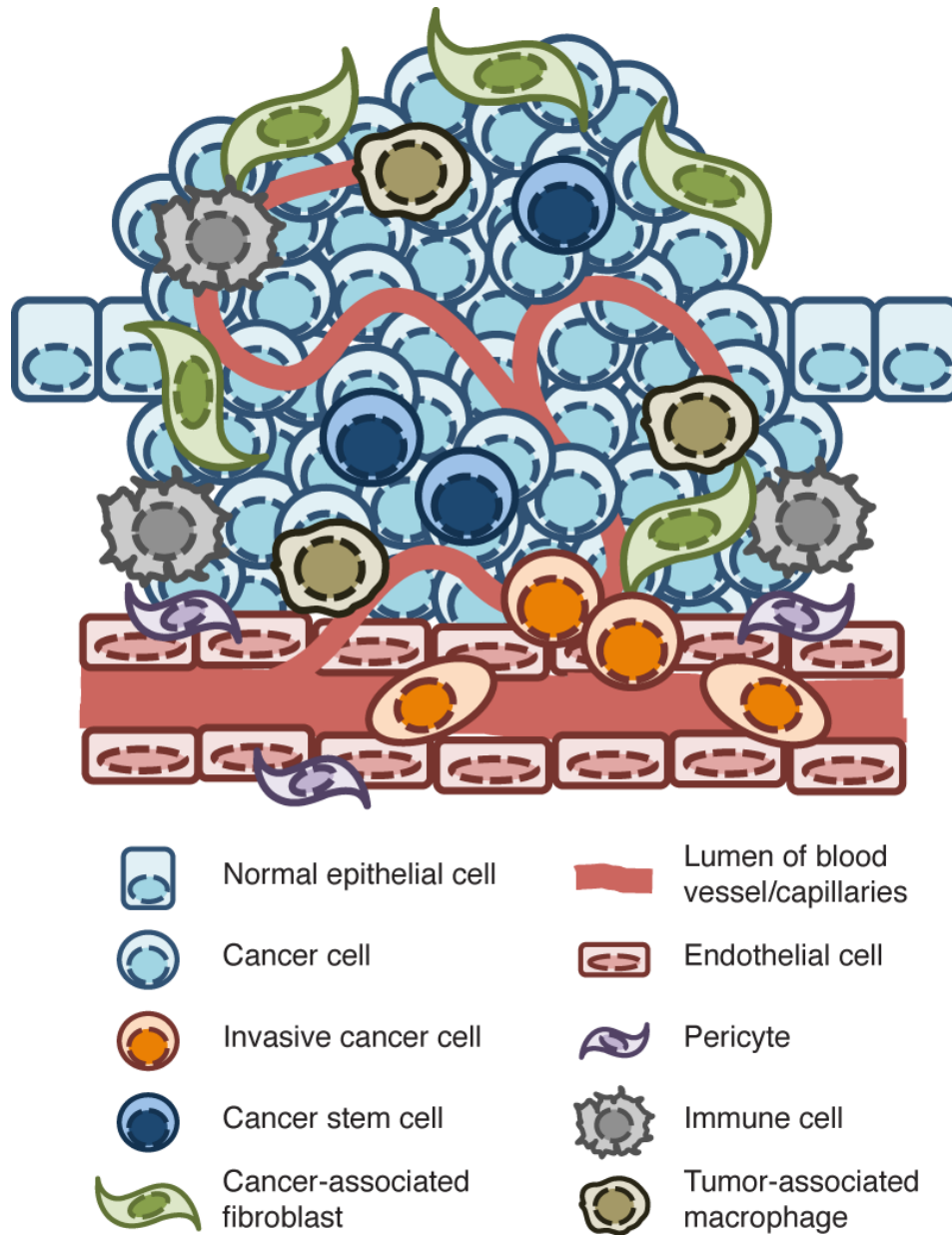
Figure 1.2 - The tumor microenvironment. The tumor microenvironment is complex and composed of several different types of cells.

and Snail, or cytokines like TGFβ, can promote tumor-initiating populations (11,16). CSCs can be identified and isolated by distinct cell markers, similar to normal stem cell populations, however the expression of these markers varies depending on context. In breast and head and neck cancers, several CSC markers have been described including cell surface glycoproteins cluster of differentiation 44 (CD44) and cluster of differentiation 24 (CD24), and intercellular protein aldehyde dehydrogenase (ALDH) (17-20).

Cancer characterization, grade v stage

During diagnosis, tumors are biopsied and evaluated by a pathologist to determine the progress of the disease defined by a grade or stage rating. This characterization aids physicians in determining the most appropriate treatment plan for the patient. Tumor grade refers to how organized the tissue appears, with well-differentiated tumors low-grade tumors (i.e. G1 tumors) being most similar to normal tissue. The more poorly differentiated or unorganized tumors are classified as highest grade, ranging from G2 to G4. Interestingly, high-grade breast cancers have a larger CSC population compared to low-grade cancers (21). Stage is also used to classify cancer progression and takes into account more aspects of the tumor including grade, the size of the primary tumor, and whether the cancer has metastasized to local or distant sites in the body. Stage ranges from I - carcinoma *in situ* to IV - distant metastases (22).

Oral cancer

OSCC is a subset of head and neck cancers that arises from the stratified squamous oral mucosa. Head and neck cancer is one of the 10 most common cancers worldwide and 90% of these are classified as OSCCs (23). These carcinomas are sometimes preceded by visible red or white pre-cancerous lesions, termed erythroplakias and leukoplakias respectively, and have a better prognosis when diagnosed in early stages (24,25). Unfortunately, a majority of oral cancers are diagnosed after they have already spread to the surrounding tissue, at which point the 5-year survival rates are around 50% (26). These patients do not respond well to treatment and have a greater chance of reoccurrence (27). Fortunately there is hope as OSCC can be prevented through early detection and reduced exposure to high risk-environmental factors such as alcohol and tobacco (28-30). Other risk factors include genetic predisposition and HPV status (31). Although current treatment options such as surgery, radiation, and chemotherapy have improved over the last few decades, targeted therapies are limited and a better understanding of molecular subtypes and biomarkers of OSCC is needed.

The accumulation of genetic mutations and aberrant signaling pathways have been implicated in the development and progression of OSCC. Similarly to many other cancers, frequent mutations in cell cycle-regulating genes are found in OSCC, including the loss of tumor suppressor genes *p53* and *Rb* and activation of *cyclin D1* (*CCND1*) (32-34). Mutations in other signaling pathways are also associated with OSCC including Notch and epidermal growth factor receptor

(EGFR), among many others. Although Notch signaling is complex in the context of tumorigenesis, generally loss of function mutations are observed in OSCC suggesting its function as a tumor suppressor in this context (35). On the other hand, EGFR signaling promotes tumorigenesis and activation occurs most commonly through either receptor overexpression or truncation, which leads to constitutive signaling (36,37). STAT3 hyperactivation is also associated with OSCC and there is evidence that STAT3 can function downstream of EGFR signaling (38,39). This is an important point to consider in using targeted EGFR therapies such as cetuximab, a recombinant monoclonal antibody that blocks extracellular EGFR from binding ligand, in OSCC treatment, as some tumors may not be responsive if downstream effectors are already aberrantly signaling (40).

Breast cancer

Breast cancer originates from the breast tissue and is the second leading cause of cancer deaths in women (2). It is a heterogeneous disease with tumors classified into various histological and molecular subtypes that aid in identification and treatment. These subtypes can be broadly defined by the spread of the tumor, indicated as either *in situ* or invasive carcinomas, and by the anatomical localization, described for instance as ductal, lobular, tubular, etc. Risk factors for developing breast cancer vary by subtype but may include age at menarche, age at first live birth, body-mass index, race, and family history (41). The histological classification of breast cancers has been in practice for several decades but does not take into account recent advances in describing molecular features (42). Gene

expression profiling and unbiased hierarchical clustering has resulted in the identification of distinct molecular subtypes of breast cancer to include luminal A (which make up approximately 40% of all breast cancers), luminal B (20%), triple negative/basal-like (15-20%), HER2 type (10-15%), and claudin-low (12-14%) (42-45). These classifications can be roughly categorized based on the expression of several genes including estrogen receptor (*ER*), progesterone receptor (*PR*), and human epidermal growth factor receptor type 2 (*HER2*). However, these classifications are generalized and can be ambiguous. For instance, luminal subtypes are ER and/or PR positive. Luminal A is HER2 negative with low cell proliferation whereas luminal B can be HER2 positive or HER2 negative with high cell proliferation. Luminal A breast cancers generally have the best prognosis out of all the subtypes (43). HER2 subtypes express high levels of HER2 whereas triple-negative breast cancers express low levels of ER, PR, or HER2. There is approximately 80% overlap between breast cancers that are considered both triple-negative and basal-like (46,47). Recently a new subtype has been identified, named claudin-low, and is defined as having high expression of EMT markers and low expression of luminal differentiation markers (44). The majority of claudin-low breast cancers are also considered triple-negative (48). HER2, triple-negative/basal-like, and claudin-low subtypes all have a higher incidence of CSC markers, such as $CD44^{high}/CD24^{low}$ and ALDH1, compared to luminal tumors (44,49). These breast cancers also have poorer survival rates and occur more often in patients with breast cancer 1 gene (*BRCA1*) mutations, although the link

8

between triple-negative/basal-like subsets and BRCA1 repair mechanisms is not clear (45,48). Tumors that retain *ER*, *PR*, and/or *HER2* expression can be treated with combinatorial hormone and trastuzumab (Herceptin) therapies with relative success. Unfortunately, triple-negative/basal-like breast cancers are not responsive to these treatments and other targeted treatment strategies are limited. Current options include surgery, radiation, and chemotherapy with potential therapeutic candidates including anti-angiogenic agents, platinum salts, and poly ADP ribose polymerase inhibitors (48). However, once a triple-negative/basal-like breast cancer has metastasized, relapse is prevalent with very poor prognosis (50).

The Hippo Pathway

Overview and pathway members

The Hippo pathway was originally discovered through forward genetic screens in *Drosophila melanogaster* that were investigating mediators of tissue overgrowth (51,52). These studies defined the Hippo pathway as a suppressor of tissue overgrowth primarily through the control of cell proliferation and apoptosis (53-58). Disruption of the upstream pathway components in flies leads to enlarged organs without major changes in overall patterning. Since these initial studies, the Hippo pathway and downstream effectors have been examined in the context of mammalian development and disease. It is clear that Hippo pathway components have critical roles in stem cell maintenance and differentiation as well as cancer. The focus of my thesis research was to investigate the roles of YAP/TAZ, the

primary effectors of the Hippo pathway in mammals, in the context of epithelial tumorigenesis and progression.

The core Hippo pathway consists of a conserved kinase cascade that results in the phosphorylation and inhibition of two homologous transcriptional co-regulators YAP and TAZ (also known as WW domain containing transcription regulator 1 or WWTR1) (YAP/TAZ; *Drosophila* Yorkie/Yki) (**Figure 1.3**). Upstream regulation is established through the phosphorylation and activation of sterile 20 (STE20) kinases mammalian ste20-like serine/threonine 1 and 2 (MST1/2, *Drosophila* Hippo/Hpo) through unknown mechanisms. With the help of adapter proteins salvador (SAV2, *Drosophila* Sav) and mps one binder protein kinase like 1 A/B (MOBKL1A/B, *Drosophila* Mats), MST1/2 phosphorylates and activates the nuclear dbf2-related (NDR) family kinases large tumor suppressor 1 and 2 (LATS1/2; *Drosophila* Warts/Wts) (59,60). LATS1/2 in turn phosphorylates YAP/TAZ on several key serine residues to promote YAP/TAZ cytoplasmic localization and degradation (**Figure 1.3**). One mechanism contributing to the sequestration of YAP/TAZ is the binding of cytoplasmic 14-3-3 proteins, an interaction that is mediated by the phosphorylation of a specific serine residue (Ser127 in human YAP, Ser89 in human TAZ) (61,62). Another is the recruitment of ubiquitin ligases β-transducin repeat-containing protein/skp1/cul1/f-box complex (β-TrCP/SCF) to promote YAP/TAZ proteasomal degradation. This is mediated by LATS1/2 phosphorylation (Ser397 in YAP, Ser311 in TAZ), which promotes further phosphorylation events by casein kinase 1 ε/δ (CK1ε/δ; Ser400/403 in YAP,
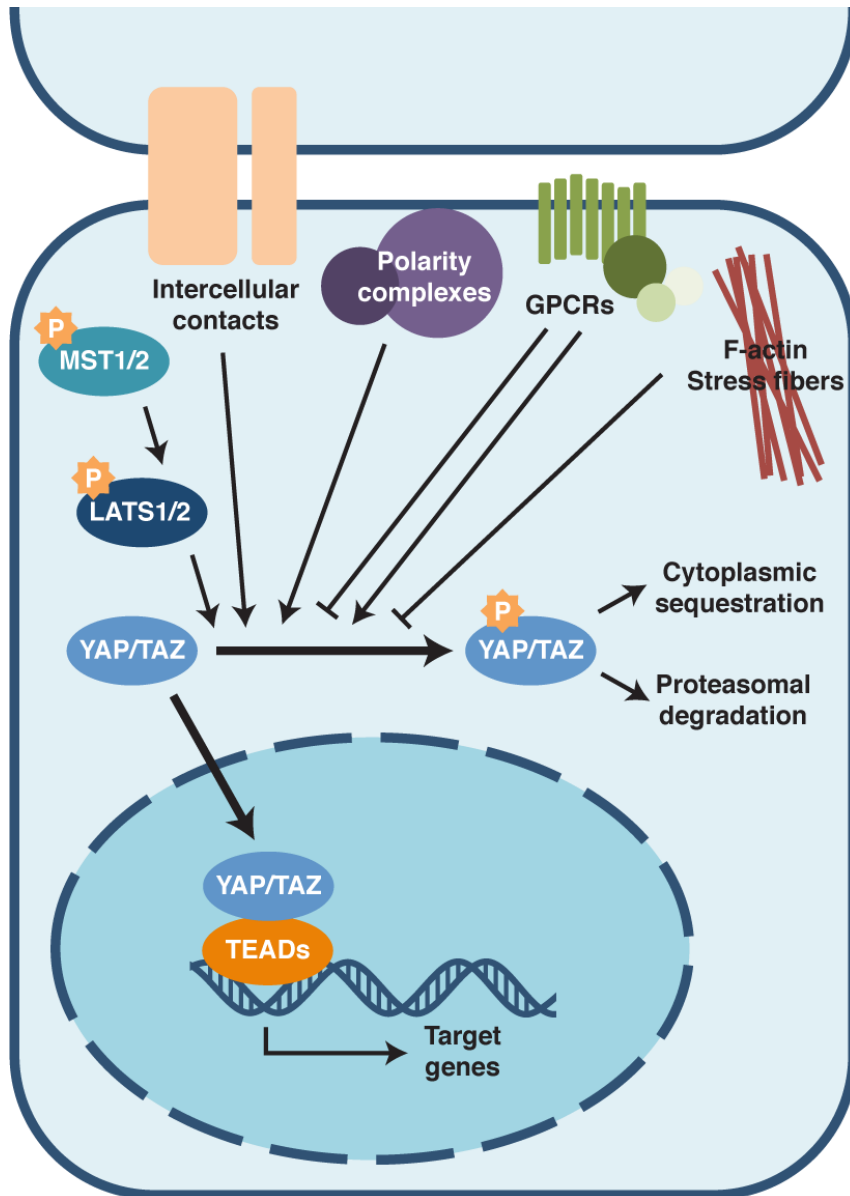
Figure 1.3 - Regulation of YAP/TAZ localization. YAP/TAZ localization can be controlled through a variety of cellular cues. MST1/2 and LATS1/2 comprise the canonical Hippo pathway kinase cascade but other mechanisms are known to affect YAP/TAZ phosphorylation status both positively and negatively. Phosphorylated YAP/TAZ results in either their cytoplasmic retention or proteasomal degradation while unphosphorylated YAP/TAZ can accumulate in the nucleus and bind to a range of transcription factors, including the TEAD family.

Ser314 in TAZ) that are recognized by β-TrCP/SCF (63,64). β-TrCP recruitment can also be mediated by glycogen synthase kinase 3β (GSK3β) phosphorylation of TAZ (Ser58 and Ser62) (65). Alanine substitutions of LATS-targeted serine residues within YAP or TAZ promote their stability and nuclear localization. Once nuclear, YAP/TAZ are able to bind and direct the activity of many different transcription factors, including the TEAD family (TEAD1-4 also known as transcriptional enhancer factors or TEFs, *Drosophila* Scalloped/Sd) (**Figure 1.3**) (1). Together with YAP/TAZ, TEADs promote cellular transformation through the expression of cell proliferation and anti-apoptotic genes (66,67). Thus overall, active Hippo pathway signaling leads to decreased YAP/TAZ nuclear localization whereas inhibition of upstream components results in nuclear YAP/TAZ and increased transcriptional activity through the association with a range of transcription factors.

Upstream regulation of the Hippo pathway

The regulation of Hippo pathway activity is complex and may differ depending on tissue context. Various cues affect Hippo signaling to control YAP/TAZ localization including mechanical and soluble signals (**Figure 1.3**). Cytoskeletal dynamics and cell contacts are the best described but their dependence on upstream kinase signaling remains unclear. A striking observation has been made regarding the dynamic nature of YAP/TAZ localization during cell compaction. Sub-confluent cells display nuclear pools that shift to the cytoplasm upon high density (68). These signals are relayed in part through the establishment

of cell contacts and polarity complexes, which may function through both the sequestration and/or phosphorylation of YAP/TAZ. Many different proteins have been implicated in this process including cytoskeletal proteins Merlin and Frmd6, adherens junction protein alpha-catenin, tight junction protein ZO-2, polarity proteins of the Crumbs complex (PatJ, Mpdz, Pals1, Lin7c, Amot), and Scribble complex (Scrib, Dlg, Lgl) and the regulation is complex (69). To highlight, loss of alpha-catenin or either Crumbs or Scribble results in nuclear YAP/TAZ accumulation (12,70-72). The matrix surface area and stiffness that adherent cells interact with can also affect YAP/TAZ localization, probably through actin cytoskeleton dynamics and reorganization. For instance, stress fiber formation results in nuclear YAP/TAZ, which is dependent on the activity of Rho-GTPases and downstream Rho-kinases (73). Secreted factors can also modulate YAP/TAZ activity through g protein coupled receptor (GPCR) signaling. Phospholipids such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) signal through Gα12/13 and can promote nuclear YAP/TAZ activity. Conversely, hormones such as epinephrine and glucagon signal to inhibit YAP/TAZ nuclear activity. It is unclear how involved upstream Hippo pathway components are as LPA and hormone signaling appear to depend on LATS1/2-mediated phosphorylation of YAP/TAZ but are dispensable for S1P signaling (74,75).

Role and regulation of transcriptional co-factors YAP and TAZ

YAP and TAZ are structurally very similar and have approximately 60% sequence similarity in humans. Redundant functions exist for YAP and TAZ,

although they also seem to have independent roles in different tissue contexts, which may be explained through their structural domains and specificity of interacting partners (**Figure 1.4**) (69). The first identified domain of YAP/TAZ was the WW motif, consisting of two tryptophans that are 20-23 residues apart and are known to bind PPxY (proline-proline-amino acid-tyrosine) containing proteins. Several binding partners of YAP/TAZ have PPxY domains, most notably LATS1/2. Alternatively spliced isoforms of YAP and TAZ exist with either one or two WW domains, named YAP1-1 or YAP1-2 and TAZ1 or TAZ2 respectively, that may allow for differences in binding affinity to PPxY proteins (76-78). Interestingly, YAP1-1 and YAP1-2 interact differently with specific proteins, as YAP1-1 cannot bind p73 or angiomotin while YAP1-2 can (79,80). In addition, YAP1-2 appears to be the major isoform both in terms of expression and transcriptional activity, however only TAZ1 has been identified in humans (81,82). Both YAP and TAZ also have an unstructured transcriptional activation domain in their C-terminal region that is required for their transcriptional activity (83,84). The last four amino acids make up a PDZ-binding motif mediating binding to PDZ domain-containing proteins that can alter YAP/TAZ localization (85,86).

YAP and TAZ cannot bind DNA directly and thus rely on transcription factors to relay their signals. They are known to bind many different transcription factors and are capable of stimulating or inhibiting the activity of these factors, although specific mechanisms are not well understood (69). YAP/TAZ interactions with TEADs are the best described, particularly in the context of tumorigenesis, and the

14
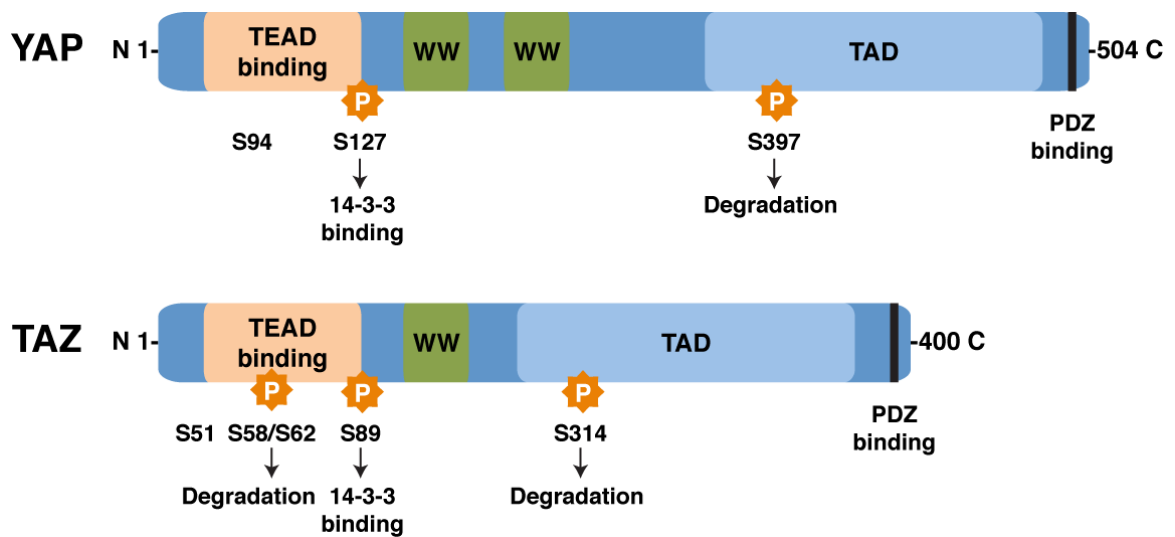
Figure 1.4 - YAP/TAZ protein domains. YAP and TAZ homologues share several important protein motifs. Most notably are the TEAD binding, WW, and transcriptional activation domains (TAD). YAP1-2 and TAZ1 isoforms are depicted, indicating the number of WW domains present. Specific serine residues and their known function are also highlighted.

oncogenic potential of YAP/TAZ relies on TEAD binding (67,87). This binding is mediated through a TEAD binding domain in the N-terminus of YAP/TAZ and can be disrupted by a single point mutation (Ser94 in YAP, Ser51 in TAZ) (67,87,88). Recently, phosphorylation by AMP-mediated protein kinase (AMPK) on this residue in YAP has been found to disrupt YAP-TEAD binding, suppressing cell proliferation and oncogenic transformation under low energy conditions (89). AMPK can also indirectly inhibit YAP by activating LATS to further restrict nuclear activity. Thus, AMPK links cellular energy stress to the Hippo pathway and is another point of regulation.

YAP/TAZ in cancer

Dysregulated YAP/TAZ activity is associated with a range of aggressive cancers and high nuclear levels correlate with high histological grade, increased invasiveness, and decreased survival rates in several cancers, including oral and breast cancers (12,90-95). Elevated YAP/TAZ also promote resistance to cancer therapies such as cisplatin and cetuximab, used to target OSCC, and paclitaxel and doxorubicin, used in breast cancer treatment (96-99). Initial studies of YAP/TAZ found that their increased nuclear activity potently drives cell transformation and tumor initiation through the control of proliferation, survival, and migration (100-102). This oncogenic activity is also associated with the acquisition of an EMT phenotype, which may explain the induced CSC properties that high expression of YAP/TAZ can confer (10,12). Transcriptional analysis of high-grade metastatic breast cancers revealed an enrichment of YAP/TAZ target gene

expression. Consistent with this observation, TAZ is required to maintain metastatic and CSC properties in breast cancer and expression of a nuclear TAZ mutant (TAZ-S89A) is sufficient to confer self-renewal of breast cancer populations. Whether YAP functions in the same capacity in this context is unclear and breast cancer tissue analysis has revealed that YAP levels change less dramatically than TAZ upon metastatic conversion, implying a more minor role for YAP in these cancer populations (12,101).

YAP/TAZ have been hypothesized to function downstream of other transcriptional regulators known to promote CSC properties (12). Such factors include the transcriptional repressors Snail and Twist, which promote EMT and CSC properties by down-regulating genes important for cell polarity and adhesion (11,103). The proper organization of cell polarity complexes, such as Crumbs and Scribble, induce YAP/TAZ cytoplasmic localization and decreased transcriptional activity through their sequestration and phosphorylation. Scribble depletion or mislocalization induces tumorigenesis in breast cancers, perhaps through the deregulation of YAP/TAZ activity (104). Snail and Twist may be exerting partial effects indirectly through the modulation of YAP/TAZ. Consistent with this interpretation, TAZ knockdown inhibits CSC properties associated with Snail or Twist expression in breast cancer cells without affecting the disruption of polarity (12). Thus, YAP/TAZ are a prominent thread linking loss of polarity, induction of EMT, and CSC properties, and the combination of invasive properties with the ability to self-renew is suggested to drive aggressive cancers.

17

Although there is evidence that *YAP* is amplified in certain cancers, mutations in Hippo pathway components are not common (90,105,106). This suggests that dysregulation of YAP/TAZ activity occurs primarily on the level of protein expression and stability through the control of subcellular localization and phosphorylation. Interestingly, another manner of regulation may occur through *YAP/TAZ* expression as hypermethylation of CpG islands in the *TAZ* promoter correlate with reduced TAZ levels and lower-grade glioblastomas (107).

Crosstalk with other pathways

There is evidence that YAP/TAZ cooperate with other oncogenic signals to promote tumorigenesis and several other pathways frequently altered in cancer have been linked to Hippo signaling including signals regulated by MAPK, ERBB4, GPCRs, WNT, and TGFβ, among others (69,90,108,109).

Recently, YAP has been linked to aberrant KRAS signaling in colon, lung, and pancreatic cancers and is necessary for KRAS-induced EMT through the activation of the FOS transcription factor (110). YAP was also shown to act downstream of KRAS-activated MAPK signaling to mediate the expression of pro-tumorigenic secreted factors such as connective tissue growth factor (*CTGF*), cysteine-rich angiogenic inducer 61 (*CYR61*), and matrix metalloproteinase 7 (*MMP7*) (111). RAS downstream of EGFR stabilizes YAP through the inhibition of suppressor of cytokine signaling 5/6. Stabilized YAP can promote the transcription of *amphiregulin* to create a positive feedback loop on EGFR signaling (112).

18

Another member of the EGFR family often dysregulated in cancer, ERBB4, has been implicated in the regulation of YAP (113). Activation of transmembrane ERBB4 by extracellular neuregulin 1 ligand can result in proteolytic cleavage and release of an intracellular domain of ERBB4 (ICD ERBB4). ICD ERBB4 translocates to the nucleus and binds YAP via PPxY-WW domain interactions to promote *CTGF* transcription and protumorigenic phenotypes (81,114). In this way, ERBB4 can act as an upstream receptor of the Hippo pathway and positively regulate YAP activity.

GPCRs have also been described as upstream regulators of YAP and are often mutated in cancers (115). LPA, a GPCR activator, is also carcinogenic, perhaps through YAP signaling (116). Cancer associated mutations in Gαq and Gα11 can activate YAP in uveal melanoma, although it is unclear if this is mediated through LATS inactivation or is independent of upstream Hippo signaling (117,118). In either case, YAP is necessary for tumorigenic GPCR-induced signals, which can be blocked with verteporfin treatment, a small molecule inhibitor of YAP-TEAD binding (119).

YAP and TAZ are known to interact with components of the wingless-related integration site (WNT) pathway to control signaling during development (69). Cytoplasmic YAP/TAZ function as an inhibitor of WNT signaling by suppressing the phosphorylation of the WNT pathway effector dishevelled 2 and by incorporating into the destruction complex to promote the degradation of β-catenin (120). In the presence of WNT, the destruction complex is relocated to the plasma

membrane and inactivated, freeing YAP/TAZ and β-catenin and allowing them to translocate to the nucleus where they can direct target gene transcription. The relationship between YAP/TAZ and WNT signaling is still incomplete and may be context dependent, which warrants further investigation (120-122). In colorectal cancers, loss of function of adenomatous polyposis coli or activating mutations in β-catenin are common and lead to unrestrained WNT signaling (123). In this context, active β-catenin can hyperactivate YAP by either physically interacting and promoting nuclear accumulation or driving *YAP* expression (124,125).

In addition to WNT pathway components, YAP and TAZ are also known to interact with TGFβ-activated SMADs (70,126). TAZ can bind SMAD2/3-4 complexes to direct their cellular localization and activity during development and YAP/TAZ-TEAD-SMAD2/3 complexes control transcriptional events necessary in the maintenance of human embryonic stem cell pluripotency (126,127). Disruption of polarity complexes in mammary epithelial cells, which promotes nuclear YAP/TAZ activity, also enhances TGFβ-induced SMAD nuclear responses through YAP/TAZ (70). YAP and TGFβ were found to function synergistically on specific gene targets in malignant mesothelioma. Specifically, YAP, TEAD4, SMAD3, and p300 form a complex that binds upstream of *CTGF* to stimulate gene expression (128). *CTGF* was previously identified as a direct gene target of both YAP and TGFβ in independent studies (67,129). CTGF is a secreted factor with known roles in promoting cell proliferation and EMT, however it is not completely clear how it exerts these effects.

The TGFβ pathway

TGFβ signaling

The TGFβ superfamily of secreted growth factors, encompassing over 40 ligands including the prototypic TGFβ, Bone morphogenetic proteins, Activins, and Nodal, regulates numerous developmental, homeostatic, and tumorigenic processes (130-132). Three mammalian isoforms of TGFβ (TGFβ1, 2, and 3) are known, which function through the same downstream signaling pathway, although TGFβ1 is the most frequently expressed in cancer (133-135). Canonical TGFβ signals are transduced through SMAD proteins to control target genes such as regulators of cell proliferation, apoptosis, and matrix synthesis (**Figure 1.5**) (136). Latent TGFβ ligand is activated, through proteolytic cleavage, integrin binding, or mechanical stress, to promote dimer formation and binding to transmembrane serine/threonine kinase receptors, TGFβ receptor type I (TGFβRI) and type II (TGFβRII) (137). Once in contact with TGFβ ligand, TGFβRII recruits TGFβRI to form heteromeric complexes that result in the phosphorylation and activation of TGFβRI. Receptor activation transduces intracellular signaling through the phosphorylation of receptor-activated SMADs, SMAD2 and SMAD3 (SMAD2/3). Phosphorylated SMAD2/3 trimerize with SMAD4 and translocate to the nucleus where the complex binds SMAD binding elements to function as a transcriptional mediator (138-141). SMAD complexes bind with poor affinity to DNA and thus cooperate with other transcription factors to initiate chromatin remodeling that either activates or represses transcription depending on context (136,142).
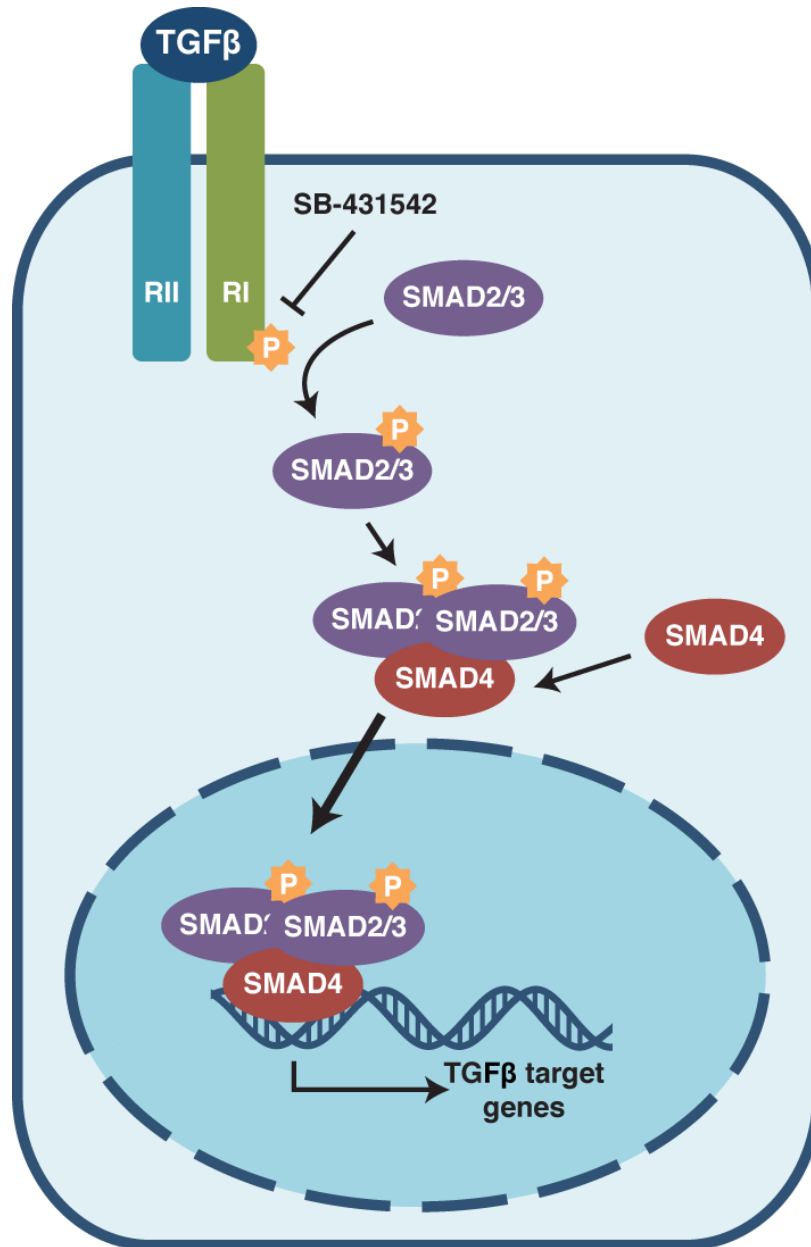
Figure 1.5 - TGFβ signaling. TGFβ ligand binding to type I and type II TGFβ receptors results in SMAD phosphorylation and complex formation. Activated SMADs are able to translocate to the nucleus and control target gene transcription. Small molecule SB-431542 inhibits receptor phosphorylation and blocks SMAD activation.

TGFβ signals can be controlled through feedback signals regulated by target genes that encode negative regulators such as *SMAD7* (143). SMAD7 can function in several different ways to inhibit TGFβ signaling through TGFβRI including directly blocking receptor phosphorylation and activation of receptor SMADs, recruiting protein phosphatase 1 to the receptor, or recruiting Smurf1/2 ubiquitin ligases to target the receptor for proteasomal degradation (136,144,145).

TGFβ in cancer

TGFβ is known to play a major role during tumorigenesis and cancer progression. Various cells in the tumor microenvironment can be responsible for TGFβ production including the cancer cells and the stroma (146). It is also accepted that the effects of TGFβ are context dependent, as TGFβ suppresses growth in normal epithelial cells and early tumors but can promote proliferation and invasion in late stage tumors leading to more aggressive cancers (**Figure 1.6**) (146,147). The mechanism responsible for the switch in TGFβ activity has not been well defined although it is clear the loss of TGFβ suppressive effects combined with the acquisition of invasive properties is important during the process.

In normal epithelial cells, TGFβ regulates cytostatic events primarily through the transcriptional regulation of genes involved in the G1/S phase restriction checkpoint, a crucial and evolutionarily maintained transition period that controls both cell division and genomic maintenance (148). For instance, TGFβ induces the expression of cyclin dependent kinase (CDK) inhibitors *p15, p21*, and *p27* (149-151). These proteins block G1/S phase cyclins from associating with their
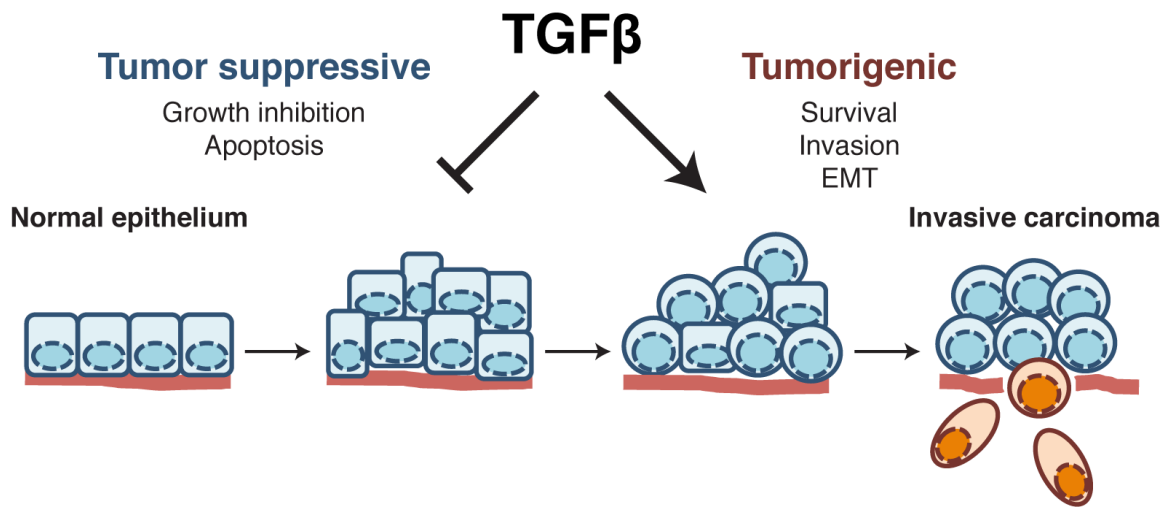
Figure 1.6 - Dual roles for TGFβ in tumorigenesis. TGFβ is known to exert different cellular effects depending on tissue context. In normal tissue and early stage tumors, TGFβ suppresses tumorigenesis by inducing cytostasis and apoptosis. However, in more advanced cancers, TGFβ activity switches to promote cell invasion and survival.

respective kinases, predominantly cyclin D-CDK4/6 and cyclin E-CDK2, preventing retinoblastoma (Rb) protein phosphorylation and entry into S phase (148). TGFβ also suppresses the expression of proliferative factors such as the transcription factor c-Myc and the inhibitor of DNA-binding family, which can inhibit Rb activity allowing for cell cycle progression (152-155). In late stage tumors, mutations in *p15* develop and the suppression of *c-Myc* or inhibitor of DNA-binding family gene targets is lost, leading to disruption of TGFβ mediated cytostasis (156-159).

TGFβ signaling can direct apoptotic events through both transcriptional and spatial regulation of various apoptotic factors. Several diverse pro-apoptotic genes are transcriptional targets of TGFβ including TGFβ-inducible early response gene transcription factor, death-associated protein kinase, and sh2-domain containing inositol-5-phosphatase, an inhibitor of AKT signaling (160-162). Other factors can directly relay TGFβ cues to modulate apoptosis like Apoptosis-related protein in the TGFβ signaling pathway, which moves from the mitochondria to the nucleus upon TGFβ stimulation to suppress inhibitors of apoptosis, or the adapter protein death domain-associated protein 6, which promotes JNK activation through its association with TGFβRII (163,164).

TGFβ promotes EMT through the regulation of *Snail*, *Slug*, and *Twist* transcription factors and the suppression of epithelial markers like *E-cadherin* (165). This is a key process in development, particularly during gastrulation, and requires properly coordinated steps that become uncontrolled in cancer (166). In

breast cancer, TGFβ confers CSC properties through the induction of EMT resulting in the expansion of CD44^{high}/CD24^{low} populations (11,167). Treatment with a TGFβRI kinase blocker induces these cells to adopt a more epithelial phenotype (167).

TGFβ plays additional roles in the tumor microenvironment besides regulating cell growth and invasion in cancer cells. Originally, TGFβ was discovered to enhance the proliferation of fibroblasts and is known to activate fibroblasts to induce extracellular matrix (ECM) remodeling (168,169). In the microenvironment, TGFβ can stimulate CAFs to secrete factors that modify the extracellular matrix, promote proliferation, invasion, and angiogenesis, which can signal to the cancer cells (170-172). Again, the source of TGFβ can come from several populations within the tumor including both the CAFs and cancer cells, allowing for intercellular communication and the potential for the generation of a feed-forward loop to promote aggressive cancers (146,173).

Significance and research goals

Deregulated Hippo pathway signaling promotes the onset of aggressive cancers (59). Through the work of several laboratories, the Hippo pathway has emerged as a central signal transduction pathway that integrates mechanical and extracellular cues to control cell proliferation, apoptosis, and cell fate (120,126,174,175). The localization and activity of the transcriptional regulators YAP/TAZ mediates Hippo pathway signaling responses, and uncontrolled nuclear YAP/TAZ activity evokes cell proliferation, tumor-initiating properties, and drug-

26

resistance in a broad range of cancers (1,174). While YAP/TAZ are essential for accurate organ growth, a gap in knowledge exists for how YAP/TAZ direct transcription to promote tumor-initiation and how they interact with other signaling pathways to accomplish this. Insight into these molecular details is critical for understanding general mechanisms of tumor suppression and is necessary for the development of effective cancer therapeutics.

The goal of this thesis research was to define the mechanisms mediating Hippo pathway-mediated tumorigenesis by 1) exploring the roles of YAP/TAZ in the formation and progression of oral cancer and 2) investigating how the convergence of YAP/TAZ and TGFβ-induced transcriptional cues promote tumorigenesis in breast cancer. I hypothesize that YAP/TAZ drive pro-tumorigenic events in oral cancer and cooperate with TGFβ signals to promote aggressive breast cancers.

CHAPTER II - MATERIALS AND METHODS

Cell culture and transfections

CAL27, Squamous cell carcinoma 2 (SCC2), Breast tumor 20 (BT20), HS578T, SKBR3 and HEK293T cells were cultured in DMEM (Hyclone) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin (P/S) (Gibco, Life Technologies). SCC9, SCC15 and SCC25 cells were cultured in DMEM/F12 (1:1) (Hyclone) supplemented with 10% FBS, 400 ng/mL hydrocortisone (Sigma) and 1% P/S. Michigan cancer foundation 10A and 12A (MCF10A and MCF12A) cells were cultured in DMEM/F12 (1:1) supplemented with 5% horse serum (HS) (Gibco, Life Technologies), 20 ng/mL EGF (PeproTech), 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin (Sigma), 10 μg/mL insulin (Sigma), and 1% P/S. Human mammary epithelial (HMLE) cells were cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL EGF, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 1% P/S. MCF7 cells were cultured in DMEM supplemented with 10% FBS, 10 μg/mL insulin, and 1% P/S. MDA-MB-231 and LM2-4 cells were cultured in Roswell park memorial institute media 1640 (RPMI 1640) (Gibco, Life Technologies) supplemented with 10% FBS and 1% P/S. SUM149 cells were cultured in Ham's F12 (Hyclone) supplemented with 5% HS, 400 ng/mL hydrocortisone, and 10 μg/mL insulin. Cell lines and culture conditions are outlined in **Table 1**.

Table 1 - Cell lines and culture conditions

Cell line	Origin	Species	From	Media	Supplements						Selection	
					Serum	EGF	Hydrocortisone	Cholera toxin	Insulin	P/S	G-418	Puromycin
CAL27	Oral	Human	ATCC	DMEM	10% FBS	-	-	-	-	1%	1 mg/mL	1 µg/mL
SCC2	Oral	Human	Weigert	DMEM	10% FBS	-	-	-	-	1%	2 mg/mL	1 µg/mL
SCC9	Oral	Human	ATCC	DMEM/F12	10% FBS	-	400 ng/mL	-	-	1%	-	-
SCC15	Oral	Human	ATCC	DMEM/F12	10% FBS	-	400 ng/mL	-	-	1%	-	-
SCC25	Oral	Human	ATCC	DMEM/F12	10% FBS	-	400 ng/mL	-	-	1%	-	-
MCF10A	Breast	Human	ATCC	DMEM/F12	5% HS	20 ng/mL	500 ng/mL	100 ng/mL	10 µg/mL	1%	1 mg/mL	1 µg/mL
BT20	Breast	Human	Kirsch	DMEM	10% FBS	-	-	-	-	1%	-	-
HMLE	Breast	Human	ATCC	DMEM/F12	-	20 ng/mL	500 ng/mL	100 ng/mL	10 µg/mL	1%	-	-
HS578T	Breast	Human	Kirsch	DMEM	10% FBS	-	-	-	-	1%	-	-
MCF7	Breast	Human	Kirsch	DMEM	10% FBS	-	-	-	10 µg/mL	1%	-	-
MCF12A	Breast	Human	ATCC	DMEM/F12	5% HS	20 ng/mL	500 ng/mL	100 ng/mL	10 µg/mL	1%	-	-
MDA-MB-231	Breast	Human	ATCC	RPMI 1640	10% FBS	-	-	-	-	1%	-	-
LM2-4	Breast	Human	Viloria-Petit	RPMI 1640	10% FBS	-	-	-	-	1%	1.5 mg/mL	2 µg/mL
SKBR3	Breast	Human	Kirsch	DMEM	10% FBS	-	-	-	-	1%	-	-
SUM149	Breast	Human	Sherr	Ham's F12	5% HS	-	400 ng/mL	-	10 µg/mL	1%	-	-
HEK293T	Kidney	Human	ATCC	DMEM	10% FBS	-	-	-	-	1%	-	-

For transfections, RNA interference was performed by transfecting siRNA using Dharmafect 1 (Thermo Scientific) according to manufacturer's protocol. siRNA sequences are outlined in **Table 2**. HEK293T cells were transfected using TurboFect (Thermo Scientific) according to manufacturer's protocol. Prior to transfection, HEK293T cells were plated on dishes coated with 1% poly-L-lysine for 30 minutes at 37°C.

Lentivirus production and stable cell lines

HEK293T cells were co-transfected with 1.5 µg/µL of ps paired box gene 2, 0.75 µg/µL of pCMV-vesicular stomatitis virus G protein, and 1.5 µg/µL of each viral plasmid. Media was changed the following day to DMEM supplemented with 10% heat inactivated FBS and cells were incubated an additional 2-3 days to produce virus. Media was collected and filtered using 0.45 µm filters. 100 µL of virus was used to infect sub-confluent cells and cells were selected 2-3 days after infection.

CAL27 and MCF10A doxycycline-inducible stable cell lines were engineered using the lentiviral Tet-On system (Clontech). 3xFLAG-tagged mutants of YAP (5SA: S61A, S109A, S127A, S164A, S97A or 5SA/S94A) or TAZ (4SA: S66A, S89A, S117A, S311A) were generated by site-directed mutagenesis and cloned into the pLVX-Tight-Puro plasmid (#632162, Clontech). Tet-On cells were selected with G-418 sulfate (Gold Biotechnology) and pLVX-Tight-Puro cells were selected with puromycin (American Bioanalytical or Invivogen). Selection conditions are outlined in **Table 1**.

Table 2 - siRNA and shRNA sequences

Target	RNA	Species	Sequence	Source
CTL	siRNA	human	GGGCAAGACGAGCGGGAAG	Thermo Scientific
TAZ	siRNA	human	siGENOME WWTR1 siRNA#1,2,4	Thermo Scientific (MQ-016083-00-0002)
YAP	siRNA	human	siGENOME YAP1 siRNA siRNA#1-4	Thermo Scientific (MQ-012200-00-0002)
YAP/TAZ	siRNA	human	UGUGGAUGAGAUGGAUACA	Thermo Scientific
TEAD1/3/4	siRNA	human	GCAAGCAGGUGGUGGAGAA	Thermo Scientific
TEAD2	siRNA	human	CGAAGGAAAUCAAGGGAAA	Thermo Scientific
NEGR1	siRNA	human	CCUCUUUCACCAGCAUAUU	Sigma
UCA1	siRNA	human	CAGCUUAAUCCAGGAGACA	Sigma
CTL	shRNA	human	GGGCAAGACGAGCGGGAAG	pLKO1 puro (Open Biosystems)
TAZ	shRNA	human	GCGATGAATCAGCCTCTGAAT	pLKO1 puro (Open Biosystems)
YAP	shRNA	human	GCCACCAAGCTAGATAAAGAA	pLKO1 puro (Open Biosystems)

SCC2-dsRED fluorescent cells were generated using lentiviral transduction of CMV-promoter driven dsRED. Stable knockdown of YAP and TAZ in SCC2-dsRED and LM2-4 cells was accomplished by lentiviral-mediated transduction of shRNA using the pLKO1-puro (a gift from Bob Weinberg (176)) and pLKO1-neo vectors (a gift from Sheila Stewart). pLKO1 vectors were engineered to express shRNA targeting control (shCTL), YAP (shYAP, pLKO1-shYAP-2 was a gift from Kun-Liang Guan, Addgene plasmid #27369 (67)), or TAZ (shTAZ (177)). Selection conditions are outlined in **Table 1** and shRNA sequences are outlined in **Table 2**.

Cell treatments

Doxycycline-inducible cells were treated with doxycycline (0.1 to 100 ng/mL or 100 ng/mL if not otherwise stated, Clontech) for at least 24 hours to induce the expression of 3xFLAG-tagged YAP-5SA, 5SA/S94A, or TAZ-4SA.

Cells were treated with TGFβ1 (500 pM, R&D) and/or SB-431542 (5 μM, Sigma) for 2 to 24 hours as indicated.

Human oral tissue specimens

Tissue specimens were obtained from patients at Boston University Medical Center, and were acquired from scalpel-generated incisional biopsies of oral epithelium (benign epithelial hyperplasia (n=7), mild (n=3) and severe (n=3) dysplasia), as well as from surgical resections of moderately differentiated (n=6) and poorly differentiated (n=4) OSCCs of the lateral tongue border and of the floor of the mouth. For each condition, cytologically normal adjacent epithelia were also obtained and analyzed. Benign epithelial hyperplasia, dysplasia, and OSCC

regions, as well as adjacent epithelia, were defined by an on-site histopathological examination by Vikki Noonan and tissues were snap-frozen at -80°C. A portion of these tissues were sectioned and used for H&E staining and immunofluorescence imaging or were lysed for biochemical analysis (see below). The institutional review board at the Boston University Medical Campus approved the use of human oral tissue specimens for these studies.

Expression analysis of The Cancer Genome Atlas (TCGA) OSCC data

Normalized Level 3 gene expression (RNASeqV2) and associated clinical data were obtained from TCGA corresponding to the Head and Neck Squamous Cell Carcinoma dataset (n=340; https://tcga-data.nci.nih.gov/tcga/). Samples were filtered so as to retain only those belonging to one of six oral cancer anatomic subtypes (Alveolar Ridge, Base of tongue, Buccal Mucosa, Floor of mouth, Oral cavity, Oral tongue), and only Caucasian patients were analyzed (filtered Oral Cancer dataset size: n=193). Box plots of the expression values were generated with respect to tumor grade/stage for YAP and TAZ (log2-transformed).

Protein isolation and co-immunoprecipitation (co-IP)

Cell monolayers were lysed in lysis buffer (50 mM Tris (pH 7.5), 150 mM sodium chloride (NaCl), 1 mM EDTA, 0.5% Triton X-100, and 1X Halt™ protease inhibitor cocktail (Thermo Scientific)) and were agitated for 10 minutes at 4°C. Human tissue samples were weighed and lysed in a relative amount of Triton X-100/β-octylglucoside buffer (10 mM imidazole, 100 mM NaCl, 1 mM magnesium chloride, 5 mM sodium EDTA, 1% Triton X-100, 0.87 mg/mL β-octylglucoside, and

1X Halt™ protease inhibitor cocktail), as previously described (178). Approximately 100 μL of buffer was used per 0.1 mg of tissue. Homogenization was performed with the Bullet Blender® (Next Advance, Inc.) using 1 scoop of 0.5 mm stainless steel beads per sample incubated on setting 9 for 4 minutes. Both cell and tissue lysates were cleared of debris by centrifuging at max speed at 4°C for 10 minutes. Supernatants were collected and samples were normalized using Pierce bicinchoninic acid protein assay kit (Thermo Scientific). Equal protein fractions were analyzed by SDS-PAGE gel followed by immunoblotting (see below).

HEK293T cells were co-transfected with the following tagged constructs driven by the CMV promoter: HA-SMAD3 (0.5 μg), FLAG-TEAD4 (0.25 μg), FLAG-TEAD2 (0.25 μg), MYC-YAP (0.5 μg) and His-TGFβR1-T240D (0.1 μg). Media was changed the following day and cells were incubated an additional 24 hours. Cells were lysed as cell monolayers (see above). Total fractions were removed from the lysate supernatant and the remaining fraction was used for co-IP. Co-Ips were performed using 20 μL anti-FLAG M2 affinity gel (Sigma) for 1 hour at 4°C. Beads were precipitated by centrifugation at 3000 RPM for 30 seconds and washed 5 times with IP wash buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and eluted in SDS-PAGE load buffer (0.1 M Tris (pH 6.8), 10% glycerol, 2% SDS, 0.01% Bromophenol blue, 100 mM DTT) for analysis by SDS-PAGE gel followed by immunoblotting (see below)

Immunoblots

SDS load buffer was added to equal protein fractions and samples were boiled at 95°C for 5 minutes. Samples were loaded and separated on freshly made 10% SDS-PAGE gels (Resolving gel: 10% acrylamide, 375 mM Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.05% tetramethylethylenediamine; Stacking gel: 3.5% acrylamide, 250 mM Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.08% tetramethylethylenediamine) in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 220 V for 1 hour and 10 minutes. Proteins were immobilized on 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories) by transferring in 1X transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol) at 100 V for 1.5 hours. Membranes were stained with Ponceau S for 5 minutes at room temperature if necessary and washed with water until bands were visible and background turned white. Images were captured to visualize total protein. Membranes were then blocked in either 5% nonfat dry milk (LabScientific, Inc.) or 5% BSA (Fisher), depending on primary antibody protocol, diluted in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 30 minutes at room temperature. Membranes were incubated in primary antibody overnight at 4°C. The following day membranes were washed 3 times 10 minutes in TBS-T. Primary antibodies conjugated to horseradish peroxidase (HRP) were immediately visualized. Membranes in unconjugated primary antibodies were incubated in secondary antibody for 1 hour at room temperature, washed again 3 times 10 minutes in TBS-T and immediately visualized. All blots were visualized using either Super Signal

West Dura Extended Substrate (Thermo Scientific) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific), exposed for 1 to 240 seconds, and captured on Chemi Doc™ XRS+ imaging station (Bio-Rad Laboratories) using Image Lab software (Bio-Rad Laboratories). Expressed proteins were compared to BLUEstain 3 protein ladder (Gold Biotechnology) to determine size. All antibodies are outlined in **Table 3**.

Immunofluorescence and Proximity Ligation Assay (PLA)

Cells were plated on coverslips for immunofluorescence or on 96-well black-walled, transparent-bottom microplates (BD Falcon) for PLA. Cells were washed one time with PBS (137 mM NaCl, 10 mM sodium phosphate (dibasic), 1.8 mM monopotassium phosphate (monobasic), 2.7 mM potassium chloride pH 7.4) and fixed in 4% paraformaldehyde-PBS for 15 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100-PBS for 10 minutes, blocked in 2% BSA-PBS for 1 hour at room temperature. For the analysis of human samples, tissues were fixed and paraffin embedded. 3 μm sections were placed on OptiPlus Positive-Charged Barrier Slides (BioGenex), deparaffinized, treated with Retrievit-6 Target Retrieval Solution (BioGenex), and blocked with 10% goat serum. Samples were incubated with primary antibodies overnight at 4°C. For cell and tissue immunofluorescence, cells were washed the following day 5 times in PBS-T (PBS with 0.1% Tween-20) and incubated in fluorescently conjugated secondary antibodies for 1 hour at room temperature. After fluorescent antibodies were added, samples were protected from light for all remaining steps. For PLA, anti-

Table 3 - Antibodies

Specificity	Source	Catalog No	Application (Concentration)
rabbit anti-YAP [EP1674Y]	Abcam	ab52771	IF (1:500), WB (1:1000)
goat anti-mouse 488 Alexa Fluor	Molecular Probes	A-11001	IF (1:500)
goat anti-rabbit 555 Alexa Fluor	Molecular Probes	A-21428	IF (1:500)
rabbit anti-phospho127 YAP	Cell Signaling	4911	WB (1:1000)
rabbit anti-YAP/TAZ	Cell Signaling	8418	WB (1:1000)
anti-FLAG HRP conjugate	Sigma	A8592	WB (1:10000)
rabbit anti-GAPDH	Sigma-Aldrich	G9545	WB (1:10000)
anti-rabbit IgG HRP conjugate	GE Healthcare Life Sciences	NA934	WB (1:10000)
anti-mouse IgG HRP conjugate	GE Healthcare Life Sciences	NA931	WB (1:10000)
mouse anti-TAZ	BD Biosciences	560235	IF (1:500); PLA (1:500)
rabbit anti-YAP	Cell Signaling	4912S	IF (1:500)
mouse anti-Human PE CD24	BD Biosciences	555428	FACS (1:6)
mouse anti-Human FITC CD44	BD Biosciences	555478	FACS (1:6)
rabbit anti-phospho (Ser465/467) SMAD2	Cell Signaling	3101	WB (1:1000)
rabbit anti-phospho (Ser423/425) SMAD3	Epitomics	1880-1	WB (1:1000)
rabbit anti-SMAD2/3	Cell Signaling	8685	WB (1:1000): PLA (1:250)
mouse anti-rabbit IgG HRP conjugate	Cell Signaling	5127	WB (1:2000)
anti-HA HRP	Santa Cruz	sc-805 HRP	WB (1:5000)
anti-cMYC HRP	Santa Cruz	sc-40 HRP	WB (1:5000)
mouse anti-TAZ/YAP	Santa Cruz	sc-101199	PLA (1:250)
mouse anti-TEAD/TEF1	BD Biosciences	610922	PLA (1:500)
rabbit anti-TAZ	Cell Signaling	2149	PLA (1:250)
rabbit anti-HA (Y-11)	Santa Cruz	sc-805	ChIP (2 μg)
rabbit anti-WWTR1 (TAZ/YAP)	Sigma	HPA007415	ChIP (2 μg)
rabbit anti-TEAD4 (also recognizes TEAD1,3)	Aviva	ARP38276-P050	ChIP (2 μg)
rabbit anti-SMAD2/3	Cell Signaling	8685S	ChIP (2 μg)

mouse MINUS and anti-rabbit PLUS PLA probes (Duolink) were used and manufacturer's protocol was followed for subsequent ligation and amplification steps. All samples were washed 5 additional times in PBS-T and counterstained with either Hoechst (diluted 1:10,000 for a final concentration of 1 µg/mL in PBS) for 15 minutes at room temperature or DAPI. Tissues analyzed in the absence of primary antibodies were used as negative controls. Coverslips were inverted and mounted on microscopy slides with Vectashield mounting media (Vector Laboratories) or Vectashield mounting media was added directly to each microplate well. Immunofluorescence and PLA were visualized by confocal microscopy (Zeiss laser scanning microscope 700 or Zeiss laser scanning microscope 510 Axiovert 200M) and images were processed using Volocity software (PerkinElmer). Images were quantitated using ImageJ software. All antibodies are outlined in **Table 3**.

Cell proliferation and cell cycle analysis

SCC2 cells were transfected with siRNA and cultured for 24 hours. Cells were then plated ($5x10^4$ cells) (Day 0) and counted by hemocytometer each day for 6 consecutive days (Day 1-6), with media changed every 2 days. CAL27 doxycycline-inducible cells were pre-treated with doxycycline (100 ng/mL) for 24 hours. Cells were plated ($5x10^4$ cells) (Day 0) in the presence of doxycycline. Cells were counted by hemocytometer every 2 days for 2 weeks (Day 2-14) and media was changed every 2 days. MCF10A doxycycline-inducible cells were plated ($5x10^4$ cells) and treated with doxycycline (100 ng/mL) with or without TGFβ1 (500

pM) (Day 0). Cells were counted by hemocytometer each day for 6 consecutive days (Day 1-6), with media changed every 2 days. All cell proliferation experiments were performed in triplicate and statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's t test.

For cell cycle analysis, MCF10A doxycycline-inducible cells were treated with doxycycline (100 ng/mL) with or without TGFβ1 (500 pM) for 48 hours. $1x10^6$ cells were fixed overnight in 100% ethanol and stained with Propidium Iodide (50 µg/mL, Sigma) and RNase A (100 µg/mL, Sigma). Samples were acquired on the FACScan (BD Biosciences), collecting $1x10^4$ events, and analyzed using FlowJo software (Treestar). Experiment was performed in triplicate and statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's t test.

Caspase 3/7 activity

SCC2 cells were transfected with siRNA and cultured for 48 hours. CAL27 doxycycline-inducible cells were treated with doxycycline (100 ng/mL) for 24 hours. Cleaved Caspase-3 and -7 activity was measured using the Caspase-Glo 3/7 kit (Promega) according to manufacturer's protocol. Experiment was performed in triplicate and statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's t test.

Wound healing and transwell migration

For wound healing, SCC2 or LM2-4 cells were transfected with siRNA. After 24 hours, media was changed and cells were treated with or without TGFβ1 (500

39

pM) or SB-431542 (5 μM) for an additional 24 hours. CAL27 and MCF10A doxycycline-inducible cells were treated with or without doxycycline (100 ng/mL) or TGFβ1 (500 pM) for 24 hours. Monolayers were wounded and photographed at 0 hours and after an additional 12 to 24 hours. Images were captured and analyzed using ImageJ software. All wound healing experiments were performed in triplicate and statistical analysis was conducted using Prism software (GraphPad) with a two-tailed unpaired Student's *t* test.

For transwell migration, LM2-4 cells were transfected with siRNA. After 24 hours, cells were trypsinized and resuspended in low serum media (0.25% FBS). Cells were plated in triplicate at 10^5 cells/mL on 8 μm transwell filters (BD BioSciences) coated for 24 hours with 1 μg/mL fibronectin (Millipore). Media supplemented with 10% FBS was used in the bottom chamber. Cells were allowed to migrate for 24 hours in the presence of TGFβ1 (500 pM) and were subsequently stained with 0.5% crystal violet.

Tongue orthotopic mouse injections and in vivo imaging system (IVIS) imaging

All experiments were approved by the Boston University Medical Center Institutional Animal Care and Use Committee. Two month old female nude mice (NCr nu/nu; Taconic Farms, Hudson, NY) were injected in the tongue with $3x10^5$ SCC2-dsRed shCTL, shYAP, or shY/T cells (n=9 mice per group) in respective groups after anesthetizing with 4% isoflurane. Primary tumors were directly measured with calipers on day 10, 15, 18, and 22 to obtain tumor volume. IVIS

40

imaging was performed on day 22 using the Caliper IVIS Spectrum Imaging System (Xenogen) to visualize fluorescence (570 nm excitation, 620 nm emission, exposed for 1.0 second). Regions of interest were quantitated for each mouse using Living Image software and background radiant efficiency in vehicle mice was subtracted. Statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's *t* test.

Microarrays

SCC2 cells were transfected with control siRNA, or siRNAs targeting TAZ, YAP, or YAP/TAZ. siRNA sequences are outlined in **Table 2**. After 48 hours, total RNA from three independent experiments carried out on separate days was isolated and purified by Rneasy Mini Kit (Qiagen), and the samples were then profiled on Affymetrix Human Gene 2.0 Chips at the Boston University Microarray Core. The microarray data are available at Gene Expression Omnibus (GEO); accession GSE66949. The expression profiles were processed and normalized using the Robust Multi-array Average procedure (179) based on a custom Brainarray Cumulative Distribution Function (180). For each of the siRNA experiments, signatures of genes differentially expressed between treatment and corresponding siRNA control with a false discovery rate (FDR) q-value ≤0.05 and a fold change ≥2 were identified as either *activated* (up-regulated in control) or *repressed* (up-regulated in treatment). The overlap between the differentially expressed gene signatures was evaluated by Fisher test. Hierarchical gene and sample clustering was performed on the top 3000 genes with highest median

absolute deviation (MAD; a robust version of the variance) across 12 samples, using "ward" as the agglomeration rule, and 1 minus Pearson correlation and Euclidean as the distance measures for genes and samples, respectively.

LM2-4 cells were transfected with control siRNA, or siRNAs targeting YAP/TAZ or all four TEADs and were treated 24 hours later with TGFβ1 (500 pM) or SB-431542 (5 µM) for an additional 24 hours. siRNA sequences are outlined in **Table 2**. Total RNA was isolated and purified by Quick-RNA MiniPrep (Zymo Research). Twelve microarrays in total were performed, with each condition carried out three times on separate days. The Boston University Microarray Core generated the data using the Affymetrix Human Gene 1.0 St Array, which covers 27,300 probesets. The microarray data are available at GEO; accession GSE56445. The data were filtered using a moderated p-value of less than 0.01, and the average fold change in expression of each gene, for each condition, relative to the siCTL+TGFβ sample was calculated. Fold-expression changes relative to siCTL+TGFβ treated cells were calculated, and statistical significance was assessed using a moderated t test and p-values. Hierarchical gene clustering was performed on overlapping genes displaying a p-value<0.01 with the open source program Cluster 3.0 (181).

Hierarchical clustering of expression signatures and projection on tumor progression

Normalized Level 3 gene expression (RNASeqV2) and associated clinical data obtained from TCGA filtered as described above to retain only samples of

Caucasian origin from one of the six oral cancer anatomic subtypes (Alveolar Ridge, Base of tongue, Buccal Mucosa, Floor of mouth, Oral cavity, Oral tongue; filtered Oral Cancer dataset size: n=193) were used for the hierarchical clustering analysis. Identified clusters were annotated by pathway enrichment based on a hyper-geometric test against the set of curated pathways (c2.cp) in the Molecular Signatures Database compendium (182). To test whether gene signatures defined by microarray experiments were up- or down- regulated with respect to tumor status or tumor grade/stage, GSEA analysis was performed to test whether the activated/repressed gene signatures were enriched in tumor versus normal or higher grade versus lower grade tumors (183).

Hyperenrichment analysis

To evaluate whether specific pathways or transcription factors might play a role in the response to targeted inhibition, enrichment analysis of the differential signatures based on a hyper-geometric test was performed. To this end, each of the up- and down-regulated signatures (with FDR≤0.05 and fold-change≥2) was tested against the Molecular Signatures Database c2.cp (canonical pathways), c3.all (TF/miR targets), and c6.all (oncogenic pathways) compendiums.

Reverse transcription quantitative PCR (RT-qPCR)

SCC2 or LM2-4 cells were transfected with siRNA. After 24 hours, media was changed and cells were treated with or without TGFβ1 (500 pM) or SB-431542 (5 µM) for an additional 24 hours. CAL27 and MCF10A doxycycline-inducible cells were treated with or without doxycycline (100 ng/mL or 0.1 to 100 ng/mL,

respectively) or TGFβ1 (500 pM) for 24 hours. Total RNA was purified using Rneasy mini prep kit (Qiagen) and complementary DNA (cDNA) synthesis was performed using 1 μg RNA and iScript cDNA synthesis kit (Bio-Rad Laboratories) according to manufacturer's protocol. Quantitative PCR (qPCR) was performed using Fast SYBR green enzyme (Applied Biosystems) and measured on ViiA 7 real time PCR system (Applied Biosystems). Transcript levels were analyzed using ΔΔCT method and normalized to GAPDH. Primer sequences are outlined in **Table 4**. All experiments were performed in triplicate and statistical analysis was conducted with Prism software (GraphPad) using a two-tailed unpaired Student's *t* test.

Flow cytometry

MCF10A, MDA-MB-231, and LM2-4 cells were dissociated, washed, and counted. Cells were resuspended at 10^6 cells/100 μL and probed with FITC-CD44 and phycoerythrin (PE)-CD24 per manufacturer's protocol, as previously described (184). Antibodies are outlined in **Table 3**. Briefly, cells were protected from light and incubated at 4 °C for 30 minutes. Cells were washed and fixed in 1% paraformaldehyde in PBS. After fixation, cells were filtered through 40 μm strainers (BD Biosciences) and analyzed on a FACScan (BD Biosciences).

Mammospheres

LM2-4 cells were transfected with siRNA, dissociated 24 hours later, and resuspended in Mammary Epithelium Growth Medium (MEGM; Lonza) supplemented with B27 (Invitrogen), 20 ng/mL EGF, 20 ng/mL basic fibroblast

Table 4 - Primer sequences

Target	Species	Application	Forward	Reverse
TAZ	human	RT-qPCR	CCATCACTAATAATAGCTCAGATC	GTGATTACAGCCAGGTTAGAAAG
YAP	human	RT-qPCR	CTCGAACCCCAGATGACTTC	CCAGGAATGGCTTCAAGGTA
GAPDH	human	RT-qPCR	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
CCNE2	human	RT-qPCR	TCACTGATGGTGCTTGCAGT	GCCAGGAGATGATTGTTACAGGA
CDK2	human	RT-qPCR	CCTGAAATCCTCCTGGGCTG	CCCAGAGTCCGAAAGATCCG
CDC6	human	RT-qPCR	GTTCGCAAAGCACTGGATGT	GACCAACCCTCTTGGGAATCA
PCNA	human	RT-qPCR	CACTCCACTCTCTTCAACGGT	TCATCCTCGATCTTGGGAGC
AURKA	human	RT-qPCR	CACCCAAAAGAGCAAGCAGC	CCCAGAGGGCGACCAATTT
PLK4	human	RT-qPCR	GCTGATAGACCACCCTCACC	GCTGTACAAACCTGGAAGCA
BIRC5	human	RT-qPCR	AGCCAGATGACGACCCCAT	TGACAGAAAGGAAAGCGCAA
TEAD1	human	RT-qPCR	GATGATGCTGGGGCTTTTTA	AGGAGCAAACTTTGGTGGAA
TEAD4	human	RT-qPCR	TTGCCAGGCCAAGCCGGAAC	CGGGCCCTGCAGGAGACTCA
ETS1	human	RT-qPCR	TTACTCAGCGCCTCGTCCT	CCAAAAGGGGTAGCAAGGTC
JUN	human	RT-qPCR	CCAAGAACTCGGACCTCCTC	CCCGTTGCTGGACTGGATTA
PBX3	human	RT-qPCR	AATTCGCCCACCACACCAAA	GTGATTGCACATTGGCTCCG
RUNX2	human	RT-qPCR	CTCCCTGAACTCTGCACCAA	CGTCATCTGGCTCAGGTAGG
SOX9	human	RT-qPCR	ACCCGCACTTGCACAACG	CCCGTTCTTCACCGACTTCCTC
TEAD2	human	RT-qPCR	TTTTGGTCTGGAGGATCTGG	CGTACCCTGGGAGGTCAGTA
TEAD3	human	RT-qPCR	TCATCCACAAGCTGAAGCAC	AGCAATGACAAGCAGGGTCT
NEGR1	human	RT-qPCR	AGAGTGAGCAGCCCTAGCAG	GGGAAGTCCACACTCTGTCC
UCA1	human	RT-qPCR	CGGGTAACTCTTACGGTGGA	TGGTCCATTGAGGCTGTAGA
CTGF	human	RT-qPCR	GCAGAGCCGCCTGTGCATGG	GGTATGTCTTCATGCTGG
OCLN	human	RT-qPCR	TTTGTGGGACAAGGAACACA	ATGCCATGGGACTGTCAACT
CYFIP2	human	RT-qPCR	GGAAGGACATGAGTATGCGGT	GGGCTGCTCGTTGCATTTC
KLRC3	human	RT-qPCR	AAGCTCCATTTCAGGAACCGA	GAGCTTTTCTGGAGGTGGCA
SPTLC3	human	RT-qPCR	ACGCAGCTGTGGAAAGAAAA	TCCAGTTGTCTCTGATTCGCA
LBH	human	RT-qPCR	TGAGATCGGCCAAGATGACTG	TGCAGCAGCGGTCAAAATCT
PMEPA1	human	RT-qPCR	TGCTGAGCCACTACAAGCTG	TTGCCTGACACTGTGCTCTC
NEGR1 promoter 1	human	ChIP	CAGTTCAGCAGGGAGGTGTT	TTTGCTTGGTGGTAGCAGTG
NEGR1 promoter 2	human	ChIP	GAACTGGGATCTGGCAAAGA	GAACCGAGGCTGAGTCGTTA
UCA1 promoter	human	ChIP	CTGGGCTTGGGGTGAGAAA	CAGAGGACAGCCTGAGATGTG
CTGF promoter	human	ChIP	TTCTGTGAGCTGGAGTGTGC	GCCAATGAGCTGAATGGAGT

45

growth factor (FGF2; Peprotech), and 1% P/S. Single cells were seeded at 5x10^3

cells/mL in 6-well ultra-low attachment plates (Corning) and treated with or without

TGFβ1 (500 pM) or SB-431542 (5 μM). Primary spheres were photographed after

7 days and either lysed for RNA by Quick-RNA MiniPrep kit to examine knockdown

or dissociated in 0.05% trypsin for 10 minutes and resuspended as single cells in

MEGM media for passage. Secondary spheres were photographed after an

additional 14 days. Images were analyzed using ImageJ software and statistics

were calculated using Prism software (GraphPad) using a two-tailed unpaired

Student's *t* test.

Three-dimensional invasion

LM2-4 cells with stable shRNA knockdown (see above) were plated as

single cells on 100% growth factor-reduced Matrigel (BD Biosciences) using the

overlay method (185). Assay media contained 2% Matrigel added to supplemented

MEGM media, and cells were cultured with puromycin and G-418 selection (see

Table 1) with media changed every 3 days. TGFβ1 (500 pM) and SB-431542 (5

μM) were added after 9 days, and cells were cultured for an additional 3 days

before being photographed.

Chromatin immunoprecipitation (ChIP)

LM2-4 cells were fixed in triplicate with ethylene glycol bis (succinimidyl

succinate) (1 mM, Thermo Scientific) for 30 minutes, 1% formaldehyde for 10

minutes, and quenched in 0.125 M glycine in PBS. Cells were collected and lysed

in Cell Lysis buffer (10 mM potassium hydroxide/HEPES pH 7.8, 85 mM potassium

chloride, 1 mM EDTA pH 8.0, 1% NP-40) with 1X Halt™ protease inhibitor cocktail (Thermo Scientific). Nuclei were lysed in Nuclear Lysis buffer (50 mM Tris pH 7.4, 1% SDS, 10 mM EDTA pH 8.0) with 1X Halt™ protease inhibitor cocktail, and genomic DNA was fragmented to <400 bp using Bioruptor bath sonicator (Diagenode) 3 times for 15 minutes each, in 15 cycles of 30 seconds on 30 seconds off. Immunoprecipitations (Ips) were performed overnight using antibodies outlined in **Table 3** (Note: anti-TEAD4 also recognizes TEAD1 and 3 (127)) followed by incubation with protein-G Dynabeads (Invitrogen) for 1 hour at 4°C. Immunoprecipitated complexes were washed sequentially 2 times each in the following buffers: Wash buffer A (20 mM Tris pH 7.6, 140 mM NaCl, 1 mM EDTA pH 8.0, 0.1% sodium deoxycholate (NaDeoxycholate), 0.1% SDS, 1% Triton X-100), Wash buffer B (20 mM Tris pH 7.6, 500 mM NaCl, 1 mM EDTA pH 8.0, 0.5% NaDeoxycholate, 1% Triton X-100), Wash buffer C (20 mM Tris pH 7.6, 1 mM EDTA pH 8.0, 0.5% NaDeoxycholate, 1% Triton X-100, 250 mM lithium chloride), and TBS (20 mM Tris pH 7.6, 50 mM NaCl, 1 mM EDTA pH 8.0). Samples were eluted in Elution buffer (50 mM sodium bicarbonate, 50 mM Tris pH 8.0, 2 mM EDTA pH 8.0, 1% SDS). Cross-links were reversed overnight at 65°C in 0.2 M NaCl in Elution buffer and DNA was purified using QIAquick PCR purification columns (Qiagen) and eluted in 50 μL deionized distilled water. Samples were analyzed by qPCR in duplicate using 2 μL of eluted IP or 2 μL 0.5% input per qPCR reaction and data were analyzed using the percent-input method. Primers were

designed based on putative TEAD sequences found in nearby upstream promoter regions and are outlined in **Table 4**.

Cell morphology analysis

Low-density MCF10A doxycycline-inducible cells were pre-treated with doxycycline (100 ng/mL) for 24 hours, then treated with or without TGFβ1 (500 pM) for an additional 24 hours before being photographed.

CHAPTER III - A YAP/TAZ-REGULATED MOLECULAR SIGNATURE IS ASSOCIATED WITH ORAL SQUAMOUS CELL CARCINOMA

Abstract

Oral squamous cell carcinoma (OSCC) is a prevalent form of cancer that develops from the epithelium of the oral cavity. OSCC is on the rise worldwide, and death rates associated with the disease are particularly high. Despite progress in understanding of the mutational and expression landscape associated with OSCC, advances in deciphering these alterations for the development of therapeutic strategies have been limited. Further insight into the molecular cues that contribute to OSCC is therefore required. In this chapter, I show that the transcriptional regulators YAP and TAZ, which are key effectors of the Hippo pathway, drive pro-tumorigenic signals in OSCC. Regions of pre-malignant oral tissues exhibit aberrant nuclear YAP accumulation, suggesting that dysregulated YAP activity contributes to the onset of OSCC. Supporting this premise, I determined that nuclear YAP and TAZ activity drives OSCC cell proliferation, survival, and migration *in vitro*, and is required for OSCC tumor growth and metastasis *in vivo*. Global gene expression profiles associated with YAP and TAZ

knockdown revealed changes in the control of gene expression implicated in pro-tumorigenic signaling, including those required for cell cycle progression and survival. Notably, the transcriptional signature regulated by YAP and TAZ significantly correlates with gene expression changes occurring in human OSCCs identified by "The Cancer Genome Atlas" (TCGA), emphasizing a central role for YAP and TAZ in OSCC biology.

Introduction

Oral squamous cell carcinoma (OSCC) originates from the epithelium of the oral cavity and represents the majority of head and neck cancers. Very poor survival rates are associated with those afflicted by OSCC (only ~50% survival over five-years), and unfortunately little progress has been made with treatment strategies over the past few decades (35). Therefore, understanding dysregulated molecular cues associated with OSCC onset and progression is an important step in the development of effective therapeutics.

Recent studies have shown that aberrant activation of the transcriptional regulators YAP and TAZ (YAP/TAZ) contributes to the onset and progression of a range of cancers (90). YAP/TAZ transcriptional activity is dependent on their recruitment to the nucleus, which promotes binding to a range of transcription factors, most notably the TEAD family (67,187). YAP/TAZ-directed transcription promotes cell proliferation, pro-survival, and cell migration signals, all of which contribute to the pro-tumorigenic roles of YAP/TAZ (100-102). Multiple signaling events restrict YAP/TAZ from the nucleus, the best characterized of which are

signals mediated by the Hippo pathway (69). In particular, Hippo pathway activation promotes the phosphorylation of YAP and TAZ on conserved serine residues that lead to sequestration and destabilization of YAP/TAZ in the cytoplasm (61,64,102). Mechanical cues and signals that affect cytoskeletal dynamics, such as those transduced by G-protein-coupled receptors (GPCRs), also control YAP/TAZ localization, both by regulating Hippo pathway activity and via Hippo pathway-independent cues (188). While the signals regulating YAP/TAZ localization are not completely understood, recent work indicates that precise control of these signals are required to maintain tissue homeostasis (189).

Dysregulated YAP/TAZ activity has been implicated in head and neck cancers. For example, YAP expression has been shown to correlate with poor patient survival in head and neck cancers (91,92), and increased YAP levels and nuclear localization are associated with high-grade OSCC (92,93). TAZ overexpression has also been shown to be significantly associated with head and neck tumor size, histopathological grade, and reduced patient survival (92). Furthermore, elevated nuclear YAP/TAZ levels are known to promote resistance to several cancer treatments, including those commonly used for OSCC therapy, such as cisplatin and cetuximab (97,98,190). While evidence supports a role for YAP/TAZ in OSCCs, little is known about the downstream events regulated by YAP/TAZ, and at what step in cancerogenesis these factors may be involved.

Given the potential importance of TAZ and/or YAP signaling, I sought to gain a better understanding of their roles in OSCC. To this end, I integrated the

use of patient tissue samples, functional assays *in vitro* and *in vivo*, genome-wide expression profiling, and analyses of publically available expression data from studies performed by "The Cancer Genome Atlas" (TCGA) groups. My observations have revealed that YAP localization is dysregulated in benign and early pre-malignant oral tissues, and that elevated YAP protein levels are evident in a subset of OSCCs. Further, I show that nuclear YAP and TAZ activity drive pro-tumorigenic signals in OSCC cells *in vitro*, and that YAP and TAZ are necessary for OSCC development and metastasis *in vivo*. A global analysis of YAP/TAZ-regulated gene expression exposed a transcriptional program associated with expression changes found in OSCC onset and progression. My data therefore highlight novel YAP/TAZ-regulated events in OSCC, and offer an important gene expression signature that may serve as a resource for OSCC detection and personalized therapeutic development strategies.

Results

Nuclear YAP accumulation marks pre-malignant dysplastic regions of the oral epithelium.

The increased activity of the transcriptional regulator YAP has been implicated in the progression of oral squamous cell carcinoma (OSCC) (92,93,191,192). In particular, increased YAP levels have been associated with OSCC and other head and neck cancers, with subsets of these cancers exhibiting elevated nuclear YAP accumulation (93,191). Dysregulated nuclear YAP is known to drive overgrowth of several tissues (90), and thus YAP-driven cues may

contribute to early events in OSCC development. Since examination of benign and pre-malignant oral epithelial tissues has been limited, we set out to characterize a potential relationship between YAP and the pathology linked to OSCC predisposition. Specifically, we examined a range of tissues characterized as benign epithelial hyperplasia, mild and severe dysplasia, as well as morphologically normal adjacent epithelium from the oral cavity of human patients. Using immunofluorescence microscopy we observed very low levels of YAP in most cells found in the tissues of adjacent epithelium, except for YAP residing in the basal cell population, which exhibited relatively high levels in both the nuclear and cytoplasmic compartments (**Figure 3.1**). We observed the emergence of cells marked with prominent nuclear YAP beyond the basal cell population in spinous regions of dysplastic tissues, with highly enriched nuclear YAP in areas with severe dysplasia pathology (**Figure 3.1**). Interestingly, nuclear YAP was evident even in regions of benign epithelial hyperplasia (**Figure 3.1**). Our observations therefore suggest that predisposition to OSCC may be related to the dysregulation of YAP localization.

Prior studies have suggested that amplification of the chromosomal region encoding *YAP* contributes to its aberrant expression in human head and neck squamous cell carcinoma cells (191,193). We therefore examined whether increased *YAP* expression may be linked to OSCC onset, and further characterized the prevalence of this potential dysregulation. For this, we made use of data from a large number of patient samples publically available from TCGA to
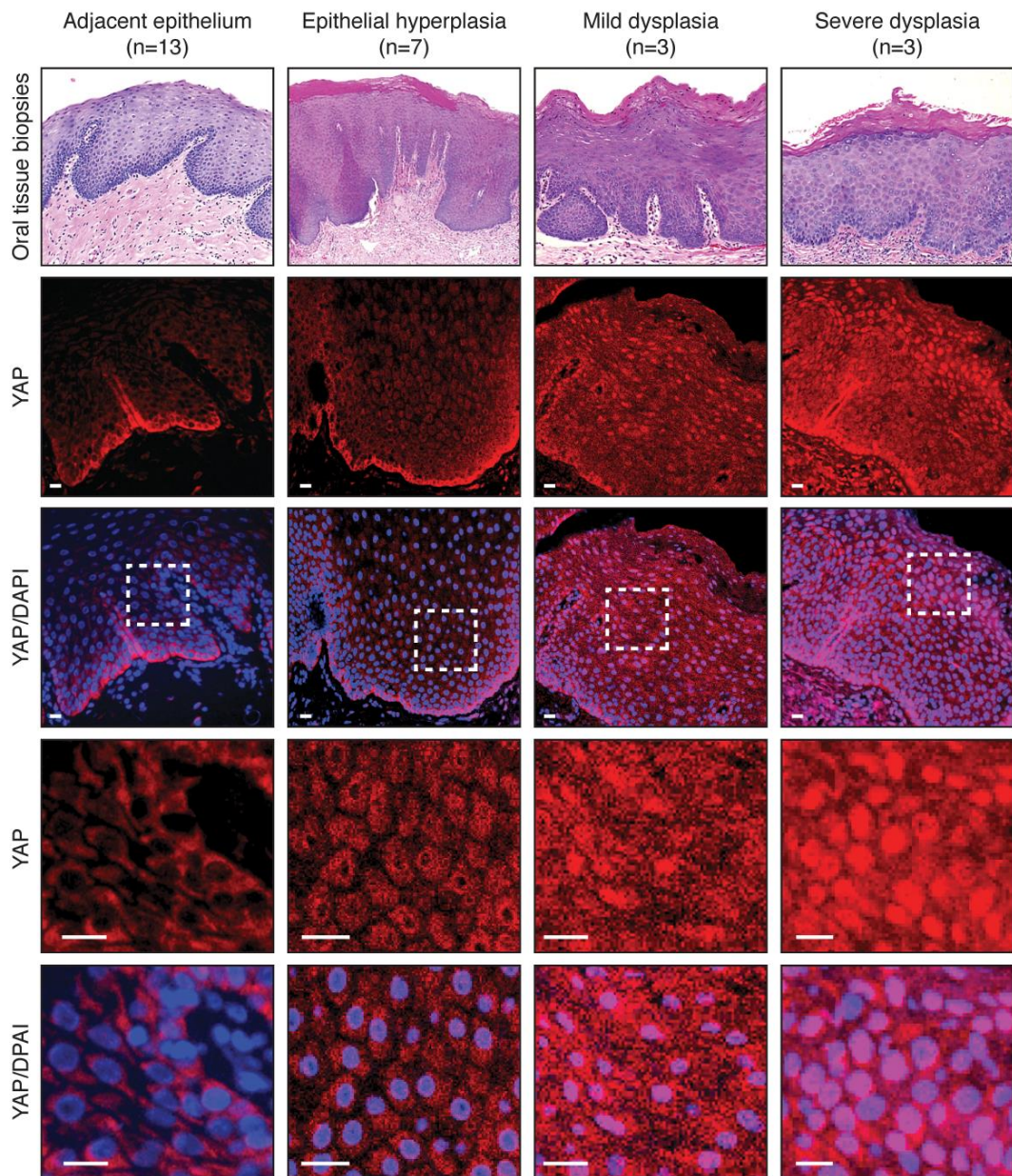
Figure 3.1 - Nuclear YAP accumulates in pre-malignant oral tissues. Tissues from patients exhibiting hyperplasia, mild dysplasia, and severe dysplasia, as well as cytologically normal adjacent epithelium, were examined by H&E staining (top) and by immunofluorescence to detect YAP localization (the number of tissues examined is indicated). DAPI was used to stain nuclei. A zoomed in image from each sample is shown in the bottom panels, highlighting the YAP localization changes observed. Scale bars, 20 μm. Tissue staining and analysis was performed in collaboration with Trevor Packer, Munirah Almershed, and Maria Kukuruzinska.

examine potential *YAP* gene expression, amplification, and/or mutations. We also included *TAZ* in our analysis of the TCGA data. We found no evidence of amplification, deletion, or mutation of the genomic region encompassing *YAP* or *TAZ* in cancers originating from the oral cavity (using the Genomic Identification of Significant Targets in Cancer tool; data not shown). Further, we found that *YAP* (**Figure 3.2A**) and *TAZ* (**Figure 3.2B**) expression was not significantly altered with tumor grade or stage.

Given that expression/mutation analysis may not reflect what is occurring with YAP protein levels, we obtained and examined protein from ten fresh OSCC tumors and their respective adjacent epithelia. Four of the tumors exhibited poorly differentiated pathology, and these tumors showed high levels of YAP compared to normal adjacent epithelia (**Figure 3.3**). Phosphorylation of YAP on Serine 127 (pS127-YAP) induces the cytoplasmic sequestration of YAP (62,102), and correlates with YAP degradation (64). This post-translational modification was relatively low in the poorly differentiated tumors (**Figure 3.3A and 3.3B**), providing a potential explanation for the observed elevated YAP protein levels. The other six tumors I examined, which were characterized as moderately differentiated, showed no differences in YAP or pS127-YAP (**Figure 3.3A and 3.3C**). These data therefore suggest that dysregulated hypo-phosphorylated YAP might contribute to the distinct pathology of a subset of OSCCs.
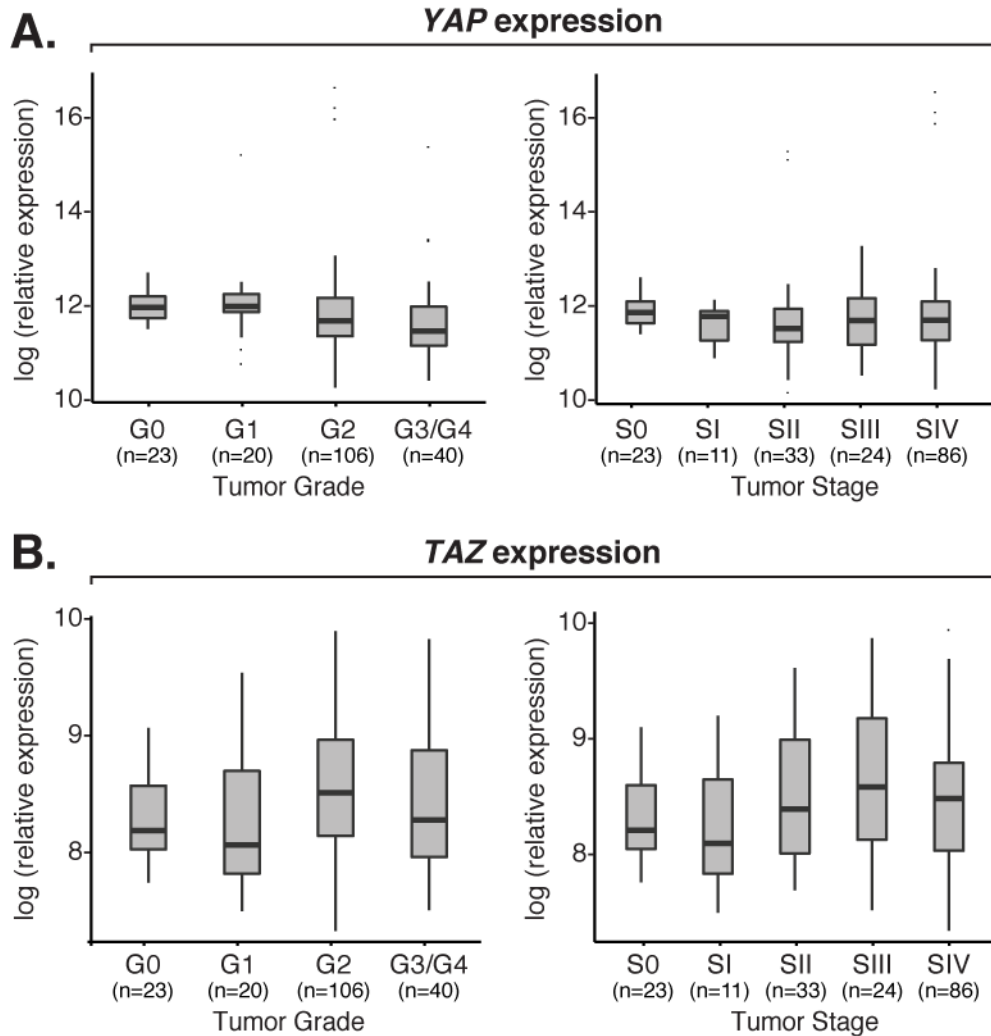
Figure 3.2 - *YAP* **and** *TAZ* **expression in OSCCs.** One-way ANOVA analysis showing (**A**) *YAP* or (**B**) *TAZ* expression with respect to TCGA OSCC tumor grade or stage data. A pairwise *t* test analysis comparing datasets revealed no significant expression changes. Analysis performed in collaboration with Liye Zhang, Vinay Kartha, and Stefano Monti.
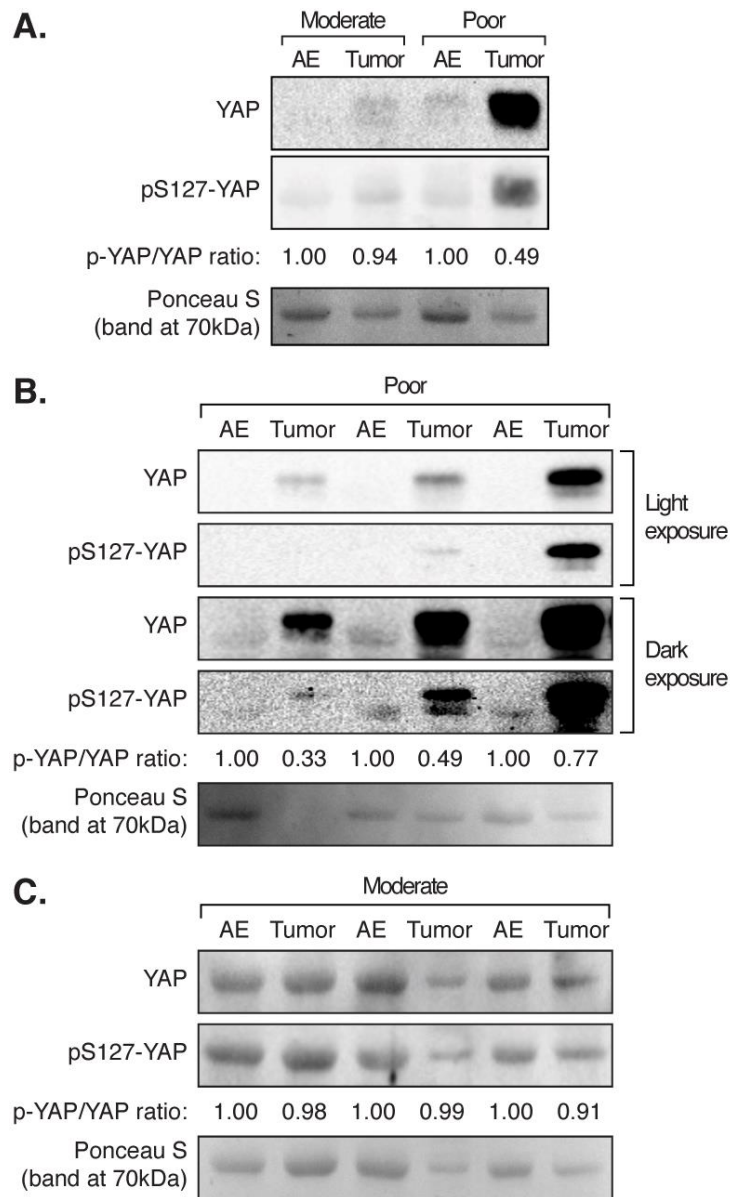
Figure 3.3 - YAP expression and phosphorylation in OSCCs. Protein was extracted from poorly and moderately differentiated tumors, and associated adjacent epithelium (AE), and examined for YAP and phospho-S127-YAP levels by immunoblotting. Ponceau-S staining of the proteins on the immunoblotted membrane is shown as a loading control. Quantitation of the relative phospho-S127-YAP to total YAP is also shown. **A.** A representative image of the observed changes in YAP expression and phosphorylation. **B.** Three poorly differentiated tumors and their associated AE after both a light and dark exposure. **C.** Three moderately differentiated tumors and their associated AE.

YAP/TAZ promote tumorigenic phenotypes in OSCC cells.

To gain further insight into the contributions of YAP to OSCC development I utilized available OSCC cell lines to carry out functional assays following repressed or induced YAP activity. I started by examining YAP and pS127-YAP levels in lysates obtained from a panel of OSCC cell lines with different tumorigenic capacities: CAL27, SCC2, SCC9, SCC15, and SCC25 cells. I found that YAP levels were highest in the SCC2, which are cells that have aggressive metastatic properties in mouse xenograft models (194). YAP in the SCC2 cells was also hypo-phosphorylated on S127 as compared to the other cells (**Figure 3.4A**). The relative differences in pS127-YAP were reflected in the compaction-induced sequestration of YAP into the cytoplasm of these cells, an event associated with contact-mediated proliferation arrest (68) (**Figure 3.4A**). In particular, nuclear YAP levels in the SCC2 cells were not altered in response to cell compaction (**Figure 3.4B**). Thus, my observations suggest that increased hypo-phosphorylated nuclear YAP may relate to the aggressive behavior of SCC2 cells.

Prior studies have indicated important roles for YAP and TAZ in the control of cell proliferation and survival (90). To test YAP activity in OSCC cells I decided to use the SCC2 and CAL27 cells as models for high and low nuclear YAP activity, respectively. First, SCC2 cells were transfected with siRNA to deplete YAP levels, and since TAZ might have complimentary roles, I also depleted TAZ, or both YAP/TAZ levels (**Figure 3.5A**). Analyses of cell numbers over time indicated that the knockdown of YAP, TAZ, or both YAP/TAZ decreased the ability of SCC2 cells
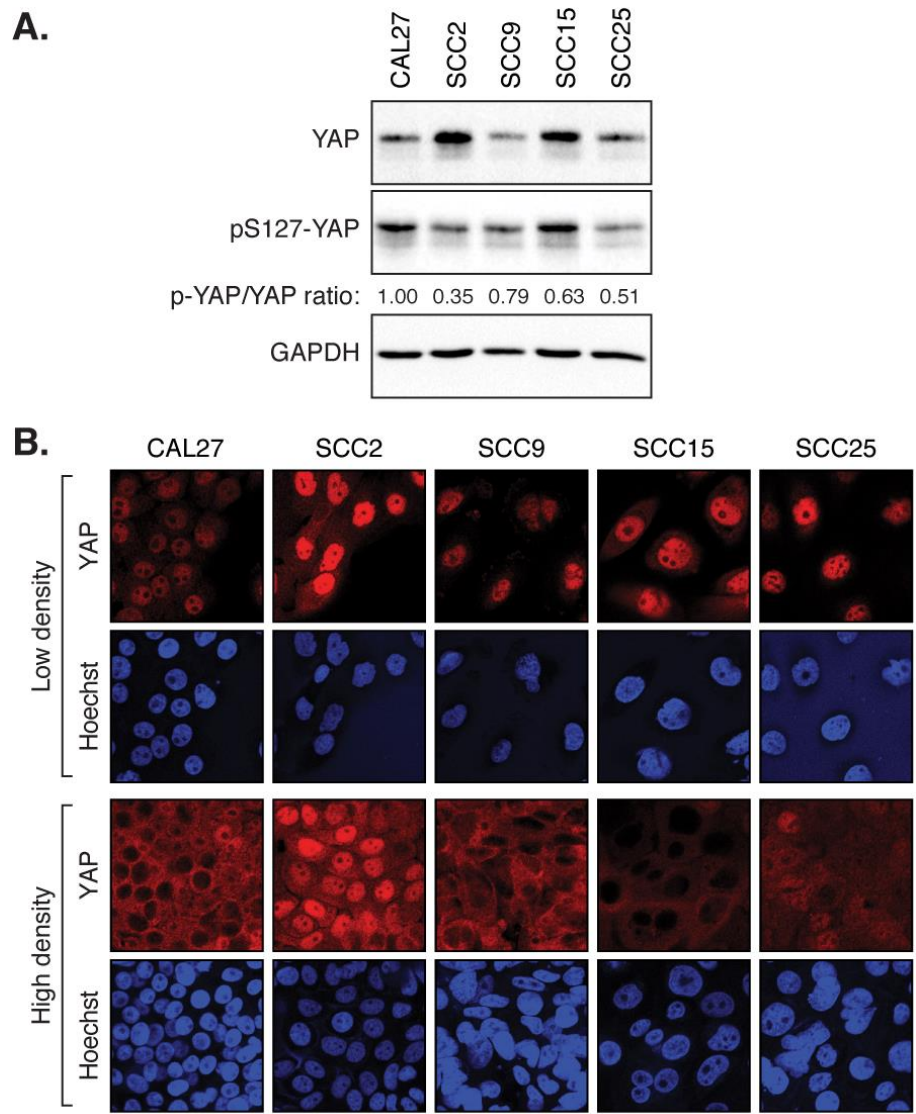
Figure 3.4 - Elevated levels of nuclear YAP are found in aggressive OSCC cells. A. A panel of oral cancer cell lines was examined by immunoblotting for endogenous YAP, phospho-S127-YAP, and GAPDH (loading control). Quantitation of relative phospho-S127-YAP to total YAP is shown. **B.** Oral cancer cell lines were examined by immunofluorescence for endogenous YAP localization at both low and high density.
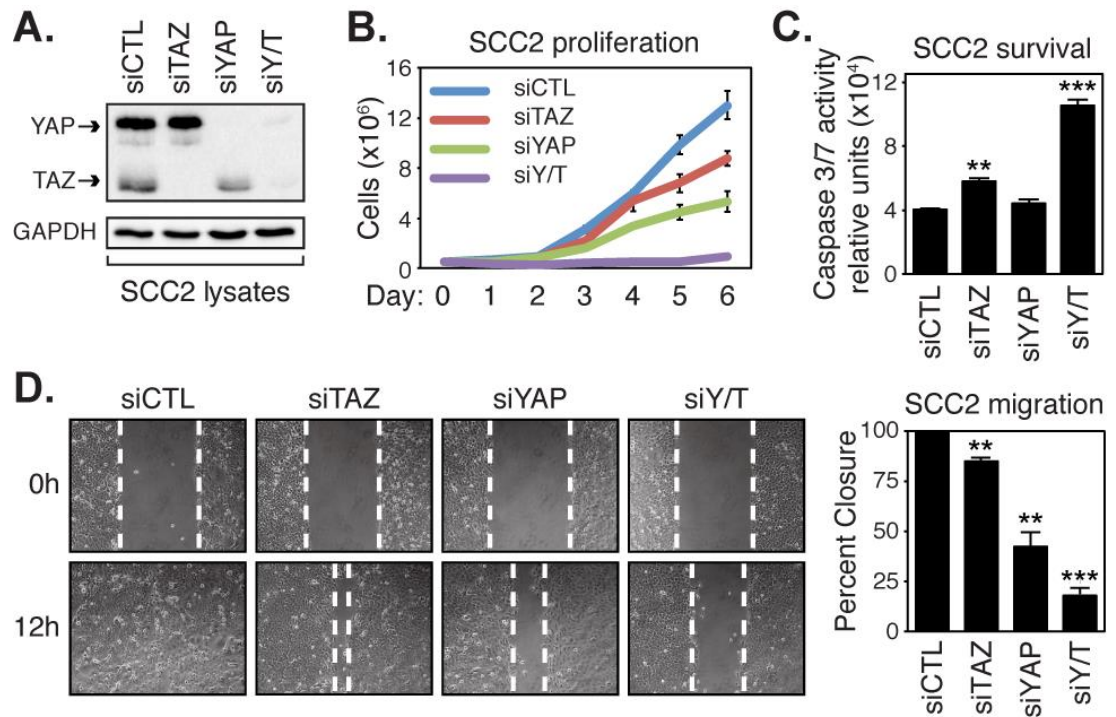
Figure 3.5 - YAP/TAZ are necessary for SCC2 proliferation, survival, and migration. A. SCC2 cells were transfected with control siRNA (siCTL) or siRNA targeting TAZ (siTAZ), YAP (siYAP), or YAP and TAZ (siY/T), and lysates from these cells were examined by immunoblotting with the indicated antibodies including GAPDH (loading control). **B.** SCC2 knockdown cells were counted over 6 days to measure their proliferative capacity. Cells from three experiments were counted and the average (± SE) for each day is shown. **C.** SCC2 knockdown cells were examined for caspase-3 and 7 activity using a Caspase-Glo 3/7 assay. The average (+SE) of three experiments is shown. **D.** Confluent monolayers of SCC2 knockdown cells were wounded and examined for their ability to migrate after 12 hours. Representative images are shown and the average wound healing (+SE) of three experiments is indicated. All statistics were calculated compared to the control sample using an unpaired Student *t* test and are represented as **, $P < 0.01$; ***, $P < 0.001$.

to proliferate, with YAP knockdown affecting the cells more than TAZ knockdown, but YAP/TAZ knockdown having the most pronounced effect (**Figure 3.5B**). Examination of SCC2 cells that were depleted of YAP/TAZ also revealed that these cells had increased Caspase-3 and -7 (3/7) activity (**Figure 3.5C**), suggesting that these cells were undergoing increased apoptosis. Furthermore, knockdown of either YAP or TAZ decreased the ability of SCC2 cells to migrate in wound closure scratch assays, with knockdown of both YAP and TAZ almost completely halting cell migration (**Figure 3.5D**).

I next examined whether induced nuclear YAP and TAZ activity could drive pro-tumorigenic behavior in non-metastatic CAL27 cells. For these studies, CAL27 cells were engineered to express, in a doxycycline-inducible manner, the nuclear-localized YAP-5SA mutant (S61A, S109A, S127A, S164A, S397A), or YAP-5SA/S94A, which has an additional mutation that disrupts binding to the TEAD transcription factors (67,68) (**Figure 3.6A**). In contrast to YAP/TAZ depletion in SCC2 cells, ectopic expression of YAP-5SA increased the ability of CAL27 cells to proliferate (**Figure 3.6B**). YAP-induced proliferation relied on TEAD binding, as expression of the YAP-5SA/S94A mutant failed to increase proliferation (**Figure 3.6B**). Expression of YAP-5SA, but not YAP-5SA/S94A, also reduced Caspase 3/7 activity in the CAL27 cells (**Figure 3.6C**), suggesting that nuclear YAP-TEAD-driven transcription induces pro-survival signals. Moreover, YAP-5SA, but not YAP-5SA/S94A, increased the ability of CAL27 cells to migrate (**Figure 3.6D**). Taken together, my observations indicate that nuclear YAP and TAZ promote the
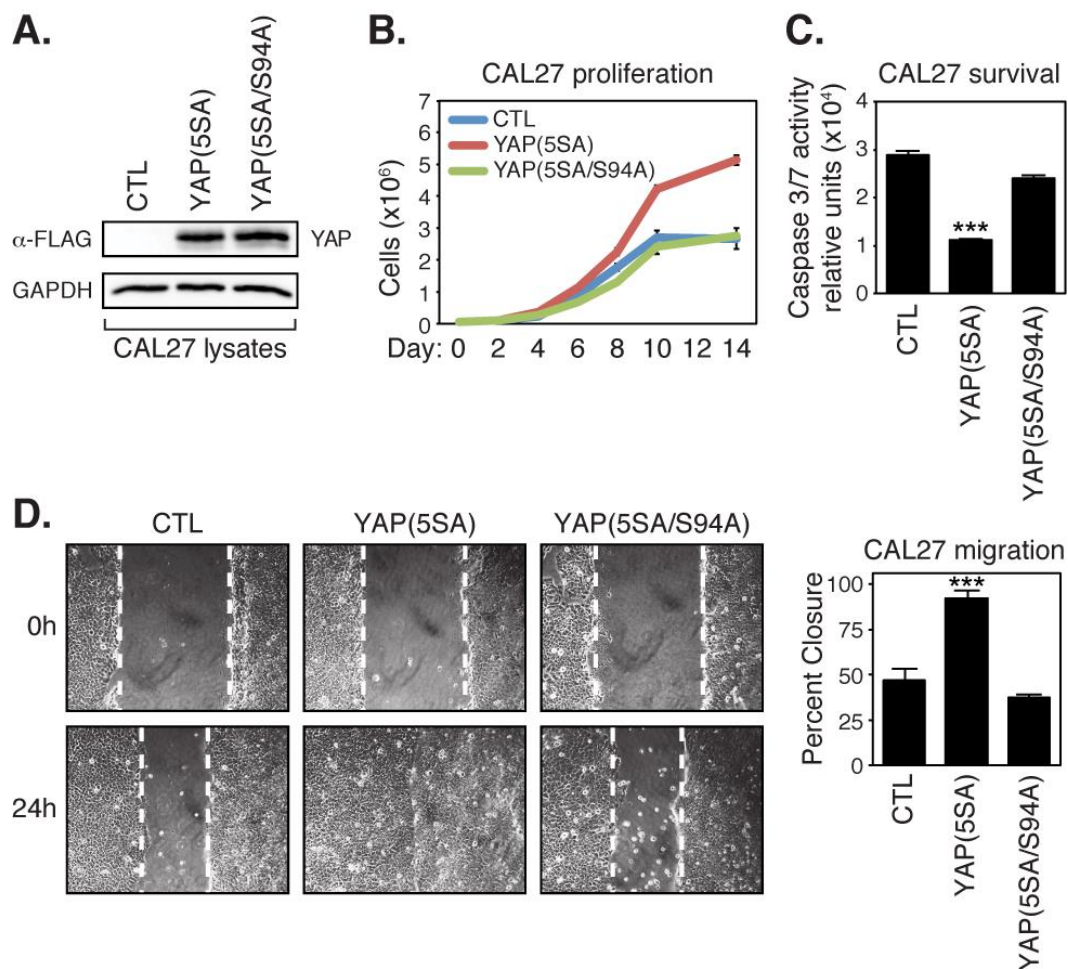
Figure 3.6 - Nuclear YAP is sufficient to promote CAL27 proliferation, survival, and migration, dependent on YAP-TEAD binding. A. Doxycycline-inducible CAL27 control cells, or cells expressing 3xFLAG-YAP(5SA) or 3xFLAG-YAP(5SA/S94A) were lysed and examined by immunoblotting with the indicated antibodies including GAPDH (loading control). **B.** CAL27-expressing cells were counted over 14 days to measure their proliferative capacity. Cells from three experiments were counted and the average (±SE) for each day is shown. **C.** CAL27-expressing cells were examined for caspase-3 and 7 activity using a Caspase-Glo 3/7 assay. The average (+SE) of three experiments is shown. **D.** Confluent monolayers of CAL27-expressing cells were wounded and examined for their ability to migrate after 24 hours. Representative images are shown and the average wound healing (+SE) of three experiments is indicated. All statistics were calculated compared to the control sample using an unpaired Student t test and are represented as **, $P < 0.01$; ***, $P < 0.001$.

proliferation, survival, and migration of OSCC cell lines *in vitro*, and suggest that the increased nuclear YAP observed in OSCC is an important contributing factor to disease progression.

To test whether YAP and TAZ drive pro-tumorigenic properties in OSCC cells *in vivo*, I knocked down YAP or YAP/TAZ in SCC2 cells and used them in tongue orthotopic xenograft tumor experiments in immune-compromised mice. I used SCC2 cells since they exhibit high nuclear YAP levels and have metastatic potential (194). To easily track tumor development and metastasis, we generated SCC2 cells expressing dsRED (SCC2-dsRED) and then engineered them to express control shRNA (shCTL), shRNA targeting YAP (shYAP), or shRNA targeting both YAP and TAZ (shYAP/TAZ). Stable knockdown was confirmed in these cells by immunoblotting (**Figure 3.7A**), and the cells were then injected into the tongue of mice (**Figure 3.7B**). Primary tumor growth was monitored with caliper measurements and also by IVIS imaging to locate dsRED-expressing cells. We found that YAP or YAP/TAZ knockdown decreased primary tumor volume, with YAP/TAZ suppressing tumor growth more dramatically (**Figure 3.7B and 3.7C**). Imaging after 22 days revealed abundant SCC2 cell metastasis, which was reduced with YAP knockdown, and almost completely ablated with YAP/TAZ knockdown (**Figure 3.7C**). Thus, our mouse experiments indicated that YAP and TAZ have important roles in OSCC tumor growth and metastasis, suggesting that dysregulated nuclear YAP in oral tissues is relevant for OSCC onset and progression.
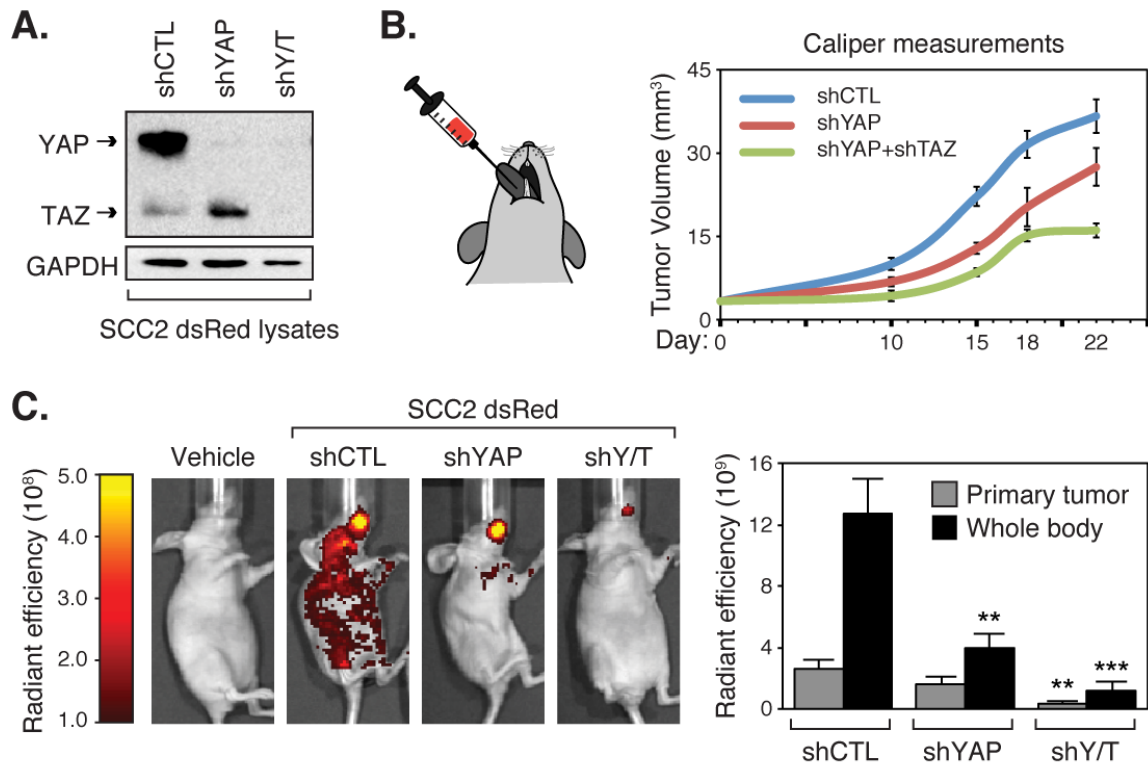
Figure 3.7 - YAP/TAZ are necessary for OSCC tumor growth and metastasis in vivo. A. SCC2-dsRED cells stably expressing control shRNA (shCTL) or shRNA targeting YAP (shYAP), or YAP and TAZ (shY/T) were lysed and examined by immunoblotting for the indicated proteins including GAPDH (loading control). **B.** SCC2-dsRED cells were injected into the tongue of nude mice and primary tumor volume was determined by caliper measurements at day 10, 15, 18, and 22, and are shown as the average (±SE; n=3 for vehicle control, n=9 for shRNA-expressing cells). **C.** SCC2-dsRED cells contributing to tumor formation and metastasis were visualized by IVIS fluorescent imaging at day 22 and representative images are shown. Total radiant efficiency of cells in the primary tumor and cells that metastasized throughout the animal body were quantitated and are shown as the average (+SE). Statistics comparing primary tumor size or metastasis of the knockdown cells to the control cells were performed using an unpaired Student t test and are represented as **, $P < 0.01$; ***, $P < 0.001$. Injections were performed in collaboration with Manish Bais.

***YAP/TAZ promote a transcriptional program that is associated with human
OSCC progression.***

The *in vitro* and *in vivo* studies suggested that the transcriptional activity of
YAP and TAZ influence pro-tumorigenic events in OSCC cells. We therefore set
out to understand YAP/TAZ-regulated transcription by using microarrays to
compare the global expression profiles of SCC2 cells transfected with either
control siRNA, or siRNA targeting TAZ, YAP, or both YAP/TAZ. Hierarchical
clustering of the top 3000 genes with the highest MAD showed that the expression
profiles from replicate samples clearly clustered next to each other. Notably, the
expression profiles from the control cells and the TAZ-depleted cells clustered
similarly, whereas those from cells depleted of YAP or YAP/TAZ had similar
expression profiles **(Figure 3.8)**. Thus, YAP appears to have more prominent
transcriptional role in SCC2 cells compared to TAZ, which was supported by my
functional experiments shown in **Figure 3.5**. Next, we carried out differential
analyses of the control siRNA-treated samples versus the knockdown samples.
For each treatment, we identified the signatures of up- (*repressed*) and down-
regulated (*activated*) genes with adjusted p-value ≤0.05 and fold-change ≥2. The
number of genes included in each signature is summarized in **Figure 3.8**.

To gain insight into whether the YAP- and TAZ-regulated gene expression
signatures relate to OSCC onset and/or progression, we used the following two
methods to make comparisons with gene expression data generated by TCGA: 1)
we tested for gene enrichment in tumor grade or stage data using Gene Set
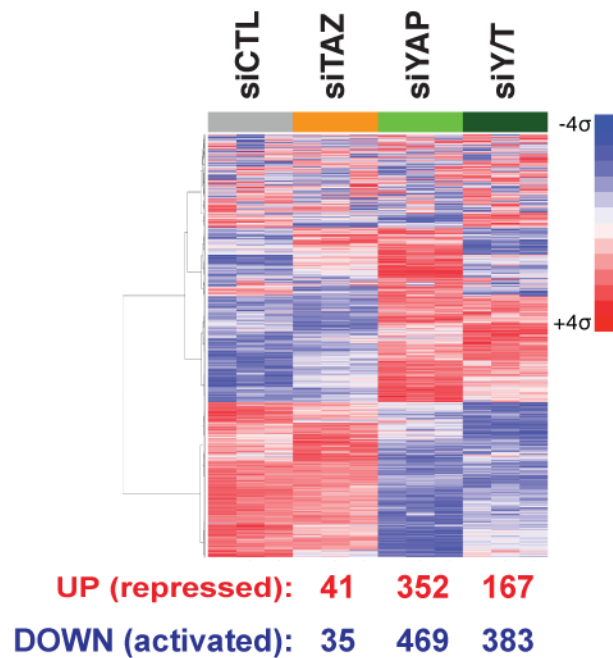
Figure 3.8 - YAP/TAZ-regulated transcriptional events in OSCC. Microarrays were performed from samples isolated from SCC2 cells transiently transfected with control siRNA (siCTL) or siRNA targeting TAZ (siTAZ), YAP (siYAP), or YAP and TAZ (siY/T). Hierarchical clustering of the top 3000 genes with the highest MAD is shown. The number of up- and down-regulated genes (genes with adjusted P ≤ 0.05 and fold change ≥ 2 compared with the control) that were identified from the microarray study is shown below. Analysis performed in collaboration with Liye Zhang, Vinay Kartha, and Stefano Monti.

Enrichment Analysis (GSEA); and 2) we used Adaptive Signature Selection and InteGratioN (ASSIGN) to capture the coordinated co-expression of YAP/TAZ targets reflecting the corresponding regulators' activity. Our GSEA analysis revealed a significant enrichment (nominal p-values ≤0.05) for genes activated by YAP or YAP/TAZ (i.e. decreasing in the microarrays with knockdown) when compared to genes with elevated expression in tumor versus adjacent epithelium (**Figure 3.9A**). Enrichment of the YAP- and YAP/TAZ-regulated gene expression signature was preserved with elevated tumor grade (**Figure 3.9A**) and tumor stage (**Figure 3.9B**), even when comparing aggressive stage IV OSCCs to earlier stages (**Figure 3.9B**), suggesting that in addition to events necessary for tumor onset, YAP/TAZ activities also control processes required for late stage OSCC progression. Similar data were obtained from ASSIGN analyses, which showed a significant upward trend of the YAP/TAZ activity score as a function of an increasing tumor grade and stage (**Figure 3.9C and 3.9D**). Together these data indicate that a subset of genes dysregulated in human OSCCs is associated with aberrant YAP/TAZ-activity.

The YAP/TAZ-regulated gene expression signature included canonical YAP/TAZ targets, such as *CTGF* or *CYR61*. However, no significant change in the expression of these genes was observed with respect to tumor onset, tumor grade, or tumor stage (**Figure 3.10A**), suggesting that the roles of YAP/TAZ in OSCCs extend beyond what has been characterized in other contexts. To better understand the cues regulated by YAP/TAZ in OSCC we clustered the identified

A.

	Tumor vs AE		≥ G2 vs G1		≥ G3 vs ≤ G2	
	AE	Tumor	G1	≥ G2	≤ G2	≥ G3
YAP activated	-	<0.001	-	0.042	-	-
YAP repressed	-	-	0.048	-	0.039	-
YAP/TAZ activated	-	<0.001	-	0.056	-	-
YAP/TAZ repressed	-	-	0.134	-	0.038	-

B.

	≥ SI vs S0		> SII vs SI-II		> SIII vs SI-III	
	S0	≥ SI	SI-II	> SII	SI-III	> SIII
YAP activated	-	0.047	-	0.029	-	0.036
YAP repressed	-	-	-	-	-	-
YAP/TAZ activated	-	0.040	-	0.017	-	0.033
YAP/TAZ repressed	-	-	-	-	-	-
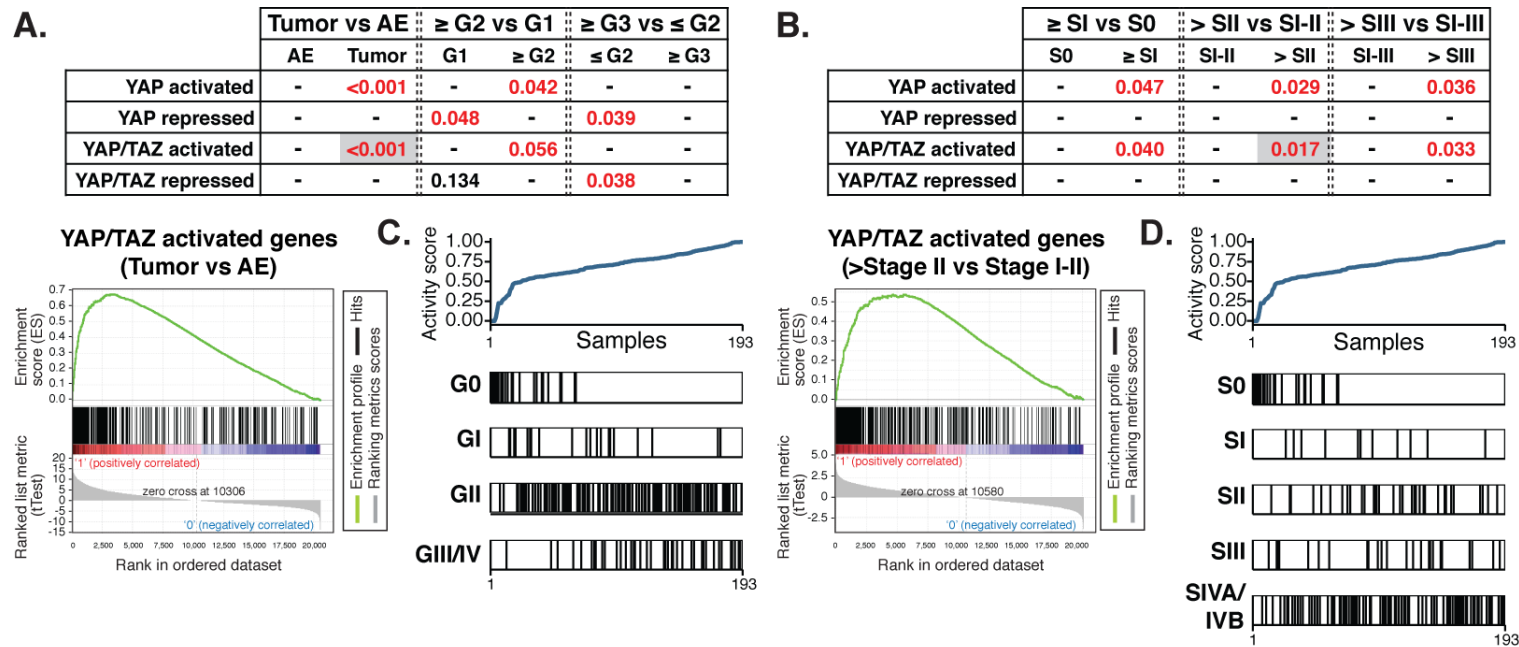
Figure 3.9 - YAP/TAZ-regulated transcriptional events correlate with OSCC tumor grade and stage. We tested for the enrichment of the YAP- and YAP/TAZ-regulated expression changes in TCGA data (n=193 samples) by GSEA. Enrichment nominal *p*-values are summarized for the analysis of (**A**) tumor grade versus adjacent epithelium (AE), with a GSEA curve for the YAP/TAZ activated genes enriched (*p*-value highlighted in grey) shown below and (**B**) for the analysis of high tumor stage versus low tumor stage, with a GSEA curve for the YAP/TAZ activated genes in > Stage II vs Stage I-II (*p*-value highlighted in grey) shown below. We also used the ASSIGN algorithm to infer the level of YAP/TAZ activity in TCGA OSCC from the coordinated expression of the set of YAP/TAZ-regulated genes identified in the microarray. ANOVA was performed to test for the association between the YAP/TAZ activity scores and tumor grade or stage, which revealed significant association of the YAP/TAZ-activity with both (p-values < 2e-16). Samples were then ranked by increasing activity score and their corresponding (**C**) tumor grade and (**D**) tumor stage information is shown. Samples with missing tumor grade or stage information were excluded from the analysis (195). Analysis performed in collaboration with Liye Zhang, Vinay Kartha, and Stefano Monti.
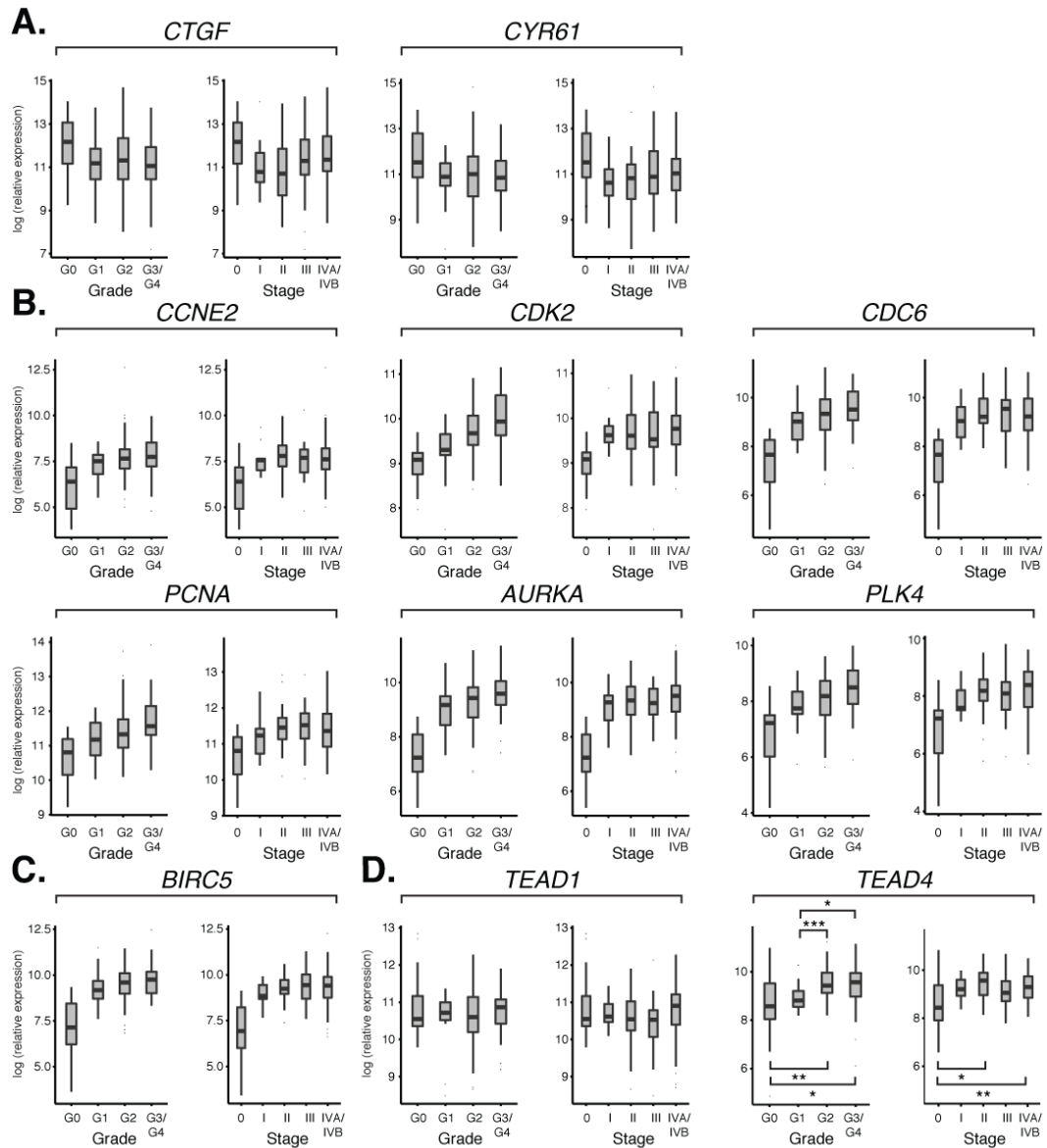
Figure 3.10 - Expression of YAP/TAZ-regulated genes in OSCC tumors. Box plots showing the expression of (**A**) canonical YAP/TAZ targets, (**B**) YAP/TAZ-regulated cell cycle genes, (**C**) YAP/TAZ-regulated survival gene *BIRC5*, and (**D**) YAP/TAZ-regulated transcription factors *TEAD1* and *TEAD4* with respect to OSCC tumor grade (G0: n=23; G1: n=20; G2: n=106; G3/4: n=40) and stage (S0: n=23; I: n=11; II: n=33; III: n=24; IVA/IVB: n=86). *TEAD4* expression is significantly induced with OSCC tumor grade (one-way ANOVA; P = 3.51 e-05) and stage (one-way ANOVA; P = 0.0012). Pairwise *t* tests of expression between groups is also shown, and represented as *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Analysis performed in collaboration with Liye Zhang, Vinay Kartha, and Stefano Monti.

gene expression signature with TCGA-derived OSCC tumor grade and stage expression changes. Clustering uncovered clear segregation of adjacent epithelium (white marked columns in **Figure 3.11A**) and tumor samples (green marked columns in **Figure 3.11A**), and further revealed tumor-associated sub-clusters of YAP/TAZ-regulated genes, the most prominent of which correlated with genes induced in expression by YAP/TAZ (yellow cluster in **Figure 3.11A**). A more focused cluster analysis of only these activated genes revealed two major sub-groups, which we termed Cluster A and Cluster B (**Figure 3.11B**). Annotation of these gene clusters by pathway hyper-enrichment analysis yielded a strong enrichment of cell cycle-related pathways in Cluster A, all of which showed increased expression with tumor grade or stage when examined in the TCGA datasets (**Figure 3.10B and 3.10C**). Cluster B showed enrichment for genes responding to signals mediated by AP1, Hippo, TGFβ, and WNT pathways. Notably, several transcription factors relevant to tumor progression were also altered in Cluster B, and included *TEAD1*, *TEAD4*, *ETS1*, *JUN*, *PBX3*, *RUNX2* and *SOX9*.

To validate whether the genes identified in Clusters A and B were indeed regulated by YAP and TAZ, I examined the expression of subset of these genes by RT-qPCR in SCC2 cells transfected with control siRNA, or siRNA targeting TAZ, YAP, or both YAP/TAZ, as well as in CAL27 cells that expressed (24 h of ectopic expression) the nuclear YAP-5SA mutant or the transcriptionally defective YAP-5SA-S94A mutant. I initially focused on genes critical for cell cycle progression

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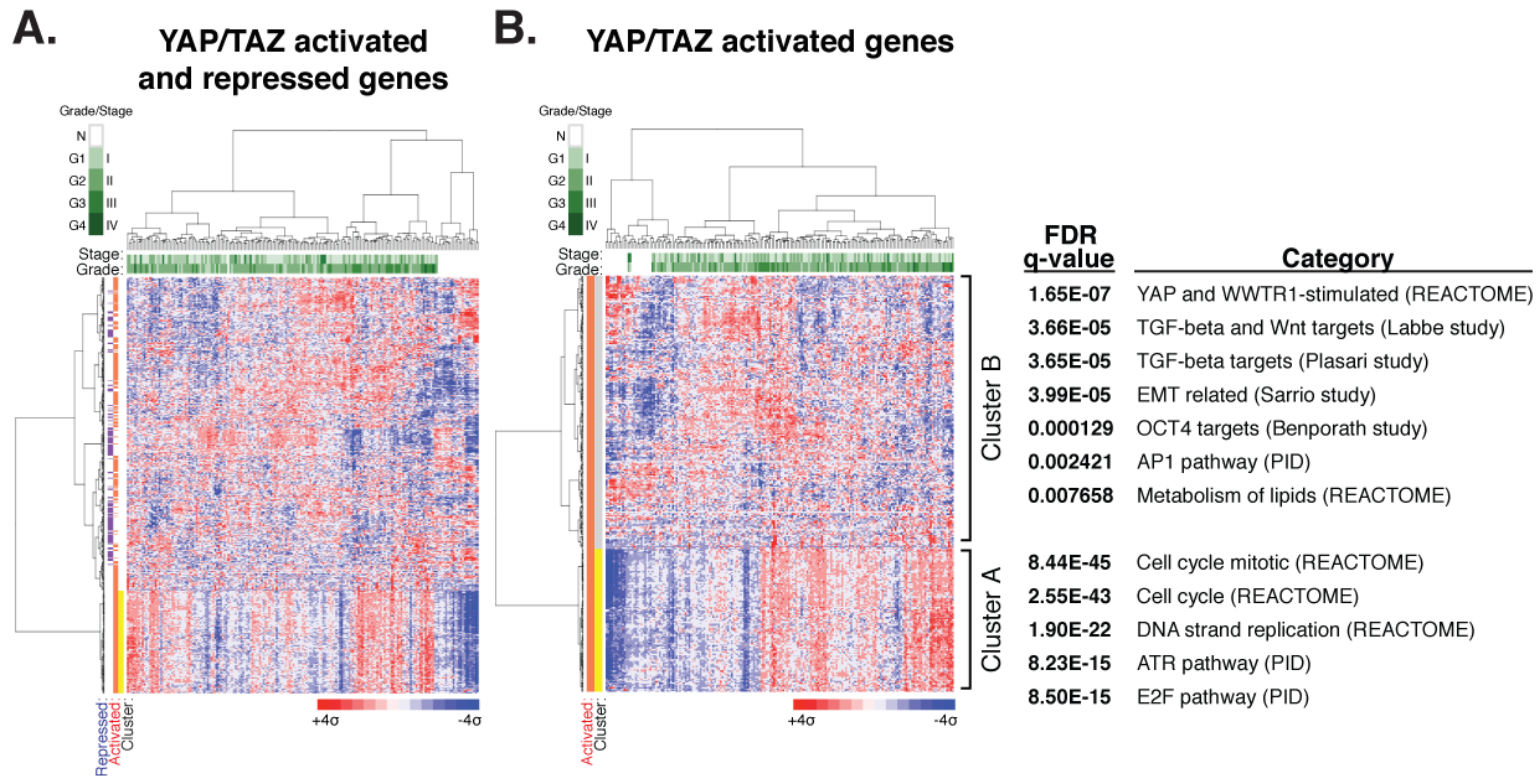
Figure 3.11 - Pathway enrichment in YAP/TAZ-regulated gene signatures. OSCC tumor grade and tumor stage data obtained from TCGA was projected onto (**A**) the entire YAP/TAZ-regulated expression signature identified from the microarray studies, or (**B**) only the YAP/TAZ-activated expression signature, and a heatmap of the clustered data is shown. Two notable YAP/TAZ-activated gene clusters were identified, and selected data from a pathway enrichment analysis of these genes are shown to the right (196-199). Analysis performed in collaboration with Liye Zhang, Vinay Kartha, and Stefano Monti.

(*CCNE2, CDK2, CDC6, PCNA, AURKA, PLK4*), and pro-survival (*BIRC5*), both of which were associated with Cluster A in **Figure 3.11B**. I found that knockdown of YAP/TAZ in SCC2 cells strikingly reduced the expression of all cell cycle and survival genes found in Cluster A (**Figure 3.12**). YAP knockdown also repressed the expression of these genes, some of which mirrored YAP/TAZ knockdown, while others were less affected, suggesting that TAZ redundantly regulates the expression of some of these genes. TAZ knockdown alone, however, had minor effects on the expression of almost all of these genes, suggesting that YAP/TAZ redundancy may only be revealed upon YAP deficiency in these cells. These same cell cycle and pro-survival genes were significantly induced by YAP-5SA expression in CAL27 cells (**Figure 3.13**), but were not affected by the expression of the transcriptionally defective YAP-5SA/S94A mutant, suggesting that nuclear YAP-TEAD activity directly regulates the expression of these genes. The transcription factors identified in Cluster B were all down-regulated following YAP/TAZ and YAP knockdown in SCC2 cells (**Figure 3.12**). However, the expression of these genes was largely unaffected following YAP-5SA expression in CAL27 cells, suggesting that some of these genes may not be direct YAP targets, or that they require additional factors or signals present in more progressed OSCCs for YAP-directed regulation. Interestingly, the exception was the regulation of *TEAD1* and *TEAD4*, as the expression of both of these TEAD family members was increased by YAP-5SA, but not by the YAP-5SA/S94A mutant, in CAL27 cells (**Figure 3.13**). Further, analysis of gene expression across

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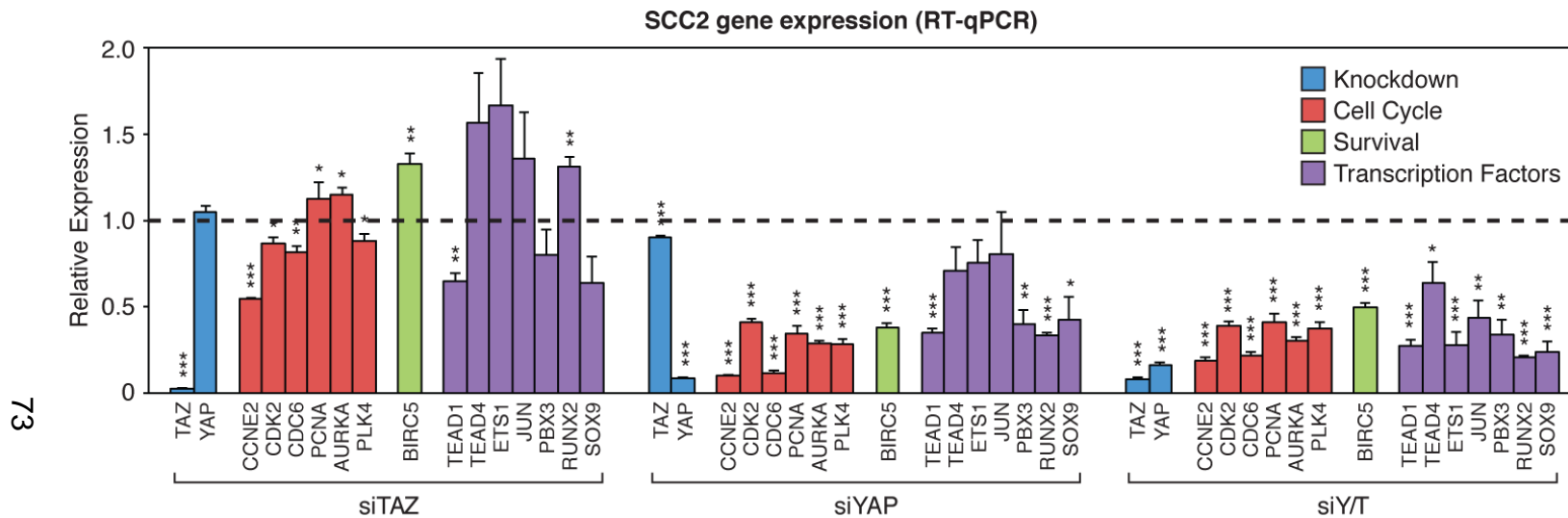
Figure 3.12 - YAP/TAZ are necessary for target gene expression in SCC2 cells. SCC2 cells were transiently transfected with control siRNA (siCTL) or siRNA targeting TAZ (siTAZ), YAP (siYAP), or YAP and TAZ (siY/T). Relative expression of genes indicated in the microarray analysis was determined by RT-qPCR. All data are relative to siCTL (dashed line) and are shown as the average of three experiments (+SE). Genes are grouped together by function including knockdown efficiency (blue), cell-cycle regulation (red), pro-survival (green), and transcription factors (purple). Statistics comparing to the control cells (dashed line) were calculated using an unpaired Student *t* test and are represented as *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
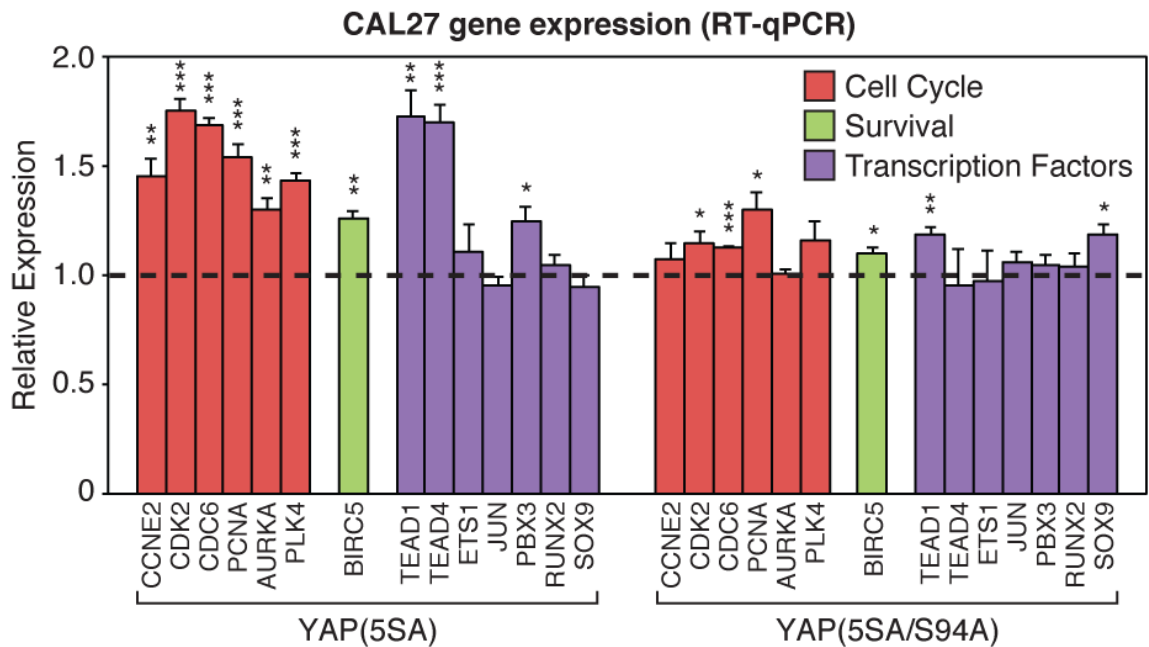
Figure 3.13 - Nuclear YAP is sufficient to promote expression of some target genes in CAL27 cells dependent on YAP-TEAD binding. Doxycycline-inducible CAL27 control cells or cells expressing 3xFLAG-YAP(5SA) or 3xFLAG-YAP(5SA/S94A) were treated with doxycycline. Relative expression of genes indicated in the microarray analysis was determined by RT-qPCR. All data are relative to siCTL (dashed line) and are shown as the average of three experiments (+SE). Genes are grouped together by function including cell-cycle regulation (red), pro-survival (green), and transcription factors (purple). Statistics comparing to the control cells (dashed line) were calculated using an unpaired Student t test and are represented as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

tumor grade using TCGA data OSCC onset, as increased nuclear YAP accumulation can be detected showed that *TEAD4* is induced in expression with tumor onset, and further increases with higher tumor grade and stage (**Figure 3.10D**). *TEAD1* did not show the same trends (**Figure 3.10D**). Thus, these data suggest that *TEAD4* may function as a relevant YAP/TAZ target that initiates a pro-tumorigenic feed-forward cascade that contributes to the onset and progression OSCC.

Discussion

This study provides evidence that the transcriptional regulators YAP and TAZ have important roles in the onset and progression of human OSCC. Notably, I have found that YAP localization is dysregulated in regions predisposed to OSCC onset, as increased nuclear YAP accumulation can be detected in epithelial cells of hyperplastic and dysplastic tissues. Thus, altered YAP localization correlates with the early transformation of oral epithelial cells, suggesting that nuclear YAP promotes their progression to a malignant state. Indeed, knockdown of YAP in SCC2 cells inhibited the ability of these cells to proliferate *in vitro*, and reduced the ability of these cells to generate tongue tumors *in vivo*. YAP knockdown in CAL27 cells also inhibited cell proliferation and anchorage-independent growth (192). Moreover, ectopic expression of a nuclear-localized YAP mutant in CAL27 cells promoted cell proliferation, indicating that nuclear YAP is sufficient to drive cell proliferation. Ectopic expression of nuclear-localized YAP also increased the ability for CAL27 cells to promote wound closure *in vitro*, and while I cannot rule out that

these differences do not relate to proliferative alterations, my observations suggest that nuclear YAP activity is also sufficient to drive cell migration. Due to technical issues we were unable to similarly examine the localization of TAZ in human tissues, but given that common regulatory signals control YAP and TAZ localization (69), and that TAZ plays an important role in other cancer cells (12,177), it is likely that dysregulated TAZ localization also contributes to early OSCC development. Supporting this premise, I found that knockdown of both YAP and TAZ in SCC2 OSCC cells severely reduced their proliferation, induced pro-apoptotic cues, and halted their wound closure potential *in vitro*, beyond the knockdown of either YAP or TAZ alone. Additionally, knockdown of both YAP and TAZ dramatically reduced primary tumor growth *in vivo*, more so than YAP knockdown alone. Thus, YAP and TAZ have redundant pro-tumorigenic roles, which may be the case for other malignancies in addition to OSCC.

While our observations indicate accumulation of nuclear YAP in tissues predisposed to form OSCC, how this dysregulation arises is less clear. Interrogation of TCGA datasets showed no indication of general increases in *YAP* (or *TAZ*) expression or genomic alterations with OSCC onset, or altered expression of core Hippo pathway components known to regulated YAP/TAZ localization. Given the close association between epithelial cell polarity cues and the control of YAP localization (70,71), and the observed epithelial polarity changes that occur with OSCC onset (200), one possibility is that altered epithelial polarity cues may contribute to the dysregulation of YAP. Another unresolved

76

question relates to why only subsets of tumors exhibit abundant levels of YAP protein. My analysis showed that four out of the ten tumors (ranging from moderately to poorly differentiated) that I examined had elevated YAP levels. While the increased YAP levels in these distinct tumors may result from amplified expression of *YAP*, as suggested by prior studies (191,193), they may also relate to alternative mechanisms of post-transcriptional control of YAP stability and localization. Phosphorylation of YAP on S127 was reduced in tumors with elevated YAP, suggesting that defective Hippo pathway signaling likely contributes to these aberrant YAP levels. Notably, the tumors I identified with high YAP levels were characterized as poorly differentiated, suggesting that YAP may contribute to tumor progression. High YAP levels with prominent nuclear localization were observed in the basal layer of adjacent histopathologically normal epithelia, which is similar to that observed in the basal progenitors of the epidermis (71), proximal lung epithelium (201,202), and a range of other epithelial stem cell populations throughout development (69). Thus, nuclear YAP activity may facilitate the oral epithelial progenitor state and possibly contribute to stem cell-like properties observed in aggressive OSCCs.

The pro-tumorigenic activity of YAP and TAZ rely on their transcriptional properties (67,88). Using global gene expression analysis following the knockdown of YAP/TAZ in OSCC cells we have identified a transcriptional program that is regulated by these factors. This YAP/TAZ expression signature correlates with gene expression changes identified by TCGA in OSCC, indicating that YAP/TAZ

is broadly dysregulated with OSCC onset. Strikingly, genes induced in expression by YAP/TAZ (i.e. genes repressed following YAP/TAZ knockdown) are significantly associated with OSCC progression, as the expression changes are maintained with advancing tumor grade and tumor stage. This includes stage IV tumors, which have the lowest 5-year survival rates (203). Our data showing that YAP/TAZ may promote OSCC cell migration and progression to a metastatic state in mouse orthotopic tongue tumor models suggest that YAP/TAZ participate in pro-metastatic events in OSCC, as is the case in other malignancies (12,88), but further work is required to clarify this possibility.

Hierarchical clustering analysis of the YAP/TAZ-induced genes with OSCC tumor grade and stage progression revealed two clusters that suggest YAP/TAZ function in OSCC. One of these clusters (Cluster A in **Figure 3.11B**) was enriched for genes critical for cell cycle progression, and likely explains the pro-proliferation roles that YAP/TAZ play in OSCC cells. Genes regulated by the ATR- and E2F-transcription factors are enriched in this cluster, suggesting that YAP/TAZ may direct the activity of these transcription factors to overcome cell cycle checkpoints. The second cluster (Cluster B in **Figure 3.11B**) was enriched for genes that respond to cancer-related signaling pathways, such as those regulated by TGFβ and WNT growth factors. Nuclear YAP and/or TAZ synergize with TGFβ-activated SMAD transcription factors to promote pro-tumorigenic events (1,126), and thus, it is likely that nuclear YAP/TAZ promote these signals in OSCC. Similarly, nuclear YAP/TAZ facilitate WNT-induced signals (120,122), which have key roles in the

development of OSCC (178). WNT and TGFβ signaling both promote EMT, which is a process implicated in the induction of tumor-initiating properties (11). EMT-related genes were enriched in the YAP/TAZ-regulated transcriptional signature, as were gene targets of the stem cell-regulating transcription factor OCT4. Thus, the YAP/TAZ-induced transcriptional program may influence tumor-initiating properties that are associated with aggressive OSCC. Additional transcription factors implicated in cancer progression were also regulated by YAP/TAZ, including *SOX9*, which has recently been described as a target of YAP in esophageal cancers (204). Notably, members of the TEAD family were induced by YAP/TAZ, and increased expression of *TEAD4* was significantly elevated with increased OSCC grade and stage. Thus, early increases in nuclear YAP/TAZ localization may initiate a feed-forward mechanism that promotes the assembly of YAP/TAZ-TEAD complexes. Given that binding to TEAD transcription factors drives YAP/TAZ nuclear accumulation (66), such a feed-forward mechanism may contribute to the elevated nuclear YAP/TAZ observed with OSCC development.

Taken together these observations indicate that YAP/TAZ are important factors contributing to OSCC biology. Our data highlight the importance of examining changes beyond single gene expression, mutation, and/or genomic alterations that correlate with cancer tissues, as our focused analysis of the YAP/TAZ-regulated signature identifies and connects tumor-associated expression changes that may be otherwise overlooked. Given that YAP/TAZ are dysregulated early in the onset of OSCC, further understanding the YAP/TAZ-

regulated transcriptional events and linking them to other cancer-related signaling networks may offer new insight into OSCC. Moreover, given the emergence of small molecules that target YAP/TAZ activity, novel therapeutic approaches may evolve that can hopefully reduce this devastating disease.

CHAPTER IV - YAP/TAZ DIRECT TRANSFORMING GROWTH FACTOR B-INDUCED TUMORIGENIC PHENOTYPES IN BREAST CANCER CELLS

Disclaimer: This chapter is adapted from Hiemer, S. E., Szymaniak, A. D., and Varelas, X. (2014) The transcriptional regulators TAZ and YAP direct transforming growth factor β-induced tumorigenic phenotypes in breast cancer cells. *Journal of Biological Chemistry* **289**, 13461-13474 (177)

Abstract

Uncontrolled Transforming growth factor β (TGFβ) signaling promotes aggressive metastatic properties in late-stage breast cancers. However, how TGFβ-mediated cues are directed to induce late-stage tumorigenic events is poorly understood, particularly given that TGFβ has clear tumor suppressing activity in other contexts. Here I demonstrate that the transcriptional regulators YAP and TAZ (YAP/TAZ), key effectors of the Hippo pathway, are necessary to promote and maintain TGFβ-induced tumorigenic phenotypes in breast cancer cells. Interactions between YAP/TAZ, TGFβ-activated SMAD2/3, and TEAD transcription factors reveal convergent roles for these factors in the nucleus. Genome-wide expression analyses indicate that YAP/TAZ, TEADs and TGFβ-induced signals coordinate a specific pro-tumorigenic transcriptional program. Importantly, genes cooperatively regulated by YAP/TAZ, TEAD, and TGFβ, such as the novel targets *NEGR1* and *UCA1*, are necessary for maintaining tumorigenic activity in metastatic breast cancer cells. Nuclear YAP/TAZ also cooperate with TGFβ signaling to promote phenotypic and transcriptional changes in non-

tumorigenic cells to overcome TGFβ repressive effects. My work thus identifies crosstalk between nuclear YAP/TAZ and TGFβ signaling in breast cancer cells, revealing novel insight into late-stage disease-driving mechanisms.

Introduction

Elevated nuclear levels of the transcriptional regulators YAP and TAZ are associated with a broad range of aggressive cancers (90). For instance, the extent of nuclear YAP or TAZ levels corresponds with breast cancer tumor grade (12,94,95). In breast cancer cells, enhanced nuclear YAP and TAZ levels promote oncogenic transformation and endow cells with tumorigenic properties, including the ability to proliferate, subvert apoptotic cues, migrate, invade, and grow under anchorage-independent conditions (10,68,101,102,105). Moreover, high nuclear TAZ levels induce CSC-like activity (12,88), and promote evasion of certain breast cancer drug therapies (12,96). Thus, understanding the roles of YAP/TAZ is critical for directing efficient breast cancer therapies.

The tumor-initiating activity of YAP/TAZ relies on their binding to the TEAD family of transcription factors (TEAD1-4) (67,87,88), indicating that together these factors direct a tumorigenic transcriptional program. Supporting this premise, YAP/TAZ-TEAD complexes directly promote the expression of oncogenic factors, such as *CTGF* and *CYR61* (67,87), which contribute to human breast cancer progression (205). Nuclear YAP/TAZ activity is highly regulated, and governed in large part by the Hippo pathway-regulated LATS1 and LATS2 kinases (59). LATS1/2 kinases phosphorylate YAP/TAZ on conserved Serine residues, which

82

promotes 14-3-3 binding and subsequent sequestration in the cytoplasm (61,62), and also prime YAP/TAZ for further phosphorylation by CK1ε/δ-kinases that evoke YAP/TAZ degradation via proteasome-dependent mechanisms (63,64). Additional phosphorylation events destabilize TAZ, including those regulated by WNT, PI3K, and GSK3β (65,121). Thus, dysregulation of multiple upstream signals likely contributes to the hypo-phosphorylation and stabilization of nuclear YAP/TAZ activity in cancer.

YAP/TAZ modify the activity of other transcription factors besides TEADs, including the Transforming growth factor-beta (TGFβ)-activated SMAD complexes (174). TGFβ is the prototypic member of a family of secreted factors that regulates numerous developmental and homeostatic processes (130). SMAD2 and SMAD3 (SMAD2/3) are the primary mediators of TGFβ-induced transcription. SMAD2/3 are phosphorylated by TGFβ-bound membrane receptors, which induces binding to SMAD4 (138,140), forming active transcriptional complexes that accumulate in the nucleus upon binding to YAP/TAZ (126). In cancer the role of TGFβ is complex, as it can suppress early oncogenic events but also promote aggressive late-stage metastatic phenotypes (146,147). Several lines of evidence indicate that TGFβ, like YAP/TAZ, promotes aggressive tumorigenic properties in late-stage breast carcinomas (11,167). What mechanistically distinguishes between TGFβ-dependent responses is poorly understood.

Given that YAP/TAZ bind to SMAD transcription factors and direct TGFβ signaling in other contexts (70,126,128), I sought to characterize whether

83

YAP/TAZ define TGFβ-mediated tumorigenic cues in breast cancer cells. My observations indicate that TGFβ-induced tumorigenic events, such as increased cell migration, invasion, and anchorage-independent growth, require YAP/TAZ. My data also indicate that like YAP/TAZ, the TEAD transcription factors interact with TGFβ-induced SMAD2/3 in the nucleus, suggesting that YAP/TAZ-TEAD-SMAD2/3 complexes coordinate transcriptional events in a concerted manner. Genome-wide microarray analysis of gene expression changes that occur upon knockdown of YAP/TAZ or TEADs, or inhibition of TGFβ signaling, revealed that YAP/TAZ, TEAD, and TGFβ regulate overlapping target genes. Interestingly, the direct gene targets *NEGR1* and *UCA1*, which are synergistically regulated by YAP/TAZ, TEAD, and TGFβ, are necessary for maintaining tumorigenic activity in metastatic breast cancer cells, suggesting that the convergence of YAP/TAZ-TEAD-TGFβ signals is critical for driving late-stage breast cancer phenotypes. Supporting this premise, expression of nuclear-localized YAP or TAZ mutants direct transcriptional events that sensitize untransformed breast cancer cells to adopt tumorigenic phenotypes in response to TGFβ, while also suppressing TGFβ-induced cytostasis. These findings reveal novel crosstalk between TGFβ and Hippo signaling that I propose is important for late stage tumorigenic events in breast cancer.

Results

Nuclear YAP/TAZ are required to promote TGFβ-induced tumorigenic phenotypes in breast cancer cells

In cancer the role of TGFβ is complex, as it can suppress early oncogenic events, such as cell cycle progression, but also promote late-stage metastatic phenotypes (146,147). What mechanistically distinguishes between TGFβ-dependent responses is poorly understood. Several lines of evidence indicate that nuclear YAP/TAZ, like TGFβ, induce tumorigenic properties in late-stage breast carcinomas (11,167). In untransformed mammary epithelium, YAP/TAZ localization is restricted to the cytoplasm by cell compaction/polarity-regulated cues (68,70). Dysregulation of cell polarity cues, which is a hallmark of cancer progression (6), induces nuclear YAP/TAZ localization. Given our prior work showing that YAP/TAZ bind to and regulate the localization and activity of TGFβ-activated SMAD transcription factors (70,126), I sought out to test whether TAZ and/or YAP promote TGFβ-induced tumorigenic events. I began my analysis by examining the relationship between YAP/TAZ localization and the TGFβ-induced cytostatic response in a panel of mammary epithelial and breast cancer cell lines. Based on published data, I divided the panel into cells that are responsive to TGFβ-induced cytostasis (MCF10A, BT20, HMLE, HS578T, MCF7, and MCF12) and cells in which TGFβ induces pro-tumorigenic signals, but not growth arrest (MDA-MB-231, MDA-MB-231-LM2-4, SKRB3, and SUM149) (206-213). Interestingly, I

observed that cells displaying high levels of nuclear YAP/TAZ correlate with those in which TGFβ induces tumorigenic cues (**Figure 4.1**).

Tumor-initiating cell properties are associated with the ratio of glycoprotein cell surface markers CD24 and CD44. High levels of CD44 to low levels of CD24 (CD44^{high}/CD24^{low}) positively correlate with the cell's ability to self-renew and differentiate (11). I have found that CD44^{high}/CD24^{low} populations also correlate with nuclear YAP/TAZ (**Figure 4.2**). In MDA-MB-231-LM2-4 cells (herein referred to as LM2-4) metastatic breast cancer cell line (214), a highly aggressive derivative of triple-negative basal subtype MDA-MB-231 cells (215), the cells have higher CD44 levels compared to the parental MDA-MB-231 cells and non-tumorigenic human MCF10A mammary epithelial cells (**Figure 4.2**). This correlates with both increased nuclear YAP/TAZ localization at high density (**Figure 4.1**), increased TAZ levels, and decreased phospho-S127-YAP levels (**Figure 4.3**).

To further investigate the relationship between YAP/TAZ and TGFβ, I sought to determine the roles of nuclear YAP/TAZ in the LM2-4 cell line. A fraction of LM2-4 cells in culture are capable of generating clonal mammospheres under anchorage-independent conditions (**Figure 4.4**), which is often used as a measure of the self-renewing potential of tumorigenic cells *in vitro* (216). TGFβ treatment of LM2-4 cells led to dramatic increases in the number and size of mammospheres observed (**Figure 4.4A and 4.4B**), similar to that observed with TGFβ treatment of other mammary cells (11). When the self-renewing properties of the cells within
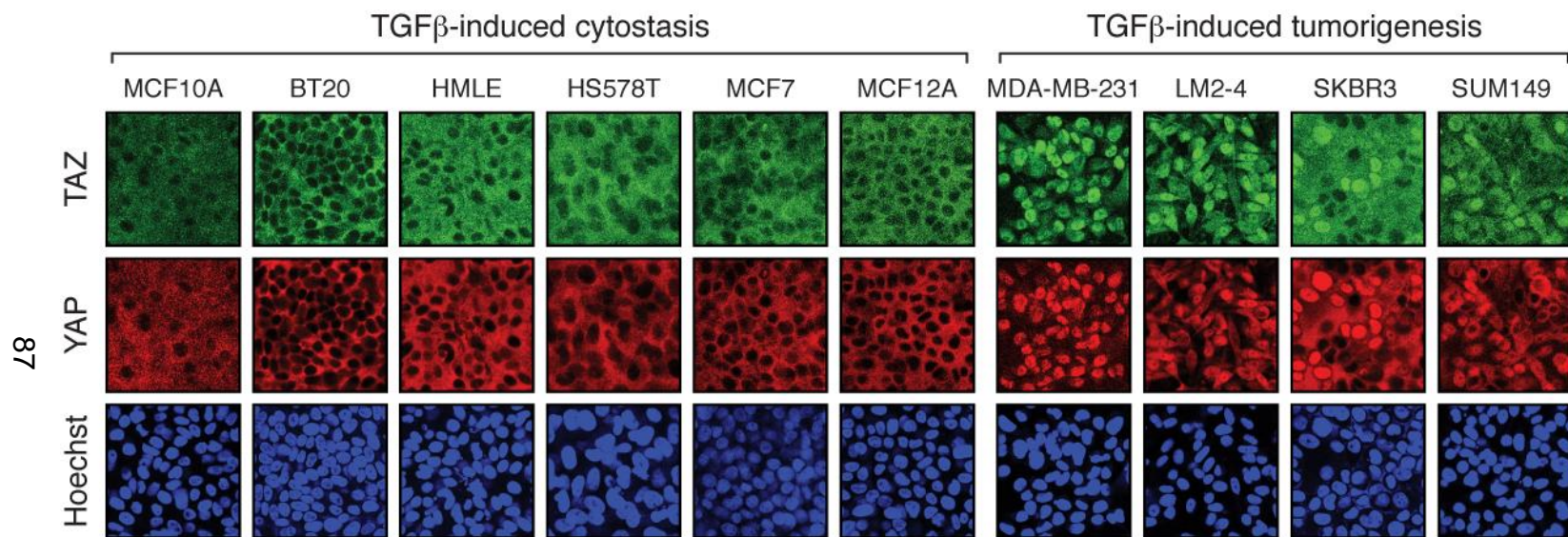
Figure 4.1 - TAZ and YAP localization in a panel of breast cancer cell lines. A panel of breast cancer cell lines was divided by TGFβ-induced cytostasis and TGFβ-induced tumorigenic responses and examined by immunofluorescence for endogenous TAZ and YAP localization.
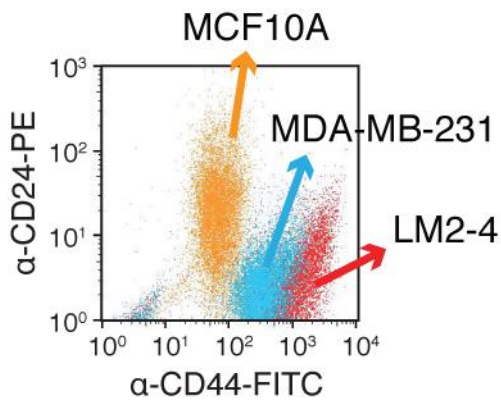
Figure 4.2 - Aggressive breast cancer cells have high CD44 and low CD24 expression. MCF10A, MDA-MB-231, and LM2-4 cells were stained for CD44-FITC and CD24-PE and analyzed by flow cytometry to determine protein expression. Data from 10,000 events from each cell line are shown.
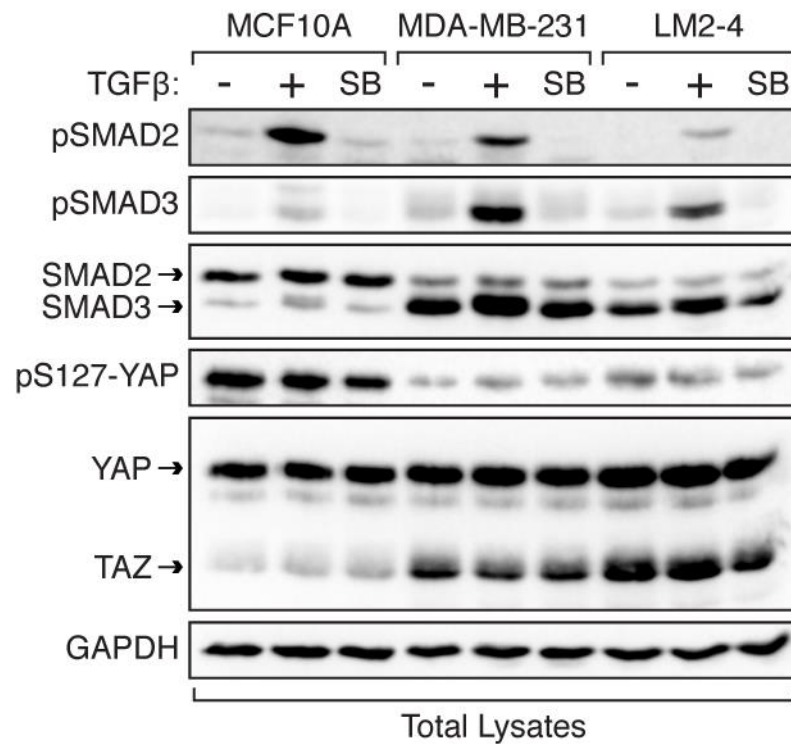
Figure 4.3 - Protein expression in MCF10A, MDA-MB-231, and LM2-4 cells.
MCF10A, MDA-MB-231, and LM2-4 cells were left untreated or treated with TGFβ
or SB-431542 (SB) for 2 hours. Cells were lysed and analyzed by immunoblotting
with the indicated antibodies including GAPDH (loading control).
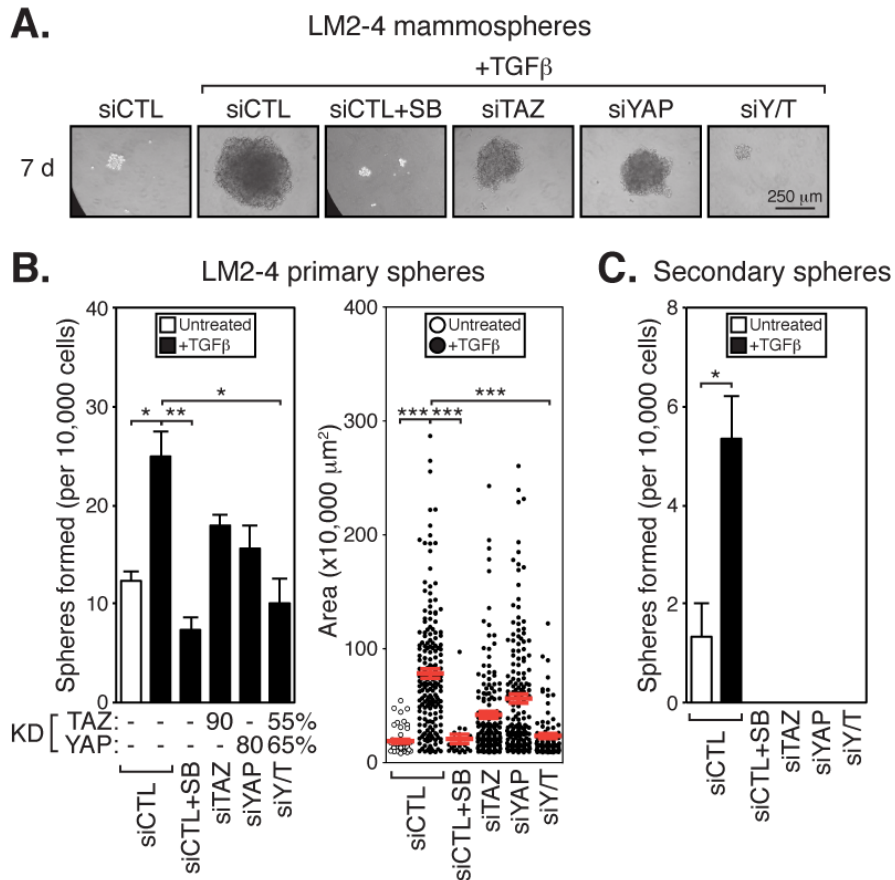
Figure 4.4 - YAP/TAZ are required for TGFβ-induced tumorisphere formation and propagation. LM2-4 cells were transiently transfect with control siRNA (siCTL) or siRNA targeting TAZ (siTAZ), YAP (siYAP), or YAP and TAZ (siY/T). Cells were left untreated, treated with TGFβ or SB-431542 (SB) +TGFβ, and grown in anchorage-independent conditions. **A.** Representative images of primary mammosphere colonies. **B.** Primary mammospheres were quantitated, measuring the number of colonies formed (left) and the size of each colony (right). Knockdown efficiency is indicated below. **C.** Primary mammospheres were passaged into secondary spheres. Secondary mammospheres following SB-431542 treatment, or transfection with siTAZ, siYAP, or siY/T were unable to be determined due to low numbers. Three independent experiments from each condition were quantitated. Black error bars represent the average (+SE) and red error bars represent the average (±SE). Statistics were performed using an unpaired Student *t* test and are represented as *, P < 0.025; **, P < 0.005; ***, P < 0.0001.

the mammospheres were assessed for their ability to form secondary clonal spheres (216), I found that TGFβ also promoted secondary mammosphere formation (**Figure 4.4C**). Co-treatment of the cells with the TGFβ-receptor agonist SB-431542 abolished the formation of primary mammospheres, validating that the observed effects are indeed generated via canonical TGFβ-receptor-mediated signals (217,218) (**Figure 4.4A and 4.4B**). As expected, SB-431542 treatment eliminated the TGFβ-induced phosphorylation of SMAD2 and SMAD3 in these cells (**Figure 4.3**). Individual TAZ or YAP knockdown also repressed the number and size of TGFβ-induced mammospheres (**Figure 4.4A and 4.4B**). However, simultaneous knockdown of both TAZ and YAP dramatically reduced primary mammosphere formation and prevented secondary mammosphere formation (**Figure 4.4**), indicating redundant roles for TAZ and YAP in transducing TGFβ-mediated cues required for anchorage-independent growth and tumor initiating properties.

I further investigated other hallmark tumorigenic properties that may be mediated by TGFβ and YAP/TAZ in metastatic breast cancers, including cell migration and invasion (6). I found that treatment of LM2-4 cells with TGFβ led to increases in cell migration in an *in vitro* wound-healing scratch assay (**Figure 4.5A**), similar to prior work (219). As expected, co-treatment with TGFβ-receptor agonist SB-431542 blocked TGFβ-induced cell migration (**Figure 4.5A**). Simultaneous knockdown of YAP/TAZ using siRNA also abolished TGFβ-induced
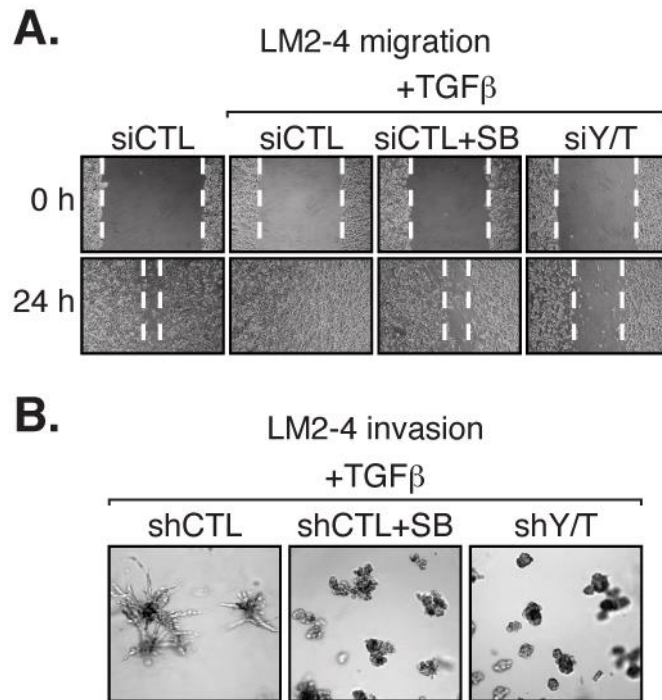
Figure 4.5 - YAP/TAZ are required for TGFβ-induced migration and invasion.
A. LM2-4 cells were transiently transfected with control siRNA (siCTL) or siRNA targeting TAZ (siTAZ), YAP (siYAP), or YAP and TAZ (siY/T). Cells were left untreated, treated with TGFβ or SB-431542 (SB) +TGFβ. Monolayers were wounded and analyzed for cell migration. **B.** LM2-4 cells stably expressing control shRNA (shCTL), or shRNA targeting YAP and TAZ (shY/T) were treated with TGFβ or SB-431542 +TGFβ and incubated in three-dimensional Matrigel culture conditions. Representative images from three independent experiments are shown.

LM2-4 cell migration (**Figure 4.5A**). Similarly, SB-431542 treatment or shRNA-mediated YAP/TAZ knockdown abolished the ability of three-dimensional colonies of LM2-4 cells to invade into surrounding Matrigel matrix in the presence of TGFβ (**Figure 4.5B**). Taken together, my observations indicate that YAP/TAZ are critical mediators of TGFβ-induced tumorigenic events, including mammosphere formation, cell migration, and invasion.

YAP/TAZ, TEADs, and SMADs converge to regulate a TGFβ-induced transcriptional program in breast cancer cells

Studies indicate that YAP/TAZ-induced cell transformation relies on the recruitment of YAP/TAZ to DNA by the TEAD family of transcription factors (TEAD1-4) (67,87). TAZ and YAP also bind TGFβ-activated SMAD complexes to control SMAD localization and activity in a variety of cell types, including mammary epithelial cells (70,126). Recent work has shown that YAP/TAZ-TEAD-SMAD2/3 complexes control transcriptional events important for maintaining human embryonic stem cell pluripotency (127). Thus, I hypothesized that similar complexes are also present in late stage breast cancers such that TEAD and SMAD transcription factors cooperatively facilitate YAP/TAZ-mediated tumorigenic activity. I found that TEAD2 and TEAD4 associate with SMAD3, as well as YAP (**Figure 4.6**), and these interactions were unaffected by stimulation with a constitutively active TGFβ receptor (TGFβR1-T240D (220)). Given that YAP/TAZ exhibit a predominantly nuclear localization in LM2-4 cells, and SMAD2/3 nuclear
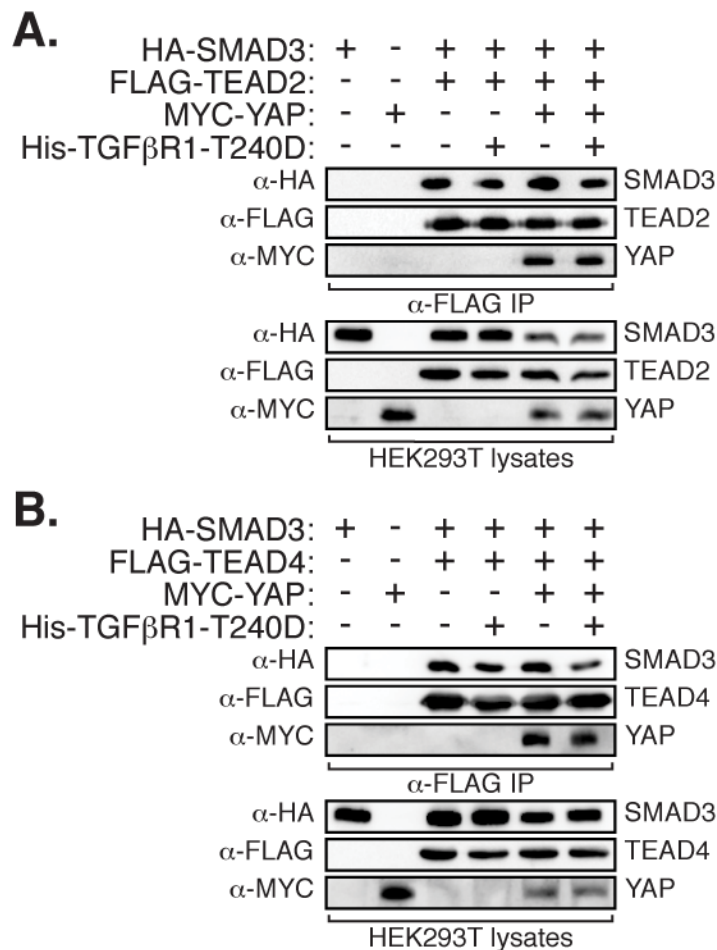
Figure 4.6 - TEAD2 and TEAD4 interact with SMAD3 and YAP. HEK293T cells expressing HA-SMAD3, MYC-YAP, His-TGFβR1-T240D, and FLAG-TEAD2 (**A**) or FLAG-TEAD4 (**B**) were lysed and subjected to IP with a FLAG antibody followed by immunoblotting with the indicated antibodies.

localization is induced upon TGFβ treatment (**Figure 4.7**), I speculated that YAP/TAZ-TEAD might be interacting with TGFβ-activated SMAD2/3 to specify pro-tumorigenic transcriptional events. To acquire both protein interaction and localization information, I performed *in situ* PLA. PLA is a sensitive technique used to visualize the localization and association of endogenous protein complexes (proteins localized within 40nm of each other) by microscopy (221). Using PLA, I observed YAP/TAZ-SMAD2/3 interactions in both the nucleus and cytoplasm of untreated LM2-4 cells (**Figure 4.8A**). Upon TGFβ treatment, nuclear YAP/TAZ-SMAD2/3 binding became much more apparent in the nucleus (**Figure 4.8A**), consistent with nuclear YAP/TAZ-SMAD2/3 complexes directing transcriptional events (70,126). I also detected endogenous TAZ-TEAD1 interactions in the nucleus of LM2-4 cells with or without TGFβ stimulation (**Figure 4.8B**), which were increased slightly upon TGFβ treatment (**Figure 4.8B**). TEAD1-SMAD2/3 interactions were readily detected in the nucleus of LM2-4 cells, particularly after TGFβ treatment (**Figure 4.8C**), suggesting these complexes stabilize upon nuclear accumulation of SMADs. Taken together, my observations indicate that YAP/TAZ, TEAD, and SMAD interact in TGFβ-stimulated metastatic breast cancer cells, and suggest that they may form transcriptional complexes that function together in the nucleus.

To explore the possible overlap in transcriptional activity by YAP/TAZ, TEAD, and SMAD complexes in tumorigenesis, I used microarrays to compare the global expression profiles of LM2-4 cells treated as follows: 1) transfected with
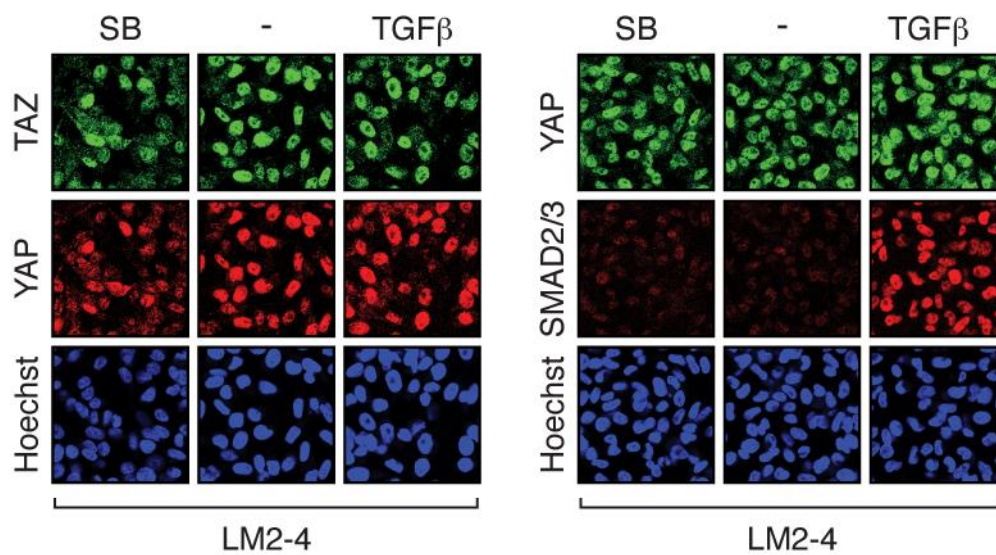
Figure 4.7 - SB-421542 or TGFβ treatment do not affect TAZ or YAP localization. LM2-4 cells were left untreated or treated with SB-431542 (SB) or TGFβ for 2 hours and were examined by immunofluorescence for endogenous TAZ or YAP localization (left) or YAP and SMAD2/3 localization (right). Nuclei were visualized with Hoechst stain. Representative images are shown.
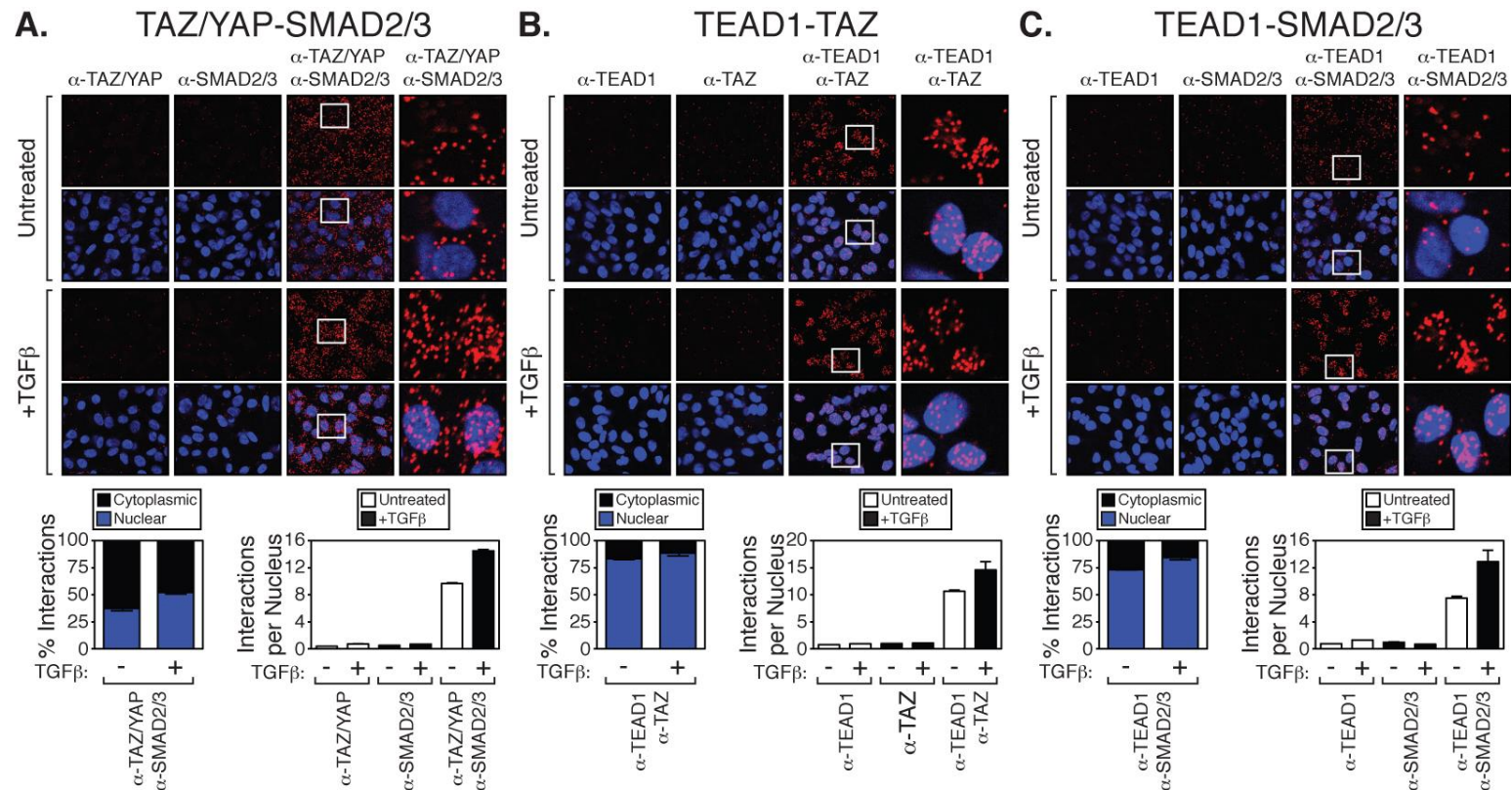
Figure 4.8 - YAP/TAZ, TEADs, and SMAD2/3 interact endogenously. LM2-4 cells left untreated or treated with TGFβ for 2 hours were probed with primary antibodies recognizing YAP/TAZ and SMAD2/3 (**A**), TEAD1 and TAZ (**B**), or TEAD1 and SMAD2/3 (**C**). *In situ* PLA) followed by confocal microscopy were performed using mouse and rabbit secondary probes. Red dots indicate endogenous interactions and nuclei were visualized with Hoechst stain. Representative images are shown, and three fields from each condition were quantitated, measuring the nuclear-cytoplasmic localization of the interactions and the number of interactions per nucleus. Black error bars either represent the average (+SE) or the average (±SE).

control siRNA (siCTL) and treated with TGFβ; 2) transfected with siRNA targeting both YAP/TAZ (siYAP/TAZ) and treated with TGFβ; 3) transfected with siRNA targeting all four TEAD (TEAD1-4) family members (siTEAD) and treated with TGFβ; and 4) transfected with control siRNA (siCTL) and treated simultaneously with TGFβ and SB-431542. In terms of significant gene expression differences (p-value < 0.01) relative to siCTL+ TGFβ treatment, 461 genes overlapped between siYAP/TAZ and siTEAD conditions (**Figure 4.9A**). This gene set displayed a high degree of correlation in expression (R=0.86). The expression of 594 genes changed following SB-431542 treatment, and of these, 176 genes overlapped with siYAP/TAZ conditions. Of these 176 genes, 80 were also altered following TEAD knockdown (**Figure 4.9A**).

Interestingly, genes for which expression was altered among all three experimental conditions exhibited distinct expression correlations. Unbiased clustering segregated YAP/TAZ-TEAD- TGFβ-regulated genes into four different groups: Group 1 – repressed following siYAP/TAZ, siTEADs, or TGFβ inhibition (therefore normally induced by the presence of these factors); Group 2 – repressed following siYAP/TAZ or siTEAD treatment, but induced by TGFβ inhibition; Group 3 – induced following siYAP/TAZ, siTEADs, or TGFβ inhibition (therefore normally repressed by the presence of these factors); and Group 4 – induced by siYAP/TAZ, siTEADs, but repressed by TGFβ inhibition. The top five genes of each group are listed in **Figure 4.9A**. RT-qPCR analysis confirmed sufficient knockdown in each
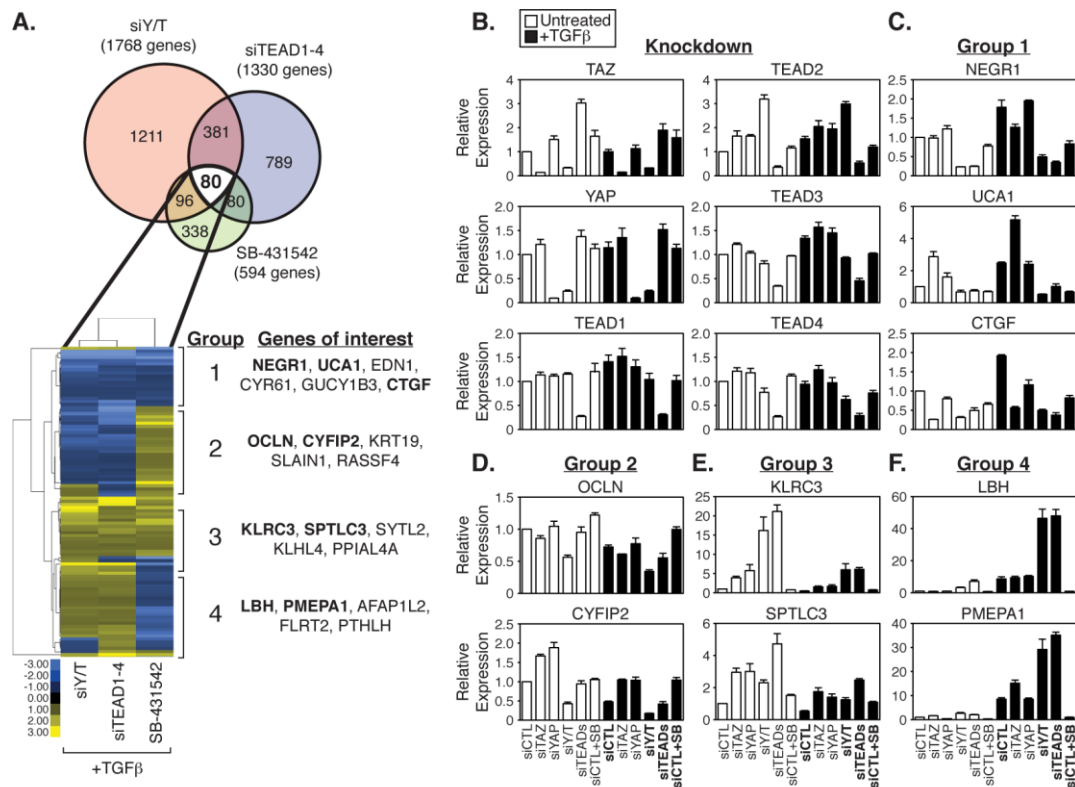
Figure 4.9 - YAP/TAZ, TEADs, and TGFβ direct different and overlapping transcriptional events. A. LM2-4 cells were transfected with control siRNA (siCTL), siRNA targeting YAP and TAZ (siY/T), or siRNA targeting all four TEADs (siTEAD1-4), and then treated with TGFβ or SB-431542 (SB) +TGFβ for 24 hours. RNA from cell lysates was harvested and global gene expression profiles were examined using Affymetrix microarrays. The Venn diagram highlights the number of genes with significant expression changes occurring for the indicated condition relative to the siCTL +TGFβ sample. Hierarchical clustering was performed on the significantly changing genes, which revealed four major clusters as indicated. Top significantly changing genes of interest are highlighted in each of the four clustering groups. **B-F.** LM2-4 cells were transiently transfected with siCTL, siTAZ, siYAP, siY/T, or siTEADs and treated with or without TGFβ or SB-431542 +TGFβ for 24 hours. Relative expression of genes indicated in the microarray analysis was determined by RT-qPCR including confirmation of knockdown (**B**), Group 1 genes, repressed by siY/T, siTEADs, and SB-431542 (SB) treatment (**C**), Group 2 genes, repressed by siY/T and siTEADs but induced by SB-431542 treatment (**D**), Group 3 genes, induced by siY/T, siTEADs, and SB-431542 treatment (**E**), and Group 4 genes, induced by siY/T and siTEADs but repressed by SB-431542 treatment (**F**). All data are shown as the average of three independent experiments (+SE).

sample (**Figure 4.9B**) and the microarray results for each group (**Figure 4.9C-F**). Notable genes for Group 1 included: *NEGR1*, *UCA1*, and *CTGF*. Elevated expression of the Group 1 genes *NEGR1*, *UCA1*, and *CTGF* relied on the presence of YAP/TAZ, TEADs, and active TGFβ signaling (**Figure 4.9C**), suggesting that YAP/TAZ-TEAD- TGFβ synergize to promote the expression of these genes. In agreement with my observations, *CTGF* has recently been confirmed as an important transcriptional target of YAP-TEAD-SMAD complexes that promotes tumorigenesis in human malignant mesothelioma (128). Interestingly, however, *NEGR1*, *UCA1*, and *CTGF* expression was abolished following YAP/TAZ or TEAD knockdown in the absence of TGFβ (**Figure 4.9C**), suggesting that while specific TGFβ signals rely on YAP/TAZ-TEAD, the basal level of YAP/TAZ-TEAD activity does not require TGFβ, and therefore YAP/TAZ-TEAD complexes may function dominantly to TGFβ signals.

Group 2 genes I confirmed by RT-qPCR included: Occludin (*OCLN*) and Cytoplasmic FMR1-interacting protein 2 (*CYFIP2*) (**Figure 4.9D**). Group 3 genes confirmed included: killer cell lectin-like receptor subfamily C protein (*KLRC3*) and serine palmitoyltransferase long chain base subunit 3 (*SPTLC3*) (**Figure 4.9E**). Confirmed Group 4 genes included: Limb bud and heart development (*LBH*) and Prostate transmembrane protein androgen induced 1 (*PMEPA1*) (**Figure 4.9F**). Notably, many genes were found to be differentially regulated by YAP/TAZ-TEADs and TGFβ, suggesting that while YAP/TAZ-TEAD complexes synergize with some

TGFβ-mediated signals (Group 1 and 3 targets), they repress others (Group 2 and 4 targets).

NEGR1 and UCA1 are direct targets of TEADs and are necessary to maintain tumorigenic breast cancer phenotypes

My analysis of LM2-4 cells indicate that YAP/TAZ, TEAD, and TGFβ co-regulate the expression of a distinct subset of genes. To examine the importance of these genes in tumorigenesis I focused my attention on Group 1 genes, as these are synergistically induced by YAP/TAZ, TEAD, and TGFβ, and include CTGF, a defined mediator of YAP/TAZ-induced tumorigenesis and CSC-like phenotypes (12,128). The top two genes synergistically induced by YAP/TAZ-TEAD and TGFβ identified in my analysis were *NEGR1* and *UCA1*. *NEGR1* encodes a cell adhesion molecule that plays a role in neuronal growth and development (222-227). *UCA1* encodes a long non-coding RNA (lncRNA) that is expressed in development, is turned off in homeostatic tissues, and has been found to be re-expressed in bladder carcinomas (228). To determine if these are direct transcriptional targets of YAP/TAZ, TEAD, and SMAD2/3, I performed ChIP. Examination of the promoter regions of *NEGR1*, *UCA1*, and *CTGF* (used as a positive control) revealed consensus TEAD binding (229) and SMAD binding motifs (230)). ChIP of YAP/TAZ, TEAD, and SMAD2/3 from LM2-4 cell lysates revealed enrichment at these sites, with SMAD2/3 recruitment only apparent after TGFβ treatment (**Figure 4.10A-C**). Thus, YAP/TAZ, TEAD, and SMAD2/3 are recruited to promoters of
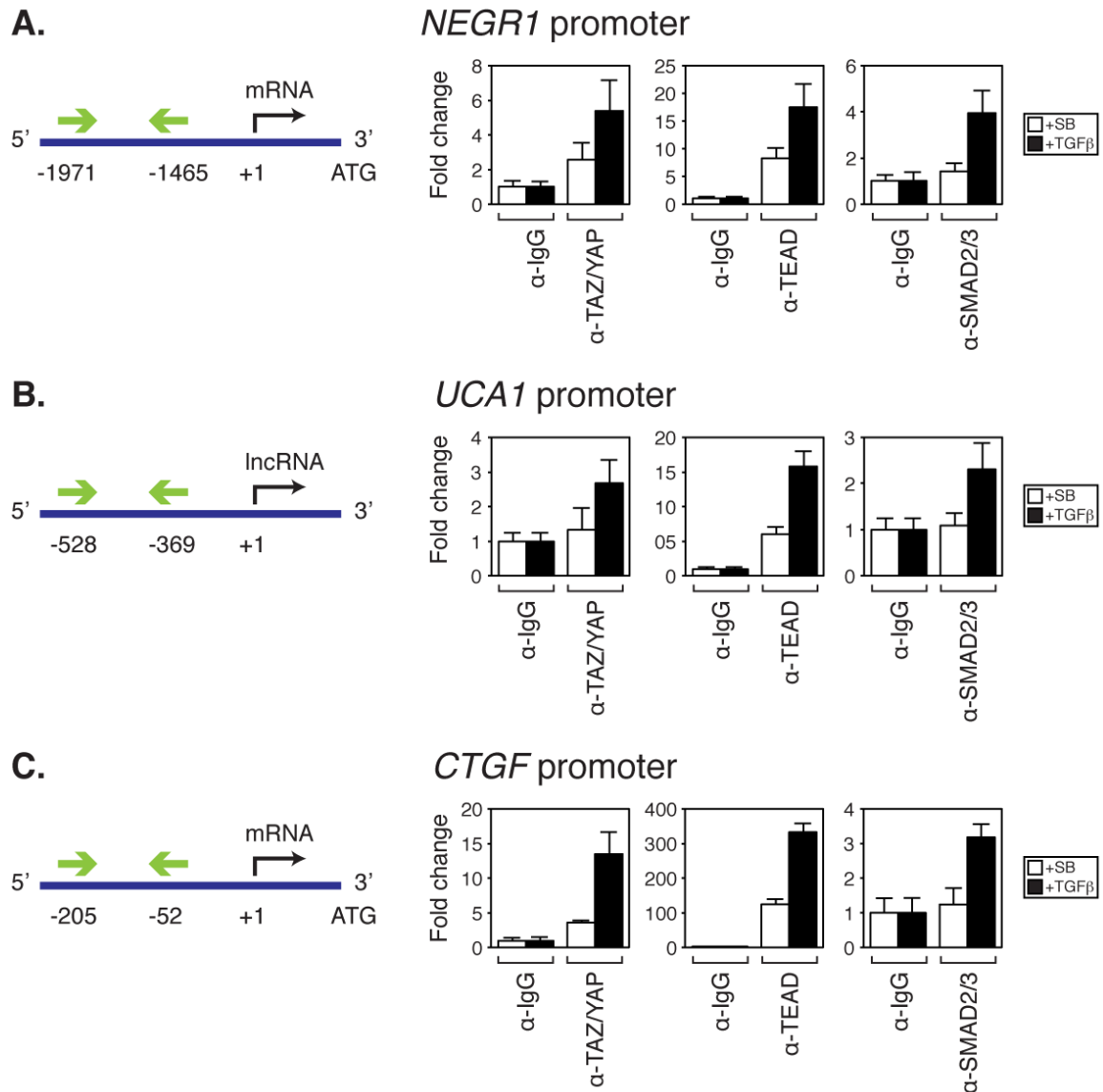
Figure 4.10 - *NEGR1*, *UCA1*, and *CTGF* are direct transcriptional targets of YAP/TAZ, TEADs, and SMADs. LM2-4 cells treated with TGFβ or SB-431542 (SB) were subjected to ChIP analysis using control rabbit IgG, YAP/TAZ, TEAD4, or SMAD2/3 antibodies. Samples were analyzed by qPCR using primers recognizing the indicated regions in the promoter of *NEGR1* (**A**), *UCA1* (**B**), or *CTGF* (**C**). Normalized values are shown as the average of three independent experiments (+SE).

genes identified in my studies, suggesting direct transcriptional regulation by these factors.

To further investigate the role of NEGR1 and UCA1 in TGFβ-mediated tumorigenesis, I found that their knockdown repressed the migration of LM2-4 cells treated with TGFβ in wound-healing scratch assays (**Figure 4.11A**) and in transwell migration assays (**Figure 4.11B**). Knockdown of either NEGR1 or UCA1 also suppressed the ability of LM2-4 cells to form large mammosphere colonies in the presence of TGFβ (**Figure 4.12**), consistent with pro-tumorigenic roles for NEGR1 and UCA1. The results of these experiments support my observations with TGFβ inhibition (SB-431542 treatment) or YAP/TAZ knockdown, suggesting that cooperative regulation of *NEGR1* and *UCA1* expression by YAP/TAZ-TEAD-SMAD complexes is necessary to promote tumorigenic phenotypes.

Nuclear TAZ and YAP cooperate with TGFβ to promote phenotypic and transcriptional changes in non-tumorigenic cells

Based on the results from my gene expression studies, I decided to test whether ectopic expression of nuclear YAP/TAZ in non-tumorigenic human mammary MCF10A cells would lead to the induction of TGFβ-dependent transcriptional events similar to those I characterized in LM2-4 cells. Stable expression of nuclear TAZ or YAP mutants can transform epithelial cells (10,12,101,105), but this occurs following weeks of stable selection. Similarly, treatment of cells with TGFβ for several days to weeks is required to observe tumorigenic events in mammary epithelial cells (11,231). To prevent confounding
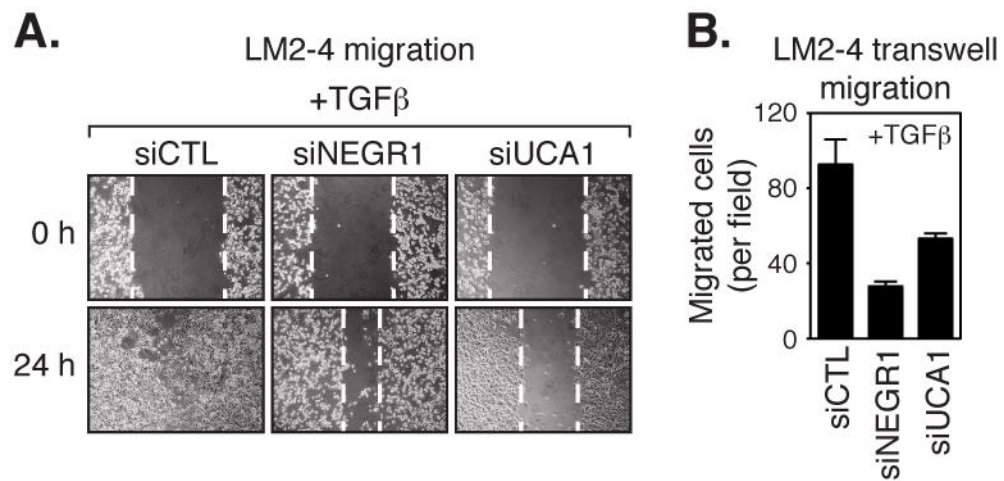
Figure 4.11 - NEGR1 and UCA1 are necessary for TGFβ-induced migration.
A. LM2-4 cells were transiently transfected with control siRNA (siCTL) or siRNA targeting NEGR1 (siNEGR1) or UCA1 (siUCA1) and treated with TGFβ. Monolayers were wounded and analyzed for cell migration. Representative images of three independent experiments are shown. **B.** LM2-4 cells transfected with siCTL, siNEGR1, or siUCA1 were plated on transwell filters to assess cell migration. Migrated cells are shown as the average number in 10 random fields over two independent experiments (+SE).
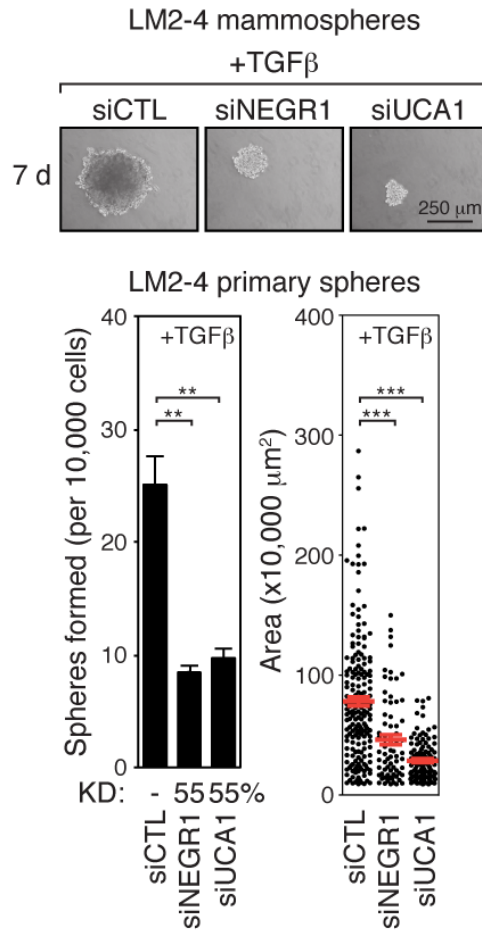
Figure 4.12 - NEGR1 and UCA1 are necessary for TGFβ-induced tumorisphere formation. LM2-4 cells were transiently transfected with control siRNA (siCTL) or siRNA targeting NEGR1 (siNEGR1) or UCA1 (siUCA1) and grown under anchorage-independent conditions in the presence of TGFβ. Representative images of primary mammosphere colonies are shown and were quantitated, measuring the number of colonies formed (left) and the size of each colony (right). Knockdown efficiency is indicated below. Three independent experiments from each condition were quantitated. Black error bars represent the average (+SE) and red error bars represent the average (±SE). Statistics were performed using an unpaired Student *t* test and are represented as **, P < 0.005; ***, P < 0.0001.

issues with long-term culture conditions, I generated MCF10A cells that express a nuclear-localized and stable TAZ mutant (TAZ(4SA)) (10) or YAP mutant (YAP(5SA)) (68) in a doxycycline-inducible manner. These YAP/TAZ mutants have the LATS kinase-induced phosphorylation sites substituted to alanines, preventing their cytoplasmic sequestration and proteasomal degradation (10,68). Titration of increasing amounts of doxycycline evoked subtle to high expression of TAZ(4SA) or YAP(5SA) in these cells (**Figure 4.13A**). High levels of TAZ(4SA) or YAP(5SA) expression for short time frames (24 hours) had minimal effects on the morphology of these cells (**Figure 4.13B**). Short treatments of TGFβ led to flattening of cells (**Figure 4.13B**), a morphology indicative of cells undergoing cell cycle arrest, as has been described for MCF10A cells post-TGFβ treatment (232). Strikingly, simultaneous doxycycline and TGFβ treatment led to rapid cell morphology changes that differed from either condition alone, with the cells becoming more spindle-like and elongated (**Figure 4.13B**). Further, TAZ(4SA)- or YAP(5SA)-expressing cells treated with TGFβ displayed much more rapid cell migration in a wound-healing scratch assay, as compared to either condition alone (**Figure 4.14**), indicating that nuclear YAP/TAZ synergize with TGFβ to promote cell morphology and cell migration changes.

In accordance with my expression analysis of LM2-4 cells, I found that nuclear TAZ or YAP function in concert with TGFβ to control transcriptional events in MCF10A cells. For example, TAZ or YAP synergized with TGFβ to promote the

A.

Dox (ng/mL): 0   0.1   1   10   100   0   0.1   1   10   100

TGFβ: - + - + - + - + - +   - + - + - + - + - +

α-FLAG _____ TAZ(4SA)   _____ YAP(5SA)

GAPDH _____   _____

MCF10A 3F-TAZ(4SA) lysates   MCF10A 3F-YAP(5SA) lysates

B.

Control +Dox   TAZ(4SA) +Dox   YAP(5SA) +Dox

Untreated   +TGFβ   Untreated   +TGFβ   Untreated   +TGFβ

24 h
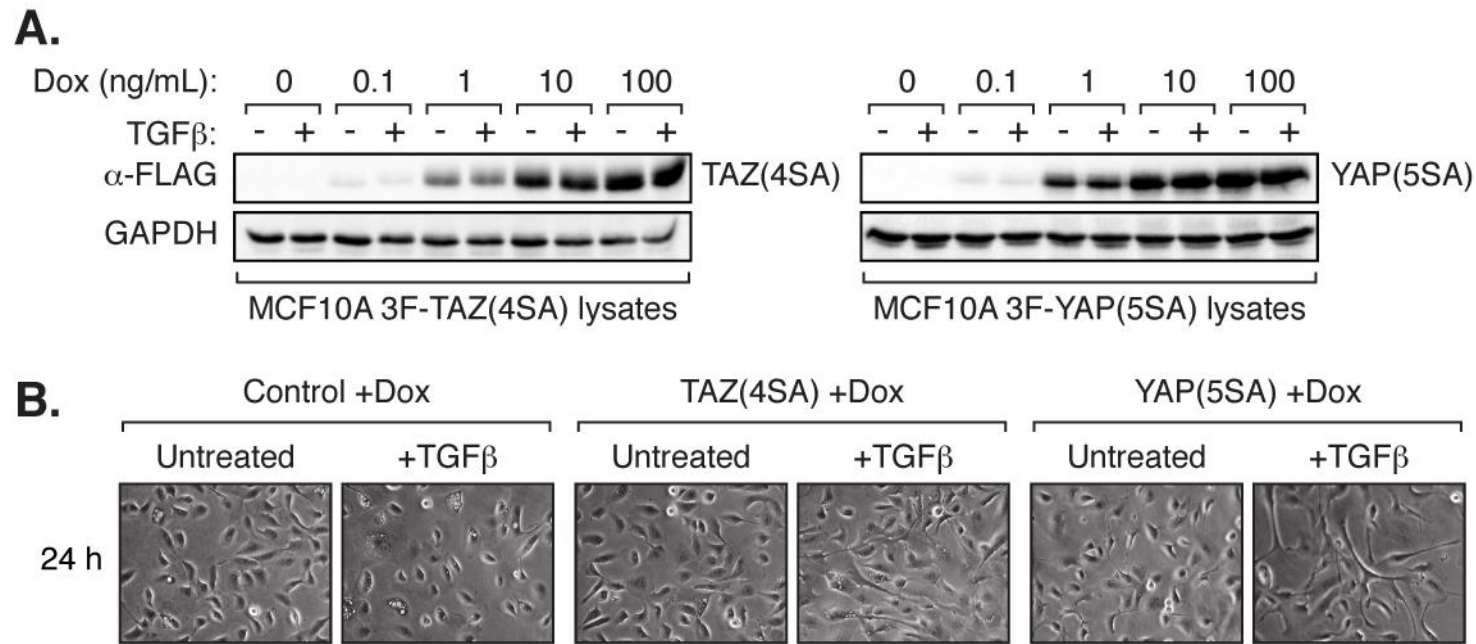
Figure 4.13 - Nuclear TAZ and YAP synergize with TGFβ to promote morphological changes. A. Doxycycline (Dox)-inducible MCF10A cells expressing 3xFLAG-TAZ(4SA) or 3xFLAG-YAP(5SA) were treated with increasing levels of doxycycline with or without TGFβ for 24 hours. Expression of TAZ or YAP was determined by immunoblotting along with GAPDH (loading control). **B.** Doxycycline-inducible MCF10A control cells or cells expressing TAZ(4SA) or YAP(5SA) were treated with doxycycline with or without TGFβ for 24 hours and examined for cell morphology.
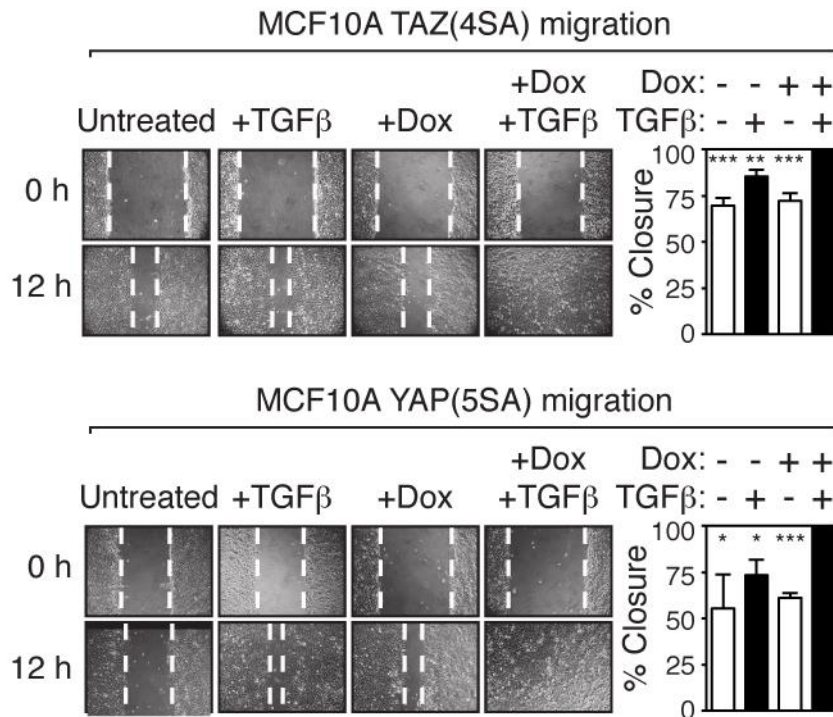
Figure 4.14 - Nuclear TAZ and YAP synergize with TGFβ to promote migration. Doxycycline (Dox)-inducible MCF10A cells expressing 3xFLAG-TAZ(4SA) or 3xFLAG-YAP(5SA) were treated with or without doxycycline and/or TGFβ for 24 hours. Monolayers were wounded and analyzed for cell migration after 12 hours. Representative images are shown and three independent experiments were quantitated. Error bars represent the average (+SE). Statistics were performed using an unpaired Student *t* test and are represented as *, P < 0.05; **, P < 0.01; ***, P < 0.0005.

transcription of Group 1 genes in an inducible fashion, including the expression of *NEGR1*, *UCA1*, and *CTGF* (**Figure 4.15A**). Nuclear TAZ or YAP expression also induced the expression of Group 2 genes (e.g. *OCLN* and *CYFIP2*), whereas TGFβ repressed this group of genes (**Figure 4.15B**). Group 4 genes on the other hand, specifically *LBH* and *PMEPA1*, were induced by TGFβ, but repressed in an inducible fashion by nuclear TAZ or YAP (**Figure 4.15C**). Intriguingly, Group 3 genes were undetectable in MCF10A cells, which may reflect the more differentiated state of these cells compared to LM2-4 cells. Together, my data indicate that the relationship between YAP/TAZ and TGFβ is conserved in mammary-derived cells, and my observations support the idea that dysregulated YAP/TAZ and TGFβ work in concert to control transcriptional events.

Nuclear TAZ and YAP overcome TGFβ-induced cytostasis in non-tumorigenic cells

A hallmark trait of TGFβ is its ability to suppress tumorigenesis in normal epithelium and early stage cancers, particularly through cell cycle inhibition. However, TGFβ signals lose their ability to induce cytostasis in late stage cancers via poorly understood mechanisms (146,147). Given that TGFβ-induced cell cycle arrest has been previously described in MCF10A cells (232), I sought to explore the relationship between TGFβ, nuclear YAP/TAZ, and cell cycle progression. I performed proliferation assays using control MCF10A cells or cells with doxycycline-inducible nuclear TAZ(4SA) or YAP(5SA) expression. TGFβ-induced cytostasis was evident in control MCF10A cells (**Figure 4.16**). Strikingly, I found
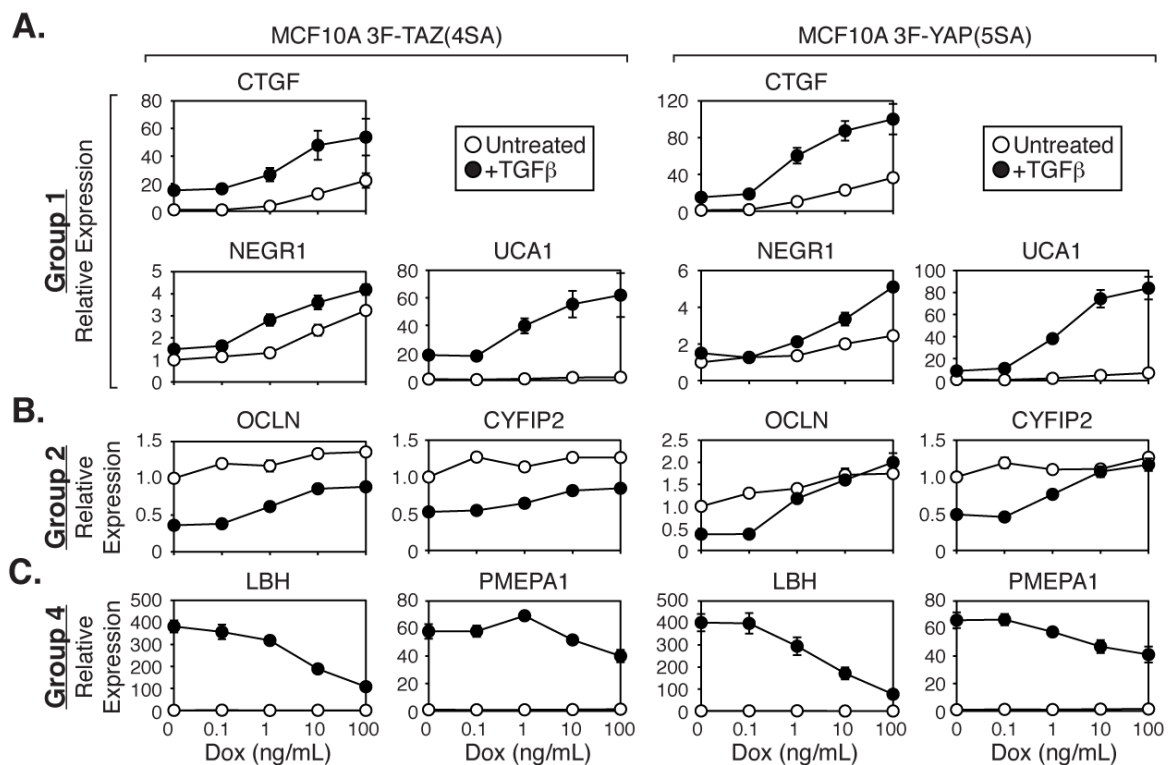
Figure 4.15 - Nuclear TAZ and YAP cooperate with TGFβ to regulate target gene transcription. Doxycycline (Dox)-inducible MCF10A cells expressing 3xFLAG-TAZ(4SA) or 3xFLAG-YAP(5SA) were treated with increasing levels of doxycycline with or without TGFβ for 24 hours. Relative expression of Group 1 genes (**A**), Group 2 genes (**B**), and Group 4 genes (**C**) were analyzed by RT-qPCR and are shown as the average of three independent experiments (±SE).
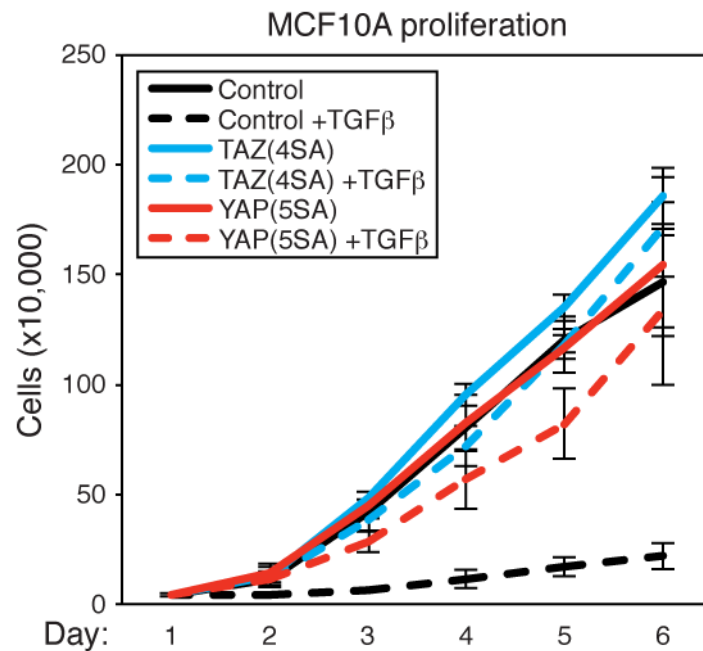
Figure 4.16 - Nuclear TAZ and YAP overcome TGFβ-induced cytostasis.
Doxycycline-inducible MCF10A control cells or cells expressing 3xFLAG-
TAZ(4SA) or 3xFLAG-YAP(5SA) were treated with doxycycline with or without
TGFβ. Cells were counted over 6 days to measure their proliferative capacity. Cells
from three experiments were counted and the average (±SE) for each day is
shown. This experiment was performed in collaboration with Aleks Szymaniak.

that expression of TAZ(4SA) or YAP(5SA) overcomes TGFβ growth arrest, as cells treated simultaneously with doxycycline and TGFβ proliferated similarly to control cells (**Figure 4.16**). To investigate whether the proliferative differences were due to cell cycle alterations, I used FACS to examine the DNA content of these cells. I found that TGFβ treatment arrests cells in the G1 phase of the cell cycle, while TAZ(4SA) or YAP(5SA) expression rescues the TGFβ induced G1 phase arrest (**Figure 4.17A and 4.17B**). Thus, my data suggest that nuclear YAP/TAZ are responsible for the switch in TGFβ activity from tumor suppressive to tumorigenic in later stage breast cancers by converging to direct a distinct transcriptional program (see model in **Figure 4.18**).

Discussion

I have found YAP/TAZ to be necessary for transduction of TGFβ-induced tumorigenic phenotypes in metastatic breast cancer cells, such as clonal anchorage-independent growth, cell migration, and invasion. Interactions between endogenous YAP/TAZ, TEAD, and SMAD2/3 in the nucleus suggest that these complexes coordinate their activities at the transcriptional level. Through genome-wide expression analysis I show that YAP/TAZ, TEAD, and TGFβ regulate individual and common gene targets both positively and negatively, implying a complex level of transcriptional regulation and crosstalk between these factors. Of those gene targets I identified, many have yet to be characterized in breast cancer and therefore my work highlights previously unrecognized factors contributing to
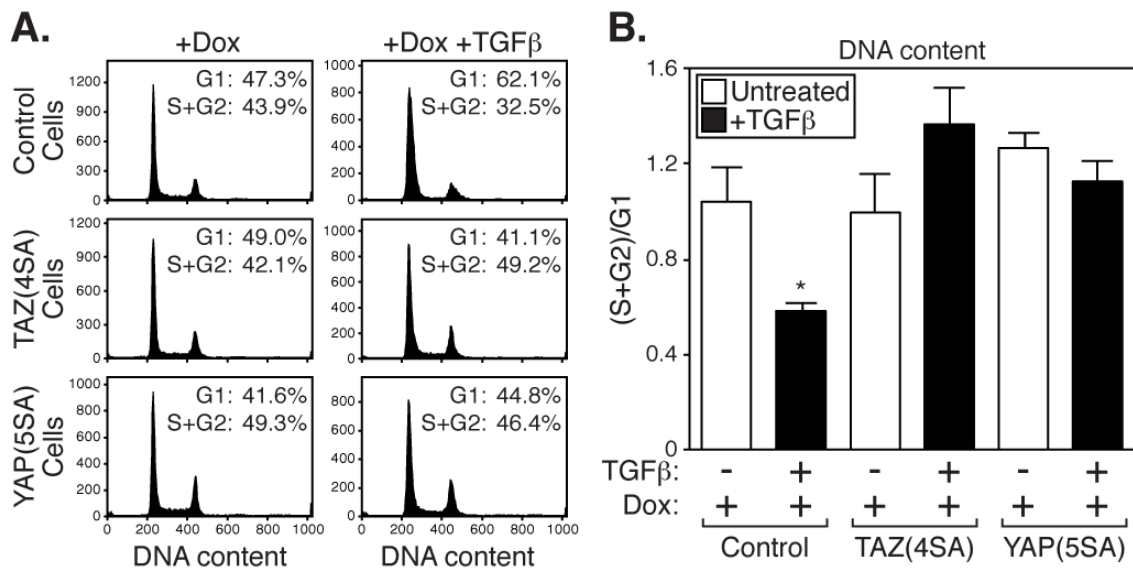
Figure 4.17 - Nuclear TAZ and YAP overcome TGFβ-induced cell cycle arrest.
Doxycycline (Dox)-inducible MCF10A control cells or cells expressing 3xFLAG-TAZ(4SA) or 3xFLAG-YAP(5SA) were treated with doxycycline with or without TGFβ. Cells were subject to propidium iodide staining and flow cytometry analysis to determine DNA content. Data from a representative experiment are shown (A) and cell cycle phase quantitation is represented as the ratio of cells in S+G2 to cells in G1 (B). The average of three independent experiments (+SE) is shown. Statistics were performed using an unpaired Student t test and are represented as *, P < 0.015.
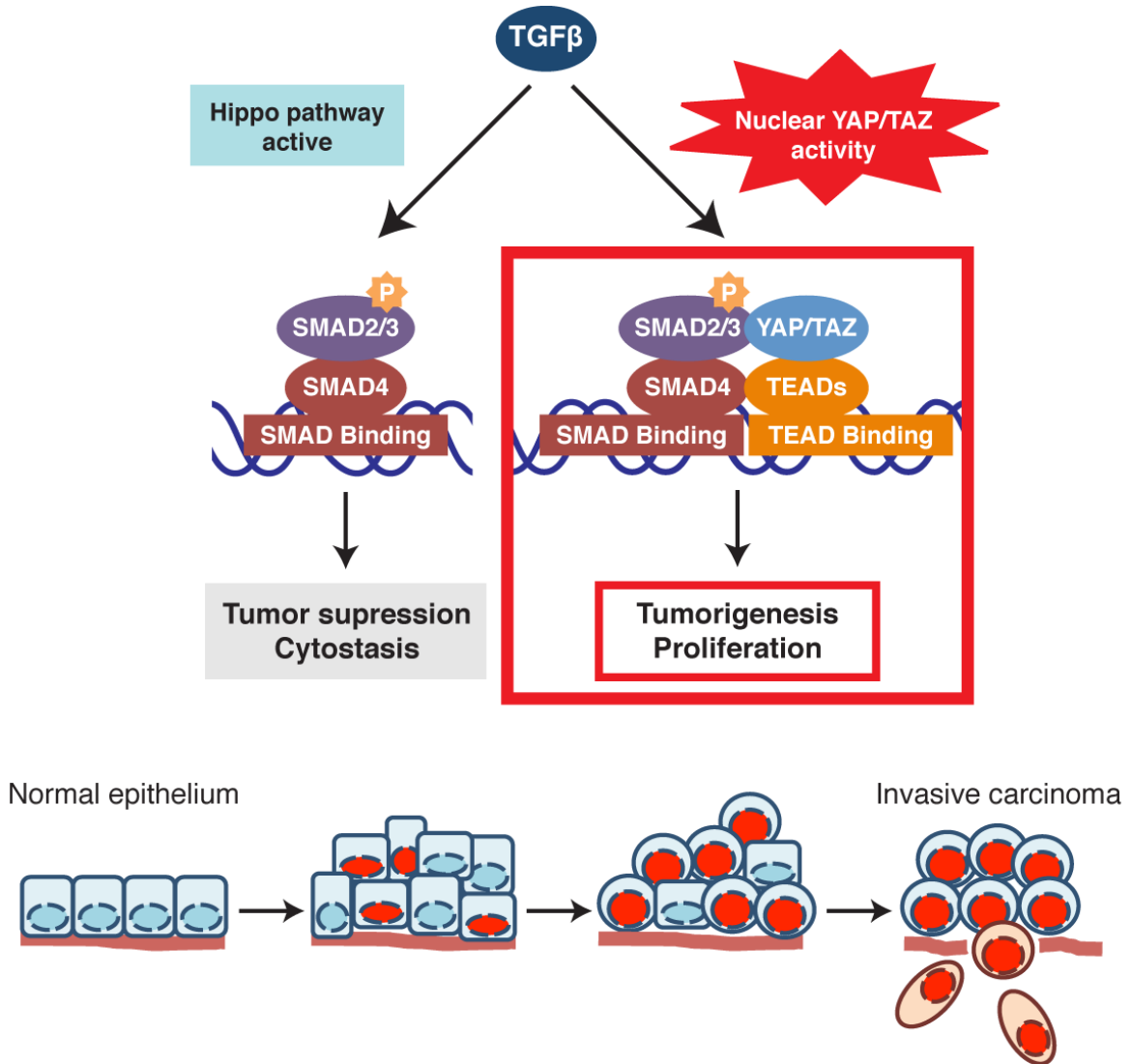
Figure 4.18 - Model for how YAP/TAZ direct TGFβ-induced tumorigenic events. I propose that increased nuclear YAP/TAZ, resulting from defects in upstream Hippo pathway signals, overcome TGFβ-mediated tumor suppressive functions (e.g. cytostasis) and concomitantly drive tumorigenic transcriptional events by promoting the activity of TEAD-SMAD complexes.

tumorigenesis. Of note, EMT-related genes were not enriched among the overlapping YAP/TAZ-TEAD-TGFβ regulated subset, indicating that the YAP/TAZ-TEAD-SMAD2/3 complex drives aggressive behaviors of metastatic breast cancer cells downstream from the loss of epithelial cell polarity. My transcriptional signature may thus reveal insight into the YAP/TAZ-mediated tumorigenic program occurring in late-stage cancers, as MDA-MB-231 cells and their LM2-4 derivatives possess mesenchymal properties. Indeed, the two genes that I characterized, *NEGR1* and *UCA1*, proved to be necessary for the anchorage-independent growth and migratory properties of LM2-4 cells. YAP/TAZ and TGFβ synergistically induce the expression of *NEGR1* and *UCA1* (Group 1 genes), and given that YAP/TAZ, TEADs, and SMAD2/3 are enriched at the promoters of these genes, direct transcriptional synergy between YAP/TAZ-TEAD-SMAD complexes likely promotes their expression in breast cancer.

Out of the 80 genes co-regulated by YAP/TAZ, TEAD, and TGFβ, 21 of them encode membrane proteins, several of which function as cell surface receptors, and 13 of them encode secreted proteins. The enrichment of such genes may reflect important non-cell-autonomous alterations that are regulated by YAP/TAZ-TEAD and TGFβ signals. Such signals are important for the pro-tumorigenic activity of TAZ and YAP (233,234), and thus I propose that crosstalk between YAP/TAZ-TEAD and TGFβ signals demarcate a distinct local cellular environment that may promote a tumor-initiating niche. The well-documented YAP/TAZ-TEAD target *CTGF* encodes for a secreted factor that is cooperatively

induced by TGFβ. *CTGF* is a well-established target of TGFβ-activated SMAD2/3 transcription factors (235), but also an important driver of YAP/TAZ-induced tumorigenic events (12,67). I observe that *CTGF* expression relies on the presence of YAP/TAZ, TEADs, and TGFβ signaling, and nuclear TAZ or YAP mutants synergize with TGFβ to strongly induce *CTGF* expression. Therefore, as in malignant mesotheliomas (128), the synergistic regulation of the *CTGF* promoter likely promotes aggressive breast cancer phenotypes.

I have additionally identified genes that are activated by both TAZ and YAP, but repressed by TGFβ signaling (Group 2 genes), and reciprocally, genes repressed by YAP/TAZ, but induced by TGFβ (Group 4 genes). These groups of genes were somewhat surprising, as they indicate that YAP/TAZ and TGFβ direct opposing transcriptional events, and therefore suggest that a subset of TGFβ-activated SMAD activity does not rely on YAP/TAZ, and vice versa. Based on the products encoded by several of these genes, I speculate that nuclear YAP/TAZ may override tumour-suppressive or negative feedback mechanisms initiated by TGFβ. For example, *PMEPA1*, which I found is induced by TGFβ and inhibited by YAP/TAZ (Group 4 gene), encodes a transmembrane protein that sequesters SMAD complexes in the cytoplasm (236). Thus, nuclear YAP/TAZ may function to overcome the induced expression of this gene to sustain pro-tumorigenic TGFβ signals.

Historically TAZ and YAP have been considered to be activators of gene transcription. However, my data indicate that YAP/TAZ play repressive roles as

well (Group 3 and 4 genes). I hypothesize YAP/TAZ-TEAD complexes execute this repressive function by various means. Recent work has shown that YAP/TAZ recruit the nucleosome remodeling and deacetylation (NuRD) complex to repress gene expression (127). Yorkie (Yki), the homolog of YAP/TAZ in *Drosophila melanogaster*, is also known to associate with chromatin-modifying proteins (237,238). Thus, YAP/TAZ-TEAD complexes likely function directly to inhibit transcription in breast cancers through similar recruitment of repressive factors to control local chromatin remodeling at promoters. However, YAP/TAZ-TEAD complexes may also function in an indirect manner, particularly in conjunction with TGFβ signaling, by binding and re-localizing SMAD complexes (70,126). SMAD redistribution by YAP/TAZ may explain why nuclear TAZ or YAP affects the expression of certain target genes (Group 2 and 4) more dramatically in MCF10A cells in the presence of TGFβ. Moreover, YAP/TAZ binding to SMADs is evident in the nucleus and in the cytoplasm (**Figure 4.8A**), suggesting that interactions between these proteins in different localizations may direct distinct events.

Of interest, nuclear TAZ or YAP is capable of overcoming TGFβ-induced cytostasis (**Figure 4.16 and 4.17**), which is a major mechanism by which TGFβ functions as a tumor suppressor in early stage cancers (147). Consistent with this, I find that constitutively nuclear YAP/TAZ is evident in breast cancer cell lines where TGFβ has lost its ability to induce cytostatic signals (**Figure 4.1**). YAP/TAZ drive the expression of cell cycle regulators (102), which may account for the ability of these factors to overcome cell cycle arrest. Indeed, my gene expression analysis

in LM2-4 cells identified several cell cycle regulators as YAP/TAZ-regulated genes (e.g. *CDKL1*, *CCNA1*, *CCNB1*, *CCND3*). However, given that YAP/TAZ bind SMAD complexes, I also speculate that YAP/TAZ may be capable of redirecting TGFβ-induced SMADs away from their cell cycle-repressive transcriptional roles towards those that promote tumorigenesis.

My phenotypic and transcriptional analysis revealed redundant functions for TAZ and YAP. For example, TAZ and YAP have redundant roles in mediating TGFβ-induced mammosphere formation. Additionally, TAZ and YAP redundantly regulate the expression of Group 1 genes *NEGR1* and *UCA1* (**Figure 4.9C**). Interestingly, TAZ knockdown alone led to increases in *UCA1* expression, which may reflect compensatory YAP hyperactivity in this context. A redundant role for these factors is further implied on account of similar effects resulting from nuclear TAZ or YAP mutant expression in MCF10A cells. Such redundancy is consistent with the overlapping roles of YAP/TAZ in early development (239). However, I also present evidence for divergent transcriptional activity, based on specific gene expression reliance on either TAZ or YAP exclusively. For example, the expression of CTGF was repressed by TAZ or YAP/TAZ knockdown in LM2-4 cells, but not by YAP knockdown alone (**Figure 4.9C**). Thus, TAZ appears to have a dominant role in regulating *CTGF* expression in LM2-4 cells. Interestingly, recent work has revealed that YAP, in cooperation with TGFβ, has critical roles in controlling the expression of *CTGF* in malignant mesotheliomas (128). Thus, it appears that context defines dominance of TAZ or YAP.

Effective treatments of late-stage breast cancers are lacking, and our current understanding of the important signals driving and maintaining proliferation and metastasis is unclear. My work has revealed critical intersections between YAP/TAZ, TEAD, and TGFβ signaling in directing pro-tumorigenic phenotypes in breast cancer, and provides novel mechanisms by which the TGFβ program may be directed towards aggressive tumorigenic phenotypes. Given the well-documented roles of TGFβ in late-stage cancers, recent efforts have been focused on optimizing new TGFβ signaling inhibitors, which are currently in pre-clinical and clinical trials (240). While advancement with such treatments is logical, my work suggests that enhanced efficacy may be achieved by treatment or co-treatment with current (119), or future YAP/TAZ-TEAD inhibitors.

CHAPTER V - FUTURE DIRECTIONS, GENERAL DISCUSSION, AND CONCLUSION

YAP/TAZ activated and repressed gene signatures

In these studies, I have shown that YAP/TAZ drive pro-tumorigenic signals in OSCC and cooperate with TGFβ signaling to promote aggressive breast cancers. My work has also identified transcriptional programs associated with YAP/TAZ activity in both contexts. Comparing these signatures may shed light on the actions of YAP/TAZ in different cancers. In SCC2 cells, the activated gene signature is enriched for proteins involved in cell cycle regulation (**Figure 5.1**). However in LM2-4 cells, although there were a few cell cycle-regulating genes affected by YAP/TAZ knockdown (such as *CCND3*), the signature was not enriched for these targets. Rather, genes important for glucose, lipid, and sterol metabolic processes were enriched (**Figure 5.1**). Both aberrant cell cycle progression and metabolic regulation are hallmarks of cancer, and therefore YAP/TAZ may direct these processes differently in various contexts (5,6). These differences in YAP/TAZ transcriptional regulation may also reflect the distinct metastatic states of the SCC2 and LM2-4 cells, as the SCC2 cells originate from a primary tumor while the LM2-4 cells are from a metastasized lung colony. Interestingly, simvastatin, a statin, has recently been shown to inhibit YAP/TAZ nuclear activity (241,242). Simvastatin has been associated with lower breast cancer reoccurrence and was effective in reducing the pro-tumorigenic effects TAZ has on OSCCs *in vitro* and *in vivo* (243,244). This suggests different therapeutic
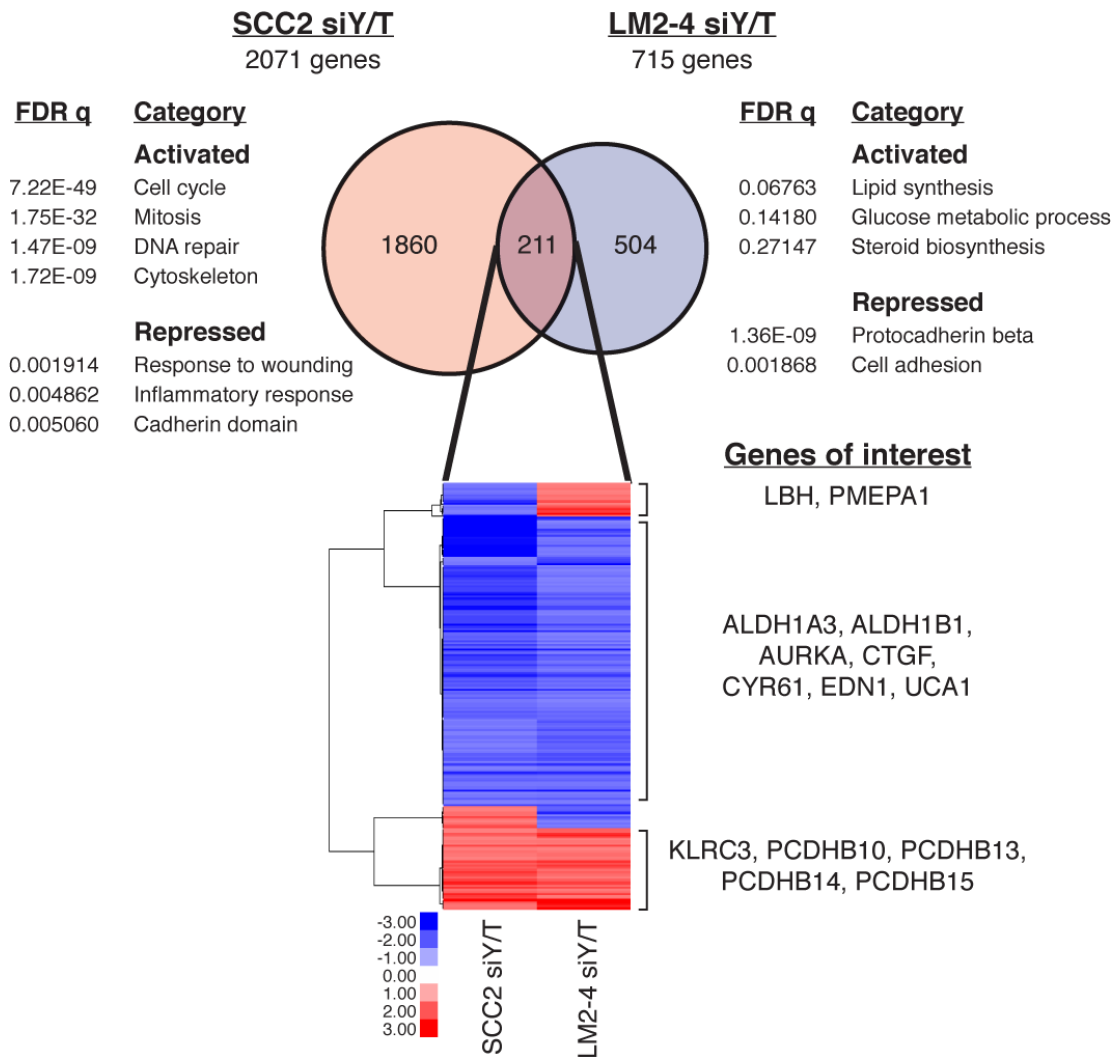
Figure 5.1 - Comparison of YAP/TAZ-regulated genes in OSCC and breast cancer. The Venn diagram highlights the number of genes with significant expression changes (genes with an FDR q-value of ≤0.05 and fold change ≥1.5 compared with the respective control) that were identified from the microarray studies performed in Figure 3.8 (OSCC; SCC2 siYAP/TAZ) and Figure 4.9 (Breast cancer; LM2-4 siYAP/TAZ +TGFβ). Enriched gene categories are shown for the activated and repressed signatures from SCC2 siY/T and LM2-4 siY/T microarrays. Hierarchical clustering was performed on the overlapping signature, which revealed the majority of the genes are regulated in the same manner by YAP/TAZ (e.g. gene expression goes down in both SCC2 and LM2-4 samples). Genes of interest are highlighted to the right for several of the clusters.

options may be available in the treatment of YAP/TAZ associated tumors and that it may be beneficial to stratify patient populations based on the YAP/TAZ transcriptional signature to determine if the most appropriate therapy is a broad cell cycle inhibitor or a statin.

Although there are clear differences in YAP/TAZ activated genes, some similarities are seen in the repressed gene signatures. Cell adhesion proteins were enriched for in both microarrays, particularly several protocadherins (PCDH) (**Figure 5.1**). Regulation of cell adhesion is complex in cancer progression, but changes in membrane bound proteins are associated with invasion and metastasis and can effect both intercellular communication as well as intracellular signaling pathways (245). For example, hypermethlyated *PCDH* clusters and reduced *PCDH* expression are associated with pediatric kidney tumors. These PCDHs function to suppress tumor growth *in vitro* partially through the inhibition of WNT signaling (246). The repression of these *PCDH* genes may reflect the control YAP/TAZ has not only over invasive properties via cell-cell contacts but may also add another layer of crosstalk with oncogenic signaling pathways. In addition, two cadherin-related proteins, Fat and Dachsous, are known to lie upstream in the *Drosophila* Hippo pathway to restrict Yki activity and homologs FAT and DCHS have been shown function similarly to direct YAP/TAZ activity in mammals (247-249). Interestingly, changes in *FAT* expression have been associated with many different types of cancer (250). In particular, loss of *FAT1* occurs in 80% of primary oral cancers and functions to suppress breast cancer progression (90,251,252).

Deregulation often occurs at the genomic level through either homozygous deletion or promoter hypermethylation. Although I did not observe changes in *FAT* or *DCHS* expression in SCC2 or LM2-4 cells, perhaps another protocadherin is responsible for regulating YAP/TAZ activity in this context. If this is true, it could illustrate a feed forward mechanism YAP/TAZ use to repress upstream Hippo pathway components resulting in increased nuclear YAP/TAZ activity and unrestrained signaling. Notably, only 20% of these protocadherin genes repressed by YAP/TAZ were the same between SCC2 and LM2-4 cells (4 genes out of 20), which suggests these overlapping targets may also be important in other situations and further demonstrates context dependent roles for YAP/TAZ.

Similarly, there was only 8% overlap in the YAP/TAZ differentially regulated genes in SCC2 and LM2-4 cells (211 overlapped out of 2575 total genes) (**Figure 5.1**). Of these overlapping genes, 87% were regulated in the same way by YAP/TAZ (e.g. expression went down with knockdown in both microarrays). Identical genes regulated by YAP/TAZ in SCC2 and LM2-4 cells were not enriched for any particular function, although several interesting genes I have previously discussed are highlighted in **Figure 5.1**. Together, this indicates that although the roles for YAP/TAZ can be broadly defined as controlling proliferation, migration, and metastasis, the specific transcriptional functions may be context dependent.

The role of YAP/TAZ in the tumor microenvironment

Angiogenesis in YAP/TAZ driven tumors

Angiogenesis, or the formation of blood vessels, is a critical event in tumor formation and metastasis and is considered to be a hallmark of cancer (5,6). These blood vessels not only provide a source of nutrients to the primary tumor, they also serve as a route by which cells can metastasize to distant sites. During the growth and development of blood vessels, various angiogenic factors are secreted (253). In my transcriptional analyses, several genes encoding proteins known to induce angiogenesis were found as regulated by YAP/TAZ: *CTGF*, *CYR61*, and *EDN1* (254-256). These factors have been previously described as targets of YAP/TAZ and YAP is necessary for the induction of *CTGF* and *EDN1* expression by TGFβ in mesotheliomas (67,74,128). This indicates that there are common targets that functionally regulate progressive tumor properties.

Other targets of YAP/TAZ in SCC2 cells may also control angiogenesis. *CXCL10* and *CXCL14*, repressed by YAP/TAZ, encode for cytokines known to inhibit angiogenesis (257,258). *CXCL14* is also absent from tongue SCCs (257). Interestingly, several *MMP* genes were found to be repressed by YAP/TAZ and may contribute to the complex roles MMPs play in cancer progression (259). Generally MMPs are thought to promote invasive properties by remodeling the ECM to allow cells to migrate and can activate pro-angiogenic factors like TGFβ and FGF (260). However, they can also promote the processing of anti-angiogenic factors such as angiostatin (from plasmin/plasminogen) and endostatin (from

collagen XVIII) (259-261). MMP12 inhibits angiogenic properties of endothelial cells and vascularization (262). Contrasting effects of MMPs have also been observed in different contexts. For instance, MMP10 is important in the invasion and metastasis of head and neck cancers but its expression decreases in breast cancer (263,264). I see *MMP10* repressed by YAP/TAZ in both OSCC and breast cancer. Although MMPs are generally thought to promote cancer progression, their tumor suppressive roles have also been revealed. MMP8 can prevent metastasis in melanoma and lung cancers and although MMP9 promotes breast cancer, it functions as a tumor suppressor in the colon by promoting Notch1 activation to suppress β-catenin signaling (265-267). These varying effects may explain why MMP inhibitors have not worked well as therapeutics (268). They may also explain why YAP/TAZ can function to repress *MMP* expression in oral and breast cancers and suggest that MMPs may play a tumor suppressive role in SCC2 and LM2-4 cells.

Although YAP/TAZ are known to control the expression of angiogenic factors, their functional ability in tumors to promote angiogenesis has not been well studied, particularly in cancer cell populations. YAP has been shown to be necessary in endothelial cells during development and in CAFs to promote angiogenesis in the tumor microenvironment (269,270). It would be interesting to investigate this in the orthotopic model to see if YAP/TAZ knockdown limits the size of the primary tumor and metastasis by inhibiting blood vessel formation.

YAP/TAZ in matrix stiffening and intercellular crosstalk

The tumor microenvironment is a crucial component in the progression of cancer, including the rigidity of the ECM (271). Fibrotic characteristics, such as stiffening of the ECM through increased collagen deposition and fiber crosslinks, are known to correlate with tumor progression (272,273). In addition, a rigid microenvironment promotes nuclear YAP/TAZ activity in normal and cancer-associated fibroblasts, which can further promote stiffening through collagen production and crosslinking via *LOX* up-regulation, creating a feed-forward loop (73,269). Nuclear YAP/TAZ have also been implicated in lung fibrosis and can overcome the limitations of a soft matrix to promote fibroblast activity (274). In SCC2 cells, YAP/TAZ promote the transcription of *COL12A1* as well as *PLOD2* and *LOXL2*, which function to crosslink collagen and elastin fibers thereby stiffening the ECM. YAP/TAZ also repress the transcription of *MMP* genes, which may function beyond angiogenesis as discussed above, and may play a role in the softness of the ECM by degrading collagen and elastin fibers.

In addition to matrix stiffness, many secreted factors may signal back and forth between cancer cells and CAFs (**Figure 5.2**). Although the sources of CAFs in the tumor microenvironment are not well understood, TGFβ signaling can promote a CAF-like phenotype in fibroblasts (275). TGFβ can also stimulate CAFs to secrete factors that signal to cancer cells to promote their activity (170-172). Similarly, secreted factors from cancer cells can signal and activate fibroblasts to create a feed-forward mechanism and promote an aggressive tumor
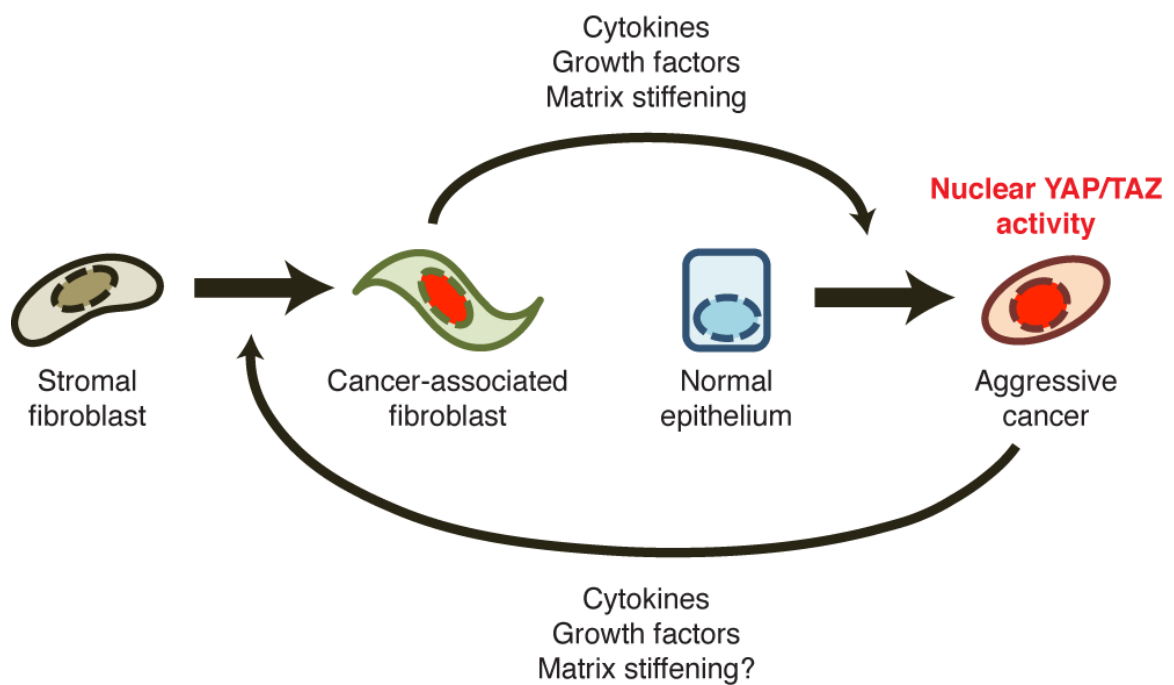
Figure 5.2 - Crosstalk between cancer-associated fibroblasts and cancer cells. Cancer-associated fibroblasts and cancer cells can signal to each other through secreted factors and mechanical cues to promote aggressive phenotypes associated with nuclear YAP/TAZ activity.

microenvironment. Candidate signaling molecules include CTGF and CYR61, which may also promote angiogenesis in addition to transformed cancer cells and CAFs. Intercellular communication within the microenvironment has not been well characterized in the context of YAP/TAZ and further studies are necessary to better understand the signaling mechanisms.

YAP/TAZ crosstalk with oncogenic signaling pathways in OSCC

YAP/TAZ modulation of TGFβ signaling

My work shows YAP/TAZ to be necessary for transduction of TGFβ-induced tumorigenic phenotypes in metastatic breast cancer cells (**Figure 4.18**). In addition, I have found several TGFβ targets to be regulated by YAP/TAZ in SCC2 cells (*CTGF* and *CYR61*). YAP/TAZ may also control TGFβ signaling in this context through the expression of a TGFβ signaling mediator, Thrombospondin1 (*THBS1*). THBS1 promotes TGFβ signaling by activating latent TGFβ complexes (276). In this way, nuclear YAP/TAZ may potentiate TGFβ signaling to promote aggressive phenotypes. Recently, TAZ was found to be necessary for TGFβ-induced EMT events and to promote CSC populations in OSCC, similarly to what I have observed in breast cancer (243). This suggests that crosstalk between YAP/TAZ and TGFβ signals may be a common occurrence in different cancer types and may be necessary in maintaining a CSC niche within the tumor. It would also be insightful to examine the role of TGFβ in the context of YAP/TAZ-mediated angiogenesis and ECM remodeling to determine if TGFβ and YAP/TAZ function synergistically to promote other hallmarks of cancer.

YAP/TAZ modulation of EGFR and WNT signaling

YAP/TAZ can interact with other oncogenic signaling pathways besides TGFβ including EGFR-MAPK and WNT, although little is known about their crosstalk in the context of cancers. In oral cancer, *EGFR* expression correlates with progression and poor prognosis (277). In *Drosophila*, Yki is necessary for EGFR induced cell proliferation and YAP knockdown sensitizes cancer cells to erlotinib, an EGFR tyrosine kinase inhibitor (278,279). EGFR also controls β-catenin localization and activity in oral cancer to promote cell cycle progression (280). I show that many of the genes induced by YAP/TAZ in SCC2 cells are cell cycle regulators and YAP/TAZ knockdown inhibits proliferation in these cells. Since YAP is known to control β-catenin localization and activity in other systems, it is possible that EGFR is signaling through YAP/TAZ and β-catenin to control cell cycle progression in SCC2 cells through the expression of *CCND3*, *CCNE2*, and *CCNF* (120,121). This may also explain why high nuclear YAP/TAZ confer chemoresistance to cetuximab, another EGFR inhibitor (97). Thus, YAP/TAZ may function downstream of EGFR and/or WNT and similar to TGFβ signaling, these signals may be dependent on nuclear YAP/TAZ activity to promote aggressive cancers.

Redundancy of YAP/TAZ

Redundancy exists between YAP and TAZ, which likely results from their striking homology and structural similarities (**Figure 1.3**) (69). My data support redundant functions for YAP and TAZ in both OSCC and breast cancer, as

knockdown of both YAP/TAZ always had a greater effect than knockdown of YAP or TAZ alone. Interestingly, I also found differential roles for YAP and TAZ, both in functional assays and in the transcriptional regulation of target genes because one generally had a greater effect than the other. In SCC2 cells, YAP has a dominant effect over TAZ while in LM2-2 cells the reverse is observed. Although YAP and TAZ are both expressed in each, this could reflect relative levels of YAP and TAZ in different cells. For instance, higher levels of YAP are seen in SCC2 cells compared to TAZ (**Figure 3.5A**). This could also result from other differences in YAP/TAZ interacting partners between these cells and subsequently the way each is regulated, similarly to the differences described between YAP1-1 and YAP1-2 isoforms binding to p73 and angiomotin (69,79,80). Perhaps this is also why different activated gene signatures are observed in the SCC2 and LM2-4 cells discussed above. LM2-4 cells have high levels of TAZ and recently CAL27 cells were shown to be responsive to simvastatin treatment, cells in which I have observed high levels of TAZ (data not shown). On the other hand, SCC2 cells have high levels of YAP and an associated cell cycle transcriptional signature. Perhaps TAZ is responsible for an aberrant metabolic profile while YAP is responsible for unrestrained cell cycle activation. However at this point it is unclear how YAP and TAZ are differentially regulated in various cancer contexts. Elucidating this regulation will lead to a better understanding of the individual and redundant roles for YAP and TAZ and how they interact with other signals, which hopefully will aid

in stratifying patient tumor populations to determine appropriate therapeutic treatments.

Summary of key findings and future directions

Uncontrolled nuclear YAP/TAZ activity is known to promote aggressive tumorigenic phenotypes in a range of epithelial cancers (59,90). While YAP/TAZ are necessary for accurate tissue patterning during development, it is unclear how dysregulated YAP/TAZ direct transcription to induce tumorigenesis and how they interact with other signaling pathways during this process. Understanding the molecular mechanisms of nuclear YAP/TAZ is essential in the development of effective targeted therapeutics.

In this thesis I present two distinct but related works examining the roles of YAP/TAZ in the initiation and progression of cancer. In Chapter III I asked: what are the actions of nuclear YAP/TAZ in oral cancers? My observations indicated that dysregulated YAP localization is an early event in the initiation of OSCC and that nuclear YAP/TAZ is necessary to drive pro-tumorigenic phenotypes both *in vitro* and *in vivo*. I also defined a YAP/TAZ-regulated transcriptional signature that correlates with changes occurring in human OSCCs. In Chapter IV I asked: do YAP/TAZ and TGFβ-induced signals cooperate to promote aggressive breast cancers and how? I found that YAP/TAZ are required for TGFβ-induced tumorigenic phenotypes and that these signals converge at the transcriptional level to control gene expression. I also defined a YAP/TAZ-TEADs-SMAD2/3-regulated transcriptional signature and identified two novel direct gene targets of YAP/TAZ-

TEADs-SMAD2/3 complexes, *NEGR1* and *UCA1*, which are necessary to maintain TGFβ-induced tumorigenic phenotypes. Further, I showed that nuclear YAP/TAZ overcome TGFβ-induced growth arrest in early cancers. Together these data indicate that nuclear YAP/TAZ can mediate the shift in TGFβ response from tumor suppressive in early stages to tumor promoting in late stage cancers.

In both Chapter III and Chapter IV, I observed that nuclear YAP/TAZ are essential to maintain and promote tumorigenic phenotypes in two different origins of cancer. I also focused on identifying YAP/TAZ-associated transcriptional signatures and in Chapter V, I have compared these signatures to offer insight into the context-dependent transcriptional roles of nuclear YAP/TAZ. Although YAP/TAZ are broadly required to induce tumorigenic phenotypes, their specific transcriptional functions vary greatly depending on context.

Additional questions remain regarding the role of YAP/TAZ in cancer initiation and progression. Particularly, it would be helpful to gain a better understanding of the context-dependent transcriptional events - do YAP/TAZ have a specific signature in every origin of cancer? What about their roles in early versus late stage tumors of the same origin? YAP/TAZ can interact with other signaling pathways and I have uncovered a relationship with TGFβ signaling in breast cancer. Investigating YAP/TAZ convergence with TGFβ and other signaling pathways across many types of cancers would be useful in understanding the molecular mechanisms responsible for tumorigenesis. Of the few overlapping YAP/TAZ-regulated gene targets, several encode for proteins that regulate

intercellular and extracellular events. These proteins could give insight into the communication occurring in the tumor microenvironment. It would also be interesting to better characterize the role of YAP/TAZ in non-epithelial derived cells in the tumor, particularly the CAF population. The last question I would like to propose is how is YAP/TAZ localization disrupted to promote their nuclear activity? Mutations in Hippo pathway components are not commonly found in cancers (90,105,106). Perhaps dysregulation occurs through the disruption of polarity complexes and upstream kinase regulation, or through mutations in other signaling pathways that can effect YAP/TAZ localization. In either case, a better understanding of both the downstream transcriptional events and the upstream regulation of YAP/TAZ localization will aid in identifying the most effective therapeutic targets.

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