YAP Regulation of RHO GTPase Activities Controls Epithelial Cell Morphology and Adhesion

A dissertation presented

by

Natalie Marie Hendrick

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

February 2019

Dissertation Advisor: Professor Joan S. Brugge Natalie Marie Hendrick

YAP Regulation of RHO GTPase Activities Controls

Epithelial Cell Morphology and Adhesion

Abstract

Yes-Associated Protein 1 (YAP) is the downstream effector of the Hippo pathway. Hippo is a kinase cascade that regulates development and tissue growth through phosphorylation of YAP and regulation of its localization. Nuclear YAP functions as a transcriptional coactivator with TEAD, regulating genes involved in tissue growth and homeostasis; when Hippo is active, YAP transcriptional activity is suppressed and YAP is localized to the cytoplasm. Previous studies from our lab have shown that loss of YAP perturbs cytokinesis in mammary epithelial cells, impairing membrane integrity and promoting genetic instability. In this thesis, we investigated the role of YAP in interphase epithelial cells.

We observed that YAP knockdown in the MCF10A cell line promotes protrusive activity from acini in reconstituted basement membrane cultures. Similar protrusive behavior was observed by timelapse microscopy in monolayer culture, where lamellipodia formation increased. Timelapse microscopy also revealed that YAP knockdown impaired collective cell migration; cells dissociated and migrated individually instead of as a single sheet. This altered migratory behavior was associated with impaired junctional maturation. YAP knockdown affected the ratio of active RHO and RAC GTPase activities; RHOA activation was significantly decreased, and RAC

activation was increased. Downstream of RHO, cellular contractility was also decreased in YAP knockdown cells.

Interestingly, we found that expression of YAP mutants defective for transcriptional activity, such as TEAD-binding mutant YAP, or cytoplasmically-localized phosphomimetic YAP, was sufficient to restore wildtype acinar structure and cell contractility in cells depleted of YAP, suggesting that this function of YAP is independent of its transcriptional activity. Our findings suggest that cytoplasmic YAP plays an important role in regulating cell-cell adhesion and membrane protrusion when the Hippo pathway is active, and that alterations in cell behavior caused by translocation of YAP to the nucleus following Hippo inactivation can be mediated in part through loss of cytoplasmic YAP. This thesis highlights a role of YAP in maintenance of cellular morphology and reveals a novel function of YAP that is consistent with its role as a tumor suppressor.

Table of Contents

Acknowledgments

First and foremost, I want to start by thanking my PI and mentor, Joan. Thank you for your continuous support, patience, and advice on this PhD journey with me. I have grown so much as a scientist and a person under your tutelage.

Thank you to those who have helped me in one way or another with YAP over the years: to Taru, for being available whenever I wanted to discuss YAP biology; to Marcin, for suggesting I film the knockdown cells and essentially inspiring the protrusivity project; to Richard, for teaching me how to do cloning and sharing your cytoskeletal expertise; to Amy, for all the YAP-mitosis studies that laid the foundation for this project; to Carman, for being a sounding block for my ideas and providing insight into invasive biology; to Clarence and the IDAC for generating countless MATLAB codes for the migration movies; to the Nikon Imaging Center for teaching me how to do microscopy. I am indebted to you all for your help. To those that helped with the immediate early response project, particularly Lisa, Laura, and Dan, thank you all.

To my dissertation advisory committee, Alex Toker, Myles Brown, and Fernando Camargo, thank you for listening to me talk about YAP biology for seven years, for offering your advice and suggestions, and for your encouragement both when work was rough and when it was progressing. To the BBS office (past and present)—Kate, Maria, Danny, and Anne—thank you for your support and guidance while navigating the PhD. To all my BBS friends, for the laughs, camaraderie, and commiseration, thank you.

To my past scientific mentors, Angelika from MIT and Sandra from Hermitage, thank you. I feel so fortunate to have surrounded myself with strong examples of women in science my entire life.

A true testament to Joan's character is the quality of people that comprise the Brugge lab. I consider you all to be my friends. To Pragya for introducing me to Zumba, changing my life, and sticking with me through thick and thin, thank you. To Patricia, for always listening to me complain about Westerns, for waiting for me in lab until 10 PM when I had a rough day, for rocking out to Taylor Swift with me in TC, and for being such a good friend—thank you. To Johan, for making me laugh and listening to me talk about puppies all the time, thank you. To Kenneth, for your fudge, your impressions, and being my American comrade, thank you. To Jason, for introducing me to Turning Leaf, for our political discussions, and for your gentle friendship, thank you. You all are my Boston family.

To my parents, who raised me to be independent and always emphasized the only constant asset one has is education, who sacrificed to give me the best childhood they could, and for their unconditional love, thank you. To Jake, who supported me and pushed me to finish this—thank you. I hope I make you proud, buddy. A la mia famiglia italiana, Osvaldo, Grazia, e Marta, grazie per il vostro sostegno.

And to my husband, Andrea, for FaceTiming with me at 1 AM when you were in Italy and I was in lab, for coming in to work with me on weekends, for sacrificing vacation time while I have been finishing this thesis, thank you. For your endless support, your patience, and your love—I love you, and every day I am grateful to have you by my side.

List of Figures

List of Tables

Chapter 1.

Introduction

Introduction

Cancer is a leading cause of death worldwide. Cancer arises due to dysregulation of cellular signaling pathways, metabolic alterations, and other cell behavior abnormalities. Common hallmarks of cancer underlie the complex biology of the disease, and include evasion of apoptosis, enhanced proliferation, evasion of growth suppression, replicative immortality, angiogenesis, and invasion into the microenvironment [1]. A fundamental step to improving therapeutics for cancer patients is expanding our knowledge of the functions of commonly altered genes in their normal cellular and physiological contexts. For example, the Hippo tumor suppressor pathway, an evolutionarily conserved kinase cascade that regulates organ size and tissue growth, was identified in the beginning of the 21st century from genetic screens in *Drosophila* [2-4]. The transcriptional coactivator YAP, which acts downstream of Hippo, has been implicated as both an oncogene and tumor suppressor in various biological settings.

Previous studies from our lab have shown that gain of YAP activity promotes phenotypes consistent with oncogenic function of YAP and loss of YAP activity results in destabilized phenotypes consistent with a tumor suppressive function of the protein using the same model system, the MCF10A breast epithelial cell line. Overexpression of YAP in MCF10A results in growth factor independent proliferation and a switch from epithelial to mesenchymal morphology; knockdown of YAP in MCF10A impairs cell division and increases genetic instability [5, 6]. In this dissertation, I describe studies aimed at examining YAP function in normal cells in the context of breast epithelial

biology. The findings presented in this thesis describe a novel function of YAP and I discuss implications of these functions in breast cancer biology.

Yes-Associated Protein 1 (YAP)

YAP, Yes-associated protein 1, was originally identified as an interactor with the SRC-family tyrosine kinase YES [7]. YAP is a 65-kilodalton protein that is primarily composed of one or two WW-domains, a TEAD binding domain (TEA domain family member), and C-terminal transactivation domain. YAP has eight splice isoforms with the most dominant forms being YAP1 or YAP2; these forms are distinguished by the presence of one or two WW-domains and consequently dictate the proteins capable of interacting with the isoform [8]. The WW-domains are important for mediating interactions with proteins containing PPxY motifs [7, 9]. YAP and its paralog TAZ (WWTR1) share approximately 46% amino acid homology [10]. YAP and TAZ canonically function as transcriptional coactivators downstream of the Hippo pathway, primarily through interaction with TEAD transcription factors, as they do not contain a DNA-binding domain themselves [11]. YAP can also interact with other transcription factors such as ERBB4, p73, RUNX, often in complex with TEAD [12].

Distinct functions of YAP and TAZ, including the precise activities of these paralogs, their functional redundancies and divergences, are not completely understood. Loss-of-function studies in mice suggest functional redundancy of YAP and TAZ during embryonic development [13]. In some contexts, compensatory signaling through YAP following decrease in TAZ activity levels has been documented, and vice versa. In Yap

knockout mice, TAZ is strongly increased at the protein level across multiple tissue types. This is consistent with observations in some cell lines, such as HeLa cervical cancer cells, where decreased YAP or decreased TAZ results in an increase in levels of the paralog, through TEAD-mediated transcriptional changes [14]. In fibroblasts, knockdown of YAP results in increased TAZ protein levels through altered proteasomal degradation; however, knockdown of TAZ in the same cells does not affect YAP protein levels [15]. There is also evidence for non-overlapping roles of YAP and TAZ. In HEK293A cells, CRISPR-mediated knockout of YAP resulted in alterations in cellular physiology, such as cell spreading, glucose uptake, and granularity. Knockout of TAZ did not produce such significant effects, and the YAP/TAZ double knockout produced mild combined phenotypic severity [16]. Tissue specific function and differential regulation of signaling may result in differences in YAP/TAZ biology.

YAP in particular is of great interest in the fields of mammary cell biology and breast cancer biology. Overexpression of YAP in the nontransformed mammary epithelial cell line MCF10A is sufficient to result in an array of transformed phenotypes, including anchorage-independent growth in soft agar, growth-factor independent proliferation, and a complete epithelial to mesenchymal transition (EMT) [5]. These transformed properties have all been linked to changes resulting from YAP transcriptional signaling. YAP transcriptional activity suppresses anoikis, or apoptosis induced by loss of proper cell-matrix attachment; as cells adhere to extracellular matrix (ECM) and YAP is activated, anoikis is suppressed. Suppression of YAP-dependent transcription under detached cellular conditions initiates anoikis; anoikis can be inhibited through knockdown of YAP [17]. Amphiregulin, an EGFR-ligand, is a YAP

transcriptional target that promotes proliferation in the absence of EGF [18]. The YAP generated EMT phenotype is also dependent on YAP interaction with TEAD proteins, and ablating this interaction diminishes the enhanced metastatic ability of the cells [19] .

Whether YAP functions as an oncogene or tumor suppressor in breast cancer is highly debated in published reports. Studies suggest that YAP functions as an oncogene in the specific case of invasive lobular breast carcinoma, ILC [20, 21]. More broadly, *YAP* is located in the 11q22 chromosomal region, a location which has been associated with both amplification and loss of heterozygosity (LOH) in breast cancers [5, 22]. Early studies on YAP revealed its ability to bind to the p53 family member p73 and recruit it to the promoters of pro-apoptotic genes, thereby increasing apoptotic pathway activation [23]. Consistent with these findings, in several breast cancer cell lines, T47D, MDA-MB-231, and BT474, knockdown of YAP protected cells from anoikis, increased migration and invasion *in vitro*, and increased resistance to the chemotherapeutic taxol [24].

Multiple studies have directly examined patient tumor samples to probe the role of YAP in cancer. One study found YAP levels correlated with favorable disease free survival and overall survival and inversely correlated with lymph node metastases, consistent with a tumor suppressive role [25]. Another study found high frequency of YAP loss in tumors, often due to aberrant promoter methylation and gene silencing, also consistent with a tumor suppressive role [26]. Yet another study identified significant differences in YAP expression amongst breast cancer subtypes (Luminal A, Luminal B, HER2, Triple Negative), generally concluding that high nuclear YAP correlated with decreased overall survival and that high cytoplasmic YAP in HER2-positive breast

cancers correlated with shorter disease free survival [27]. These observations are more consistent with an oncogenic role of YAP. Further examination of YAP and upstream components of the Hippo pathway will help elucidate the complex biology of YAP in breast cancer.

The Hippo-YAP pathway

The Hippo tumor suppressor pathway is at its core a kinase cascade composed of MST1/2 kinases that phosphorylate LATS1/2 that phosphorylation and YAP and TAZ, resulting in their transcriptional inactivation through binding to 14-3-3 proteins and sequestration in the cytoplasm [4] (Figure 1.1). The Hippo pathway was initially characterized in *Drosophila melanogaster* from mutagenesis screens searching for an overgrowth phenotype. Hippo is strongly conserved from the fly to mammals, as expression of the Human ortholog of *Drosophila* Hippo kinases, MST1/2, was sufficient to rescue the overgrowth resulting from loss of *hpo* in the fly [4]. Interestingly, the Hippo kinases themselves are conserved in *Saccharomyces cerevisiae,* budding yeast, where they function to regulate the transition from mitosis to G1 of the cell cycle and are known as the "Mitotic Exit Network" [28].

Beyond the core kinases, Hippo is a highly interconnected pathway. Both core kinase families have adaptor proteins that promote their enzymatic function, Salvador (SAV) for MST1/MST2, and MOB1A/MOB1B for LATS1/LATS2. Additionally, Hippo responds to and directly interacts with proteins involved in cell-cell adhesion, cell polarity, the cytoskeleton, G protein-coupled receptors, and more [29].

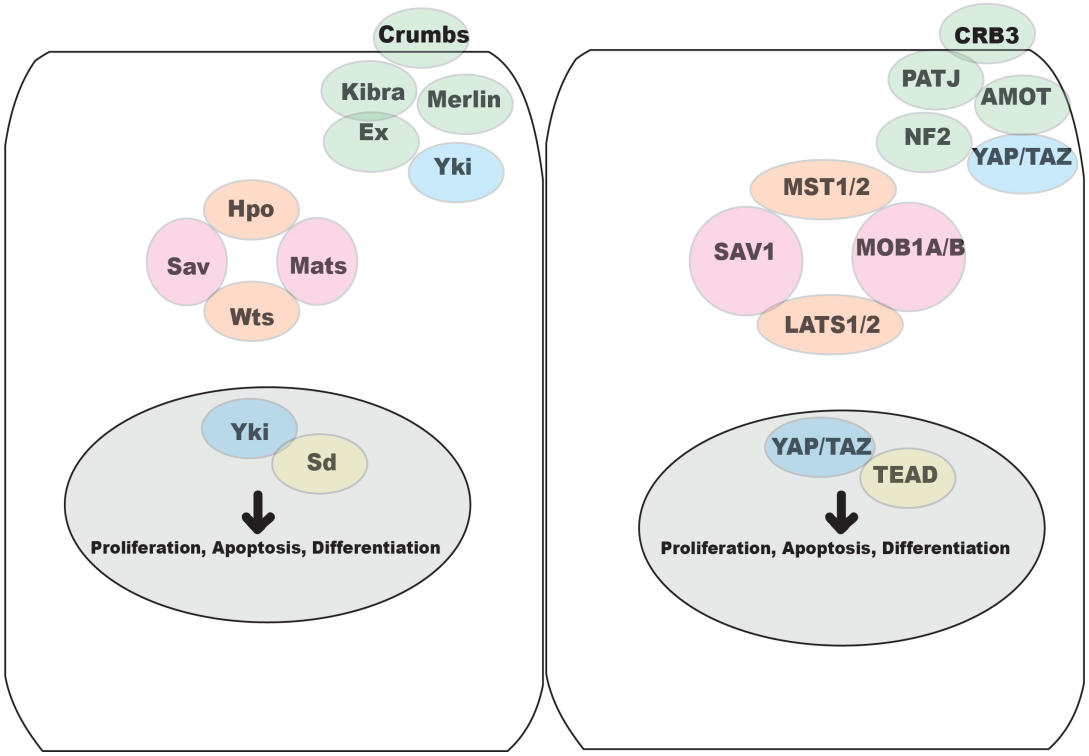

Figure 1.1 Diagram of core Hippo pathway components in *D. melanogaster* and mammalian cells. The Hippo kinases in the cytosol phosphorylate Yorkie or YAP/TAZ when activated, leading to its binding to 14-3-3 or tight junction proteins. When Hippo signaling is off, Yorkie or YAP/TAZ translocates into the nucleus to activate its target genes via interaction with TEAD. *Adapted from Wang et al., 2016.*

YAP localization is determined by its phosphorylation state. LATS phosphorylates YAP on five HXRXXS motifs: Serine 61, Serine 109, Serine 127, Serine 164, and Serine 381 [30, 31]. YAP phosphorylation at S127 is both necessary and sufficient for its binding to 14-3-3 proteins and sequestration in the cytoplasm [32]. Phosphorylation at S381 primes YAP to become a target of CK1 kinase within a phosphodegron[31]. Precise function of the other serines remains unclear, though it is evident that they do influence YAP activity, as mutation of all five sites produces the greatest effect on YAP activation[33]. Once phosphorylated and sequestered in the cytoplasm, the fate of YAP diverges; it can be polyubiquitinated and targeted to the proteasome for degradation [31] or it can translocate to the cellular cortex and interact with adherens junction or tight junction proteins. Hippo is responsive to contact inhibition of proliferation as cells form a confluent monolayer. Following changes in cell structure and tension as cell-cell junctions mature, Hippo is activated, leading to phosphorylation of YAP and relocalization from the nucleus to the cytoplasm [32]. Overexpression of YAP or mutation of the LATS phosphorylation site at S127 results in evasion of contact inhibition. Specifically, the adherens junction is an upstream regulator of Hippo signaling, and both E-cadherin and α-catenin are necessary for transduction of the contact-inhibition signal from the junction to YAP [34, 35]. As previously mentioned, YAP localization has been detected at the adherens junction in dense cultures and *in vivo* in mouse skin. Indeed, when sequestered by 14-3-3 proteins, the YAP/14-3-3 complex can bind directly to α-catenin at the cell membrane [35].

NF2, the ERM-like protein Merlin, is also a critical regulator of YAP and Hippo in the context of cell-cell contact [32]. NF2 localizes to adherens and tight junctions,

where it acts as a scaffold to promote interactions between proteins such as KIBRA and WILLIN, which interact with and activate LATS [36]. As deletion of cadherins is seldom sufficient to generate a hyperproliferative phenotype, it is possible that Merlin or other adherens junction proteins play a critical role in inhibiting YAP transcriptional function [37]. The nuclear export signal (NES) of Merlin is also involved in YAP nuclear export; under high cellular tension, nuclear Merlin binds nuclear YAP and the complex translocates to the cytoplasm [38]. Merlin can also function in a Hippo-dependent manner to inhibit YAP transcription, by acting as a scaffold between the Hippo kinases MST1/2 and LATS1/2 [39].

YAP and Growth Factor Signaling

As cells become confluent and undergo contact inhibition, they stop proliferating, even in the presence of pro-proliferative growth factors. This suggests an inhibitory role for cell-cell contact in growth factor signaling, highlighting a potential link between Hippo and YAP in growth factor signaling. Indeed, in confluent MCF10A cells, treatment with EGF inhibits Hippo and results in rapid translocation of YAP to the nucleus [40]. Studies in nonconfluent, proliferating MCF10A and breast cancer cell lines demonstrate that serum and/or growth factor starvation is sufficient to activate LATS, resulting in YAP phosphorylation and often polyubiquitination and degradation [41]. Evidence suggests that YAP is responsive to Insulin and PDGF stimulation, as well, through PI3K and SRC signaling, respectively [42, 43]

The role of YAP transcriptional programing in growth factor signaling has yet to be fully explained, though the evidence of a link is abundant. YAP is upregulated in cells with mutant KRAS, has been implicated to function downstream of KRAS in neoplastic transformation, and YAP in complex with the AP-1 transcriptional complex substitutes for oncogenic KRAS signaling addiction [44-46]. Interestingly, YAP and Serum Response Factor (SRF), a transcriptional effector of growth factor signaling and master regulator of the immediate early response gene (IEG) program, coimmunoprecipitate in 293T cells [47]. In fibroblasts, MRTF-SRF and YAP-TEAD signaling interact indirectly through their target genes (contractility and cytoskeletal genes for SRF, TGFβ for YAP) [48]. Further examination of the role of YAP in growth factor signaling and defining the genes that are YAP targets in response to these stimuli will provide greater insight into YAP's transcriptional coactivator function.

YAP and The Actin Cytoskeleton

YAP is a key element in cellular mechanotransduction. In addition to responding to changes in cell shape and size relating to cellular confluency, YAP responds to substrate stiffness. When cells were plated on hydrogels of varying stiffness to mimic physiological conditions, stiff hydrogels resulted in a nuclear translocation of YAP/TAZ and corresponding increase in YAP-TEAD target gene expression [49]. YAP is specifically responsive to RHO GTPase activity and changes in the actin cytoskeleton and cellular contractility. In cases of stiff ECM, cell spreading activates RHO, myosin contractility, and actin remodeling into bundles known as stress fibers; disruption of

RHO or downstream effectors suppresses YAP nuclear translocation [49]. In cancer associated fibroblasts (CAFs), YAP transcriptional activation is a hallmark of tumor stroma. Upon knockdown of YAP, CAFs demonstrated perturbed actin bundling, fewer focal adhesions to matrix, and impaired contractile ability of a collagen matrix [50]. Interestingly, decreased total myosin light chain (MLC2) was observed in addition to decreased phosphomyosin light chain (pMLC2), though MLC is not a direct transcriptional target of YAP, perhaps due to the dependence of MRTF-SRF signaling on YAP [48]. This suggests existence of a feedback loop where YAP is required for contractile function of CAFs and promotion of matrix stiffness, and matrix stiffness serves to further activate YAP dependent transcription.

Non-canonical, Non-transcriptional Functions of YAP

Multiple non-transcriptional functions of YAP have been described (Figure 1.2). Our lab previously identified a role for YAP in cytokinesis, where loss of YAP resulted in hyperdynamic mitoses and enhanced membrane blebbing, impaired polarity of the daughter cells, and often resulted in failed cytokinesis, leading to altered chromosomal copy number. This function of YAP was independent of its transcriptional coactivator activity. YAP was required for positioning of mitotic machinery, orienting daughter cells, and localizing contractile forces. Proper cell division required the interaction of YAP and the Crumbs polarity component factor PATJ [6]. This work laid the foundation for our follow-up studies regarding YAP regulation of morphology and cell contractility in non-dividing cells, described in Chapter 2.

A study in *Drosophila* found that Yorkie, the homolog of YAP, accumulated at the apical junction region. This tethering of Yorkie at the membrane results in strong activation of cortical actomyosin through interaction of Yorkie and Stretchin, a myosin light chain kinase [51]. The domain of Yorkie required for the interaction with Stretchin is not conserved in mammals, but the possibility of a similar biological function of YAP in mammals exists. For example, a study in mice found that loss of YAP resulted in hydrocephaly through impaired apical junction integrity, and restoration of phosphomimetic YAP was sufficient to reduce the observed junctional defects [52]. A role for cytoplasmic YAP in regulation of CDC42 activity in angiogenesis was also observed [53].

Several studies have implicated cytoplasmic YAP and TAZ in sequestration of effector proteins. Examination of crosstalk between Hippo and TGF-β pathways revealed direct interaction of YAP/TAZ and SMAD proteins and demonstrated that YAP restricts SMAD nuclear translocation [54]. Similarly, YAP/TAZ can interact with Dishevelled (DVL) during development to restrict nuclear translocation of DVL, dampening Wnt/β-catenin signaling [55, 56]. Furthermore, a link between Hippo and microRNA processing, where YAP binds to and sequesters the miRNA regulator p72 in the nucleus and subsequently cytoplasmic YAP releases p72 leading to activation of Microprocessor, an initiating protein of the pathway which cleaves pri-miRNA to generate pre-miRNA, suggests that YAP functions in a diverse range of biochemical processes in the cell [57]. These studies highlight the complexity of YAP signaling and reveal that non-transcriptional functions of YAP are an important factor in the biology of this protein.

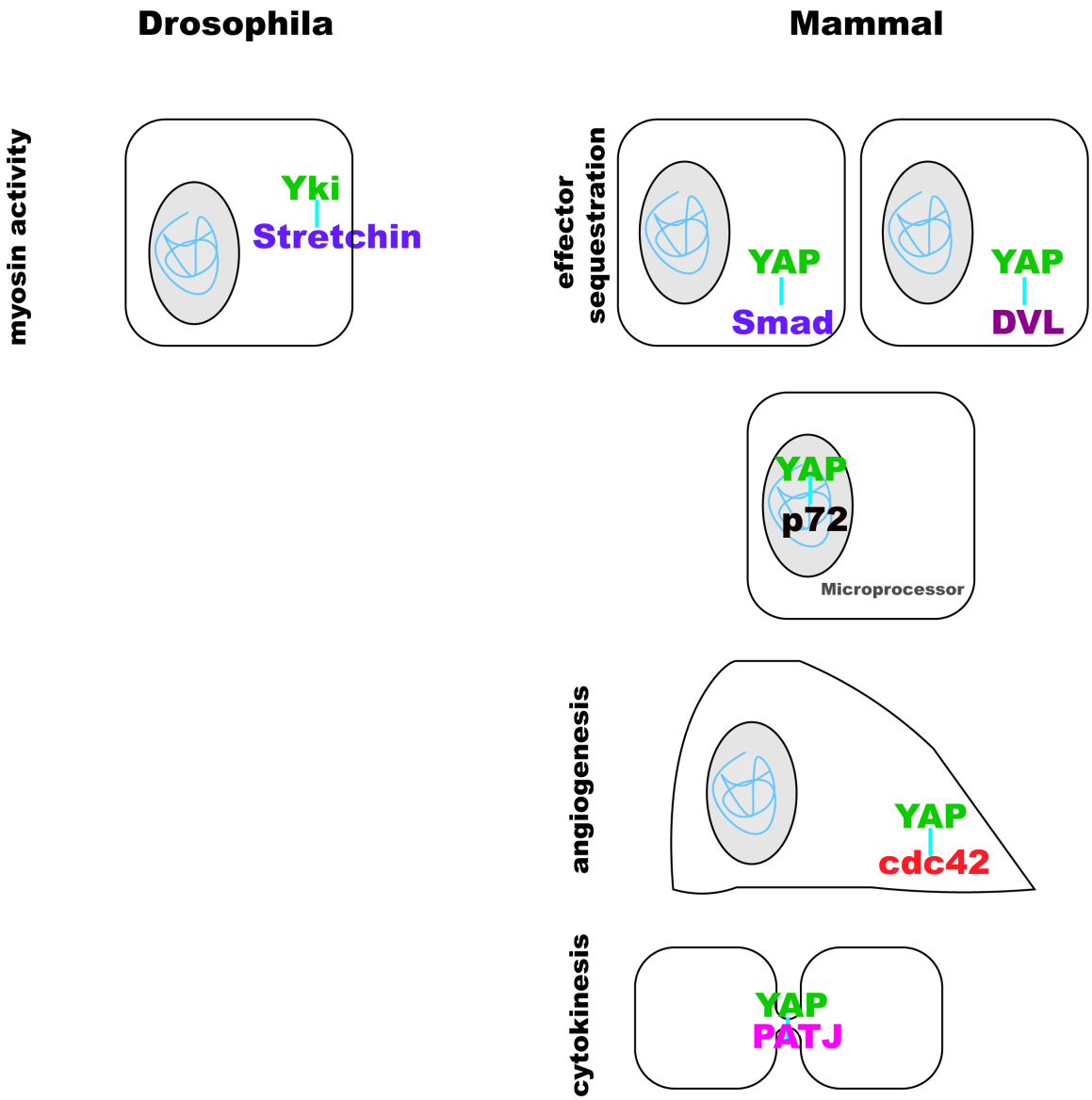

Figure 1.2 Non-transcriptional functions of Yorkie *(Drosophila)* or YAP (mammalian). Cortical Yki regulates myosin activity through interaction with the myosin light chain kinase Stretchin. YAP sequesters effector proteins such as Smad or Dishevelled in the cytoplasm, or p72 in the nucleus. YAP interacts with CDC42 in endothelial cells to regulate angiogenesis, and interacts with PATJ during cytokinesis to regulate the cleavage furrow.

Epithelial cellular adhesion, migration and invasion

Epithelial cells form sheets of adherent cells that form distinct physiological spaces *in vivo*. As such, the strength of the cell-cell adhesion is a key feature of epithelia. The cadherin family of proteins, including the classical epithelial cadherin E-cadherin, generally mediates these adhesions. A cadherin switch, such as from expression of E-cadherin to expression of N-cadherin, signifies a change in adhesiveness; the specific example of E-cadherin to N-cadherin is a feature of epithelial-to-mesenchymal transition (EMT), a process often associated with cancer metastasis [58].

Cadherins form adherens junctions between adjacent cells. Each classical cadherin contains a calcium-dependent ectodomain, transmembrane domain, and cytoplasmic domain that binds other components of the adherens junction, such as the adaptor proteins α-catenin and β-catenin [59]. The catenins link the cadherin to the actin cytoskeleton. p120-catenin also binds to the cadherin and stabilizes it on the cell cortex. The catenins additionally have interactions with intracellular kinases and phosphatases, RHO-family GTPases, and transcription factors. As previously discussed, both α-catenin and β-catenin can interact with YAP [35, 55, 60]. β-catenin also serves as a transcription factor; upon its translocation into the nucleus it regulates Wnt-dependent gene expression [61].

The strength of epithelial adhesion allows for collective cell migration, a property where cells maintain their cell-cell junctions and migrate directionally in sheets or clusters. Collective cell migration is observed *in vivo* during processes such as embryonic development and wound healing. During wound healing, cells at the wound

edge (the leading edge) extend lamellipodia forward and crawl into the space [62]. Wound healing is easily simulated *in vitro* using a wound-healing or scratch assay in standard monolayer cell culture, where a confluent monolayer is either scratched or a barrier in the cells is removed, resulting in a gap. Impairing cell-cell junctions ablates collective cell migration, resulting in single cells dissociating from the leading edge [63, 64].

Migration of a single cell is a complex process involving intracellular biochemical and biomechanical changes, as well as interaction with the external environment. Migration involves cellular protrusion followed by engagement of the protrusion with the ECM via integrin-mediated adhesion, and cell tail engagement with the cytoskeleton through adaptor proteins such as Talin [65]. Cellular contractility mediated by the actomyosin cytoskeleton matures the matrix-adhesions, eventually weakening the rear adhesions, causing retraction of the cell tail and resulting in translocation of the cell forward [66]. As the process repeats in a polarized manner, the cell is able to move directionally.

Cell invasion is a process that occurs in three dimensions. A cell must recognize the physical constraints blocking its migration (cell-cell contacts, ECM contacts), alter the ECM, weaken existing cell-cell adhesions, and migrate from its original location [67]. Alteration of the ECM largely occurs through extracellular proteases, often matrix metalloproteinases (MMP), which often play a critical role in cancer metastasis and disease progression [68]. MMPs act as critical instigators of tumor invasion, particularly in mammary epithelial cells, where they have been implicated in inducing EMT directly,

by enhancing migratory abilities of the tumor cells [69]. These cellular processes are all regulated by cell structure.

RHO GTPases and cellular contractility

Changes in cell morphology, such as those that occur during cell migration or cell division, are tightly regulated by the actin cytoskeleton [70]. A key family of proteins that regulate intracellular actin dynamics is the RHO family of small GTPases. There are three RHO GTPases: RHO, RAC, and CDC42. RHO regulates cell contractility, stress fiber and focal adhesion formation, while RAC regulates cell spreading, membrane ruffling and lamellipodium formation, and CDC42 regulates filopodium formation [71]. RHO and RAC are the main effectors of cellular motility, and they function as reciprocal antagonists of each other. These GTPases are activated by binding to GTP which is mediated by a Guanine Nucleotide Exchange Factor (GEF), and are inactivated when GTP is hydrolyzed into GDP, an intrinsic process of the GTPase catalyzed by a GTPase Activating Protein (GAP) [72]. RHO GTPases associate with the cell cortex and act as molecular switches that trigger signaling cascades regulating cellular morphology via the cytoskeleton (Figure 1.3).

RHO regulates myosin phosphorylation both through activation of RHO associated kinase (ROCK) and Myosin Light Chain Kinase (MLCK), and inhibition of Myosin Light Chain Phosphatase (MLCP). Phosphorylation of the motor protein myosin is required for actomyosin bundling into contractile substrates; as actin filaments are pulled by neighboring myosin, the increase in filament overlap causes a net reduction in

length. The extent of F-actin crosslinking is an important determinant in how efficiently the contractile force is transmitted [73]. Many additional proteins are involved in actin bundling, such as formins that promote the growth of F-actin by preferential binding to the barbed end of the actin filament and recruiting myosin, or filamin that functions in the aforementioned F-actin crosslinking [74].

Cell contractility is critical for proper maintenance of epithelial cell structure and many characteristic properties of epithelial cells. A thin network of cortical actomyosin defines the shape of a cell, and this network is perturbed during morphological changes arising from cell division or cell migration [75]. Cellular migration is inherently dependent on contractility, for maturation of focal adhesions in the lamellipodium and intracellular tension to disassemble focal adhesions in the cell rear. As previously described, when cells migrate the tail detaches due to increasing directional contractile force; in cells with reduced RHO activity, tail retraction is frequently inhibited due to the decrease in actomyosin contractility [76]. Additionally, mature adherens junctions are sites that are both assembled by actomyosin based recruitment and are stabilized by intracellular contractility [77].

In opposition to RHO is RAC. Where active RHO is required at the back of a migrating cell for contractile force, active RAC at the front of the cell promotes the large ruffled protrusions known as lamellipodia [78]. Nascent focal complexes are laid down in the protrusion due to RAC activity and require RHO activity to mature into focal adhesions. RAC stimulates actin polymerization through Arp2/3 mediated actin nucleation and branching, a process required for lamellipodium extension in response to stimulation by growth factors, cytokines, or ECM [79]. RAC has a critical function in

regulation of actin polymerization at the leading edge of the cell, as well as regulation of invadopodial extension and turnover in 3D culture systems [80]. Abnormal regulation of both RHO and RAC has been implicated in disease biology, particularly in cancer.

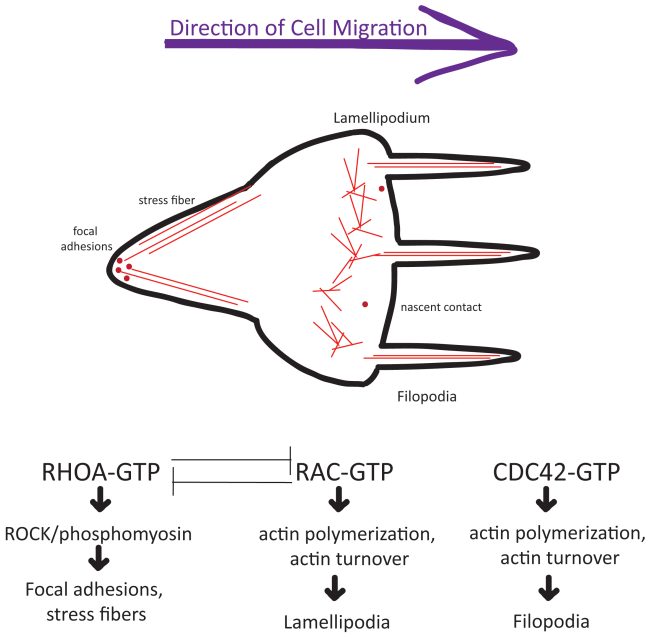

Figure 1.3 Simplified diagram of RHO-GTPase activity in cell migration. RHOA activity, mediated by myosin phosphorylation, regulates focal adhesion maturation and stress fiber formation. RAC activity regulates lamellipodial extension, and CDC42 activity regulates filopodial extension. *Adapted from Mayor and Carmona-Fontaine, 2010.*

The MCF10A Model System

The MCF10A cell line is an optimal model system for studying epithelial cell biology *in vitro*. MCF10A is a non-tumorigenic breast epithelial cell line that spontaneously immortalized in culture. The line is near diploid, excluding minor chromosomal rearrangements [81]. When grown in reconstituted basement membrane (Matrigel), a single MCF10A cell will proliferate into a structure resembling mammary acini in that they are spherical, hollow, polarized structures similar to those within the mammary gland [82]. Beyond the ability to form acini in 3D, MCF10A cells also exhibit many properties of normal epithelial cells, including expression of canonical epithelial markers, lack of anchorage independent survival, growth factor dependent proliferation, and collective cell migration due to their strong epithelial cell-cell adhesion [83].

Many studies utilize the MCF10A cell line to recapitulate the biology of the mammary gland due to these properties, including the YAP overexpression studies conducted by our lab that offered great insight into the array of transformed phenotypes driven by increasing YAP activity [5]. These studies provided the rationale for use of MCF10A cells as a model system in this thesis to examine effects of loss of function in interphase cells.

YAP as a target in Invasive Lobular Carcinoma

Breast cancer is a leading cancer diagnosis amongst women worldwide, accounting for approximately 25% of cases [84]. One method of classifying breast

cancer cases is by histological subtype. The two main classes of histologic subtypes are invasive ductal carcinoma (IDC) and invasive lobular carcinoma. ILC accounts for 10-15% of all breast cancers, and its prevalence is increasing [85]. ILC is diagnosed by a characteristic appearance of linear, single filing of small, non-adherent cells. This morphology is a result of deregulated cell-cell adhesion, and, more specifically, aberrant E-cadherin function, which is lost in approximately 90% of ILC cases [86].

ILC cases are typically Luminal A, displaying ER+ status and a low level of proliferation [87]. ILC cases are more often multifocal and bilateral, often resulting in multiple tumor sites in both breasts [88]. ILC tumors are generally responsive to hormone-based therapeutics, but are less responsive to traditional chemotherapies due to their low proliferative index [86]. Interestingly, the metastasis pattern of ILC tumors is distinct from the majority of other breast tumors, including high incidence of bone, liver, lung, and brain metastases [89, 90]. Increasing our understanding of underlying ILC biology will lead to advances in precision medicine for patients diagnosed with the disease.

Many studies examining ILC biology have used mouse models of ILC (mILC) generated by mammary specific loss of E-cadherin in combination with loss of the tumor suppressors *TP53* or *PTEN*. These models greatly recapitulate human ILC (hILC) morphologically, though they do not reflect the hormone positivity so often associated with human ILC [91, 92]. Additionally, an immune-reactive mILC model was generated through mutation of E-cadherin and an activating mutation of PI3K; this model was created specifically to examine interactions with the microenvironment and immune system, interactions that offer great insight into potential therapeutic avenues for patients

[21]. Beyond *in vivo* models, however, there are limited cell culture systems to study ILC *in vitro*. The cancer cell lines SUM44PE, MDA-MB-134VI, and IPH-926 were all derived from pleural effusion at metastatic sites (either pleural effusion or ascites), and with the exception of SUM44PE proliferate at a rate that makes *in vitro* studies technically challenging [93].

A well-established hallmark of ILC is the loss of cell-cell adhesion due to alterations in E-cadherin. This molecular alteration greatly affects ILC cellular biology. Loss of E-cadherin perturbs the adherens junction complex and results in localization of p120 catenin to the cytosol. This relocalization of p120 indirectly activates RHO/ROCK signaling, leading both to anoikis resistance and anchorage independent growth occurring in parallel with the tumor initiating event of E-cadherin [94]. This distinguishes ILC from other cancers where these epithelial properties are lost later in tumor progression during the metastatic cascade. Additionally, the relocalization of p120 results in extracellular changes for the tumor as well, increasing sensitivity of the tumor cells to growth factor signaling, stimulating secretion of inflammatory cytokines, and increasing infiltration of pro-inflammatory immune cells into the tumor microenvironment [95]. These studies highlight how a single event, loss of E-cadherin, alters the cellular biology of ILC and also impacts the tumor microenvironment of the disease.

Loss of E-cadherin would be expected to alter localization and subsequent transcriptional activity of YAP. One histology-based study found that E-cadherin expression inversely correlates with nuclear expression of YAP, and nuclear YAP positively correlated with both hILC and mILC [20]. Additionally, the immune-reactive mILC model shows high expression of YAP target genes and high sensitivity to the YAP

transcriptional inhibitor verteporfin [21]. These studies raise questions regarding the precise role that oncogenic YAP plays in ILC progression, and what transcriptional targets of YAP may contribute to ILC progression. We initiated studies to examine YAP transcriptional activity in tumors lacking E-cadherin, such as ILC. These findings are preliminary and included in Appendix I.

Goals and Overview of the Dissertation

The goals of this thesis were to examine the effects of loss of YAP expression in both normal and transformed breast epithelial cells. We examined normal YAP function in non-transformed cells using MCF10A as a model system and characterizing phenotypes observed upon YAP knockdown. Chapter 2 describes a novel, TEAD-independent function of YAP that regulates breast epithelial cell integrity in the MCF10A system. Cells with decreased YAP showed increased protrusive activity in Matrigel culture and increased lamellipodia formation in monolayer culture. Furthermore, decreased YAP expression impairs collective cell migration by altering epithelial cell-cell adhesion and impeding junctional maturation. Interestingly, decreased YAP expression alters RHO-GTPase activity, decreasing RHOA and increasing RAC activity. These altered RHO levels are accompanied by changes in cellular contractility. Restoration of wildtype RHO and RAC activity levels is sufficient to inhibit the aberrant protrusive behavior of YAP knockdown cells. Additionally, we find that this function of YAP in suppressing protrusive behavior and regulating cellular contractility is independent of its transcriptional activity with TEAD. These studies suggest a tumor suppressive function

of YAP. Our findings suggest that cytoplasmic YAP plays an important role in the regulation of cell-cell adhesion and membrane protrusion when the Hippo pathway is active, and that alterations in cellular behavior caused by translocation of YAP to the nucleus following Hippo inactivation can be mediated through the loss of cytoplasmic YAP and gain of nuclear YAP transcriptional activity.

References

1. Hanahan D WR. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.

2. Harvey KF PC, Hariharan IK. The Drosophila Mst Ortholog, hippo, Restricts Growth and Cell Proliferation and Promotes Apoptosis. Cell. 2003;114:457-67.

3. Tapon N HK, Bell DW, Wahrer DC, Schiripo TA, Haber D, Hariharan IK. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell. 2002;110(4):467-78.

4. Wu S HJ, Dong J, Pan D. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell. 2003;114:445-56.

5. Overholtzer M ZJ, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, Haber DA. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. PNAS. 2006;103(33):12405-10. doi: 10.1073/pnas.0605579103.

6. Bui DA LW, White AE, Harper JW, Schackmann RC, Overholtzer M, Selfors LM, Brugge JS. Cytokinesis involves a nontranscriptional function of the Hippo pathway effector YAP. Science Signaling. 2016;9(417). doi: 10.1126/scisignal.aaa9227.

7. Sudol M BP, Einbond A, Kastury K, Druck T, Negrini M, Huebner K, Lehman D. Characterization of the Mammalian YAP (Yes-associated Protein) Gene and Its Role in Defining a Novel Protein Module, the WW Domain. Journal of Biological Chemistry. 1995;270(24):14733-41.

8. M. S. YAP1 oncogene and its eight isoforms. Oncogene. 2013;32:3922.

9. Iglesias-Bexiga M CF, Cobos ES, Oka T, Sudol M, Luque I. WW domains of the yes-kinase-associated-protein (YAP) transcriptional regulator behave as independent units with different binding preferences for PPxY motif-containing ligands. PLoS One. 2015;10(1):e0113828.

10. Hong W GK. The YAP and TAZ transcription coactivators: key downstream effectors of the mammalian Hippo pathway. Seminars in Cell & Developmental Biology. 2012;23(7):785-93.

11. Vassilev A KK, Shu H, Zhao Y, DePamphilis ML. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. Genes and Development. 2001;15(10):1229-41.

12. Kim MK JJ, Bae SC. DNA binding partners of YAP/TAZ. BMB Reports. 2015;51(3):126-33. doi: 10.5483/BMBRep.2018.51.3.015.

13. Nishioka N IK, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, Morin-Kensicki EM, Nojima H, Rossant J, Nakao K, Niwa H, Sasaki H. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Developmental Cell. 2009;16(3):398-410.

14. Moroishi T PH, Qin B, Chen Q, Meng Z, Plouffe SW, Taniguchi K, Yu FX, Karin M, Pan D, Guan KL. A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. Genes and Development. 2015;29:1271-84.

15. Finch-Edmondson ML SR, Passman AM, Sudol M, Yeoh GC, Callus BA. TAZ Protein Accumulation Is Negatively Regulated by YAP Abundance in Mammalian Cells. Journal of Biological Chemistry. 2015;290(46):27928-38.

16. Plouffe SW LK, Moore JL 3rd, Tan FE, Ma S, Ye Z, Qiu Y, Ren B, Guan KL. The Hippo pathway effector proteins YAP and TAZ have both distinct and overlapping functions in the cell. Journal of Biological Chemistry. 2018;293(28):11230-40.

17. Zhao B LL, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes and Development. 2012;26(1):54-68.

18. Zhang J JJ, Yu M, Overholtzer M, Smolen GA, Wang R, Brugge JS, Dyson NJ, Haber DA. YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. Nature Cell Biology. 2009;11(12):1444-50.

19. Lamar JM SP, Liu H, Schindler JW, Jiang ZG, Hynes RO. The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. Proceedings of the National Academy of Science. 2012;109(37):E2441-50.

20. Vlug EJ vdVR, Vermeulen JF, Bult P, van Diest PJ, Derksen PW. Nuclear localization of the transcriptional coactivator YAP is associated with invasive lobular breast cancer. Cellular Oncology. 2013;36(5):375-84.

21. An Y AJ, Hollern DP, Zhao A, Chang SG, Gams MS, Chung PED, He X, Jangra R, Shah JS, Yang J, Beck LA, Raghuram N, Kozma KJ, Loch AJ, Wang W, Fan C, Done SJ, Zacksenhaus E, Guidos CJ, Perou CM, Egan SE. Cdh1 and Pik3ca Mutations Cooperate to Induce Immune-Related Invasive Lobular Carcinoma of the Breast. Cell Reports. 2018;25:702-14.

22. Steinhardt AA GM, Klein AP, Dong J, Maitra A, Pan D, Montgomery EA, Anders RA. Expression of Yes-associated protein in common solid tumors. Human Pathology. 2008;39(11):1582-9.

23. Strano S ME, Rossi M, Castagnoli L, Shaul Y, Sacchi A, Oren M, Sudol M, Cesareni G, Blandino G. Physical interaction with Yes-associated protein enhances p73 transcriptional activity. Journal of Biological Chemistry. 2001;276(18):15164-73.

24. Yuan M TV, Lara R, Holliday D, Chelala C, Harada T, Gangeswaran R, Manson-Bishop C, Smith P, Danovi SA, Pardo O, Crook T, Mein CA, Lemoine NR, Jones LJ, Basu S. Yes-associated protein (YAP) functions as a tumor suppressor in breast. Cell Death and Differentiation. 2008;15(11):1752-9.

25. Cao L SP, Yao M, Jia M, Gao H. Expression of YES-associated protein (YAP) and its clinical significance in breast cancer tissues. Human Pathology. 2017;68:166-74.

26. Real SAS PF, Rehman AU, Khan MA, Deo SVS, Shukla NK, Husain SA. Aberrant Promoter Methylation of YAP Gene and its Subsequent Downregulation in Indian Breast Cancer Patients. BMC Cancer. 2018;18(711).

27. Kim SK JW, Koo JS. Yes-associated protein (YAP) is differentially expressed in tumor and stroma according to the molecular subtype of breast cancer. International Journal of Clinical and Experimental Pathology. 2014;7(6):3224-34.

28. Rock JM LD, Stach L, Ogrodowicz RW, Keck JM, Jones MH, Wong CC, Yates JR 3rd, Winey M, Smerdon SJ, Yaffe MB, Amon A. Activation of the yeast Hippo pathway by phosphorylation-dependent assembly of signaling complexes. Science. 2013;340(6134):871-5.

29. Kwon Y VA, Sun X, Dephoure N, Gygi SP, Hong P, Perrimon N. The Hippo Signaling Pathway Interactome. Science. 2013;342(737):737-40.

30. Zhao Y KP, Savage P, She YM, Cyr TD, Yang X. YAP-Induced Resistance of Cancer Cells to Antitubulin Drugs Is Modulated by a Hippo-Independent Pathway. Cancer Research. 2014;74(16):4493-503.

31. Zhao B LL, Tumaneng K, Wang CY, Guan KL. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). Genes and Development. 2010;24(1):72-85.

32. Zhao B WX, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC, Guan KL. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes and Development. 2007;21(21):2747-61.

33. Zhao B KJ, Ye X, Lai ZC, Guan KL. Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. Cancer Research. 2009;69(3):1089-98.

34. Kim NG KE, Chen X, Gumbiner BM. E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. Proceedings of the National Academy of Science. 2011;108(29):11930-5.

35. Schlegelmilch K MM, Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR, Camargo FD. Yap1 acts downstream of α-catenin to control epidermal proliferation. Cell. 2011;144(5):782-95.

36. Moleirinho S CN, Sims AH, Tilston-Lunel AM, Angus L, Steele A, Boswell V, Barnett SC, Ormandy C, Faratian D, Gunn-Moore FJ, Reynolds PA. KIBRA exhibits MST-independent functional regulation of the Hippo signaling pathway in mammals. Oncogene. 2013;32:1821-30.

37. McClatchey AI YA. Contact inhibition (of proliferation) redux. Current Opinion in Cell Biology. 2012;24(5):685-94.

38. Furukawa KT YK, Sakurai N, Ohno S. The Epithelial Circumferential Actin Belt Regulates YAP/TAZ through Nucleocytoplasmic Shuttling of Merlin. Cell Reports. 2017;20(6):1435-47.

39. Yin F YJ, Zheng Y, Chen Q, Zhang N, Pan D. Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. Cell. 2013;154(6).

40. Fan R KN, Gumbiner BM. Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1. Proceedings of the National Academy of Science. 2013;110(7):2569-74.

41. Adler JJ JD, Heller BL, Bringman LR, Ranahan WP, Conwell MD, Sun Y, Hudmon A, Wells CD. Serum deprivation inhibits the transcriptional co-activator YAP and cell growth via phosphorylation of the 130-kDa isoform of Angiomotin by the LATS1/2 protein kinases. Proceedings of the National Academy of Science. 2013;110(43):17368-73.

42. Hao F XQ, Zhao Y, Stevens JV, Young SH, Sinnett-Smith J, Rozengurt E. Insulin Receptor and GPCR Crosstalk Stimulates YAP via PI3K and PKD in Pancreatic Cancer Cells. Molecular Cancer Research. 2017;15(7):929-41.

43. Smoot RL WN, Sugihara T, Hernandez MC, Yang L, Mehner C, Graham RP, Bronk SF, Truty MJ, Gores GJ. Platelet-derived growth factor regulates YAP transcriptional activity via Src family kinase dependent tyrosine phosphorylation. Journal of Cell Biochemistry. 2018;119(1):824-36.

44. Muranen T SL, Hwang J, Gallegos LL, Coloff JL, Thoreen CC, Kang SA, Sabatini DM, Mills GB, Brugge JS. ERK and p38 MAPK Activities Determine Sensitivity to PI3K/mTOR Inhibition via Regulation of MYC and YAP. Cancer Research. 2016;76(24):7168-80.

45. Shao DD XW, Krall EB, Bhutkar A, Piccioni F, Wang X, Schinzel AC, Sood S, Rosenbluh J, Kim JW, Zwang Y, Roberts TM, Root DE, Jacks T, Hahn WC. KRAS and YAP1 converge to regulate EMT and tumor survival. Cell. 2014;158(1):171-84.

46. Zhang W NN, Shi Y, Manzano M, Smith A, Graham G, Gupta S, Vietsch EE, Laughlin SZ, Wadhwa M, Chetram M, Joshi M, Wang F, Kallakury B, Toretsky J, Wellstein A, Yi C. Downstream of mutant KRAS, the transcription regulator YAP is essential for neoplastic progression to pancreatic ductal adenocarcinoma. . Science Signaling. 2014;7(324).

47. Kim T YS, Hwang D, Song J, Kim M, Kyum Kim S, Kang K, Ahn J, Lee D, Kim MY, Kim S, Seung Koo J, Seok Koh S, Kim SY, Lim DS. A basal-like breast cancer-specific role for SRF–IL6 in YAP-induced cancer stemness. Nature Communications. 2015;6:10186.

48. Foster CT GF, Treisman R. Mutual dependence of the MRTF-SRF and YAP-TEAD pathways in cancer-associated fibroblasts is indirect and mediated by cytoskeletal dynamics. Genes and Development. 2017;31(23-24):2361-75.

49. Dupont S ML, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S. Role of YAP/TAZ in mechanotransduction. Nature. 2011;474(7350):179-83.

50. Calvo F EN, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, Harrington K, Williamson P, Moeendarbary E, Charras G, Sahai E. Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. Nature Cell Biology. 2013;15(6):637-46.

51. Xu J VP, Ludwig M, Sherzod TS, Tokamov A, Fehon RG. Yorkie Functions at the Cell Cortex to Promote Myosin Activation in a Non-transcriptional Manner. Developmental Cell. 2018;46(3):271-84.

52. Park R MU, Park JY, Hughes LJ, Johnson RL, Cho SH, Kim S. Yap is required for ependymal integrity and is suppressed in LPA-induced hydrocephalus. Nature Communications. 2016;7.

53. Sakabe M FJ, Odaka Y, Liu N, Hassan A, Duan X, Stump P, Byerly L, Donaldson M, Hao J, Fruttiger M, Lu QR, Zheng Y, Lang RA, Xin M. YAP/TAZ-CDC42 signaling regulates vascular tip cell migration. Proceedings of the National Academy of Science. 2017;114(41):10918-23.

54. Varelas X S-TP, Narimatsu M, Weiss A, Cockburn K, Larsen BG, Rossant J, Wrana JL. The Crumbs Complex Couples Cell Density Sensing to Hippo-Dependent Control of the TGF-b-SMAD Pathway. Developmental Cell. 2010;19:831-44. doi: 10.1016/j.devcel.2010.11.012.

55. Varelas X MB, Sopko R, Song S, Gregorieff A, Fellouse FA, Sakuma R, Pawson T, Hunziker W, McNeill H, Wrana JL, Attisano L. The Hippo pathway regulates Wnt/beta-catenin signaling. Developmental Cell. 2010;18(4):579-91.

56. Barry ER MT, Butler BL, Shrestha K, de la Rosa R, Yan KS, Fuchs CS, Magness ST, Smits R, Ogino S, Kuo CJ, Camargo FD. Restriction of intestinal stem cell expansion and the regenerative response by YAP. Nature. 2013;493(7430):106-10.

57. Mori M TR, Mohseni M, Schlegelmilch K, Shrestha K, Camargo FD, Gregory RI. Hippo signaling regulates microprocessor and links cell-density-dependent miRNA biogenesis to cancer. Cell. 2014;156(5):893-906.

58. JP. T. Epithelial-mesenchymal transitions in tumor progression. Nature Reviews Cancer. 2002;2:442-54.

59. Nollet F KP, van Roy F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. . Journal of Molecular Biology. 2000;299:551-72.

60. Azzolin L PT, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, Fassina A, Cordenonsi M, Piccolo S. YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response. Cell. 2014;158(1):157-70.

61. BM G. Regulation of cadherin-mediated adhesion in morphogenesis. Nature Reviews Molecular Cell Biology. 2005;6:622-34.

62. Grasso SJ HJ, Chifflet S. Roles of wound geometry, wound size, and extracellular matrix in the healing response of bovine corneal endothelial cells in culture. American Journal of Physiology, Cell Physiology. 2007;293:C1327-37.

63. Simpson KJ SL, Bui J, Reynolds A, Leake D, Khvorova A, Brugge JS. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. Nature Cell Biology. 2008;10(9):1027-38.

64. Gallegos LL NM, Sowa ME, Selfors LM, White A, Zervantonakis IK, Singh P, Dhakal S, Harper JW, Brugge JS. A protein interaction map for cell-cell adhesion regulators identifies DUSP23 as a novel phosphatase for β-catenin. Scientific Reports. 2016;6. doi: 10.1038/srep27114.

65. Lock JG W-HB, Stromblad S. Cell-matrix adhesion complexes: master control machinery of cell migration. Seminars in Cancer Biology. 2008;18:65-76.

66. Nobes CD HA. Rho GTPases control polarity, protrusion, and adhesion during cell movement. Journal of Cell Biology. 1999;144:1235-44.

67. Wells A KJ, Solava J, Turner T, Lauffenburger DA. Growth Factor-Induced Cell Motility in Tumor Invasion. Acta Oncologica. 2002;41(2):124-30.

68. Radisky ES R-SM, Radisky DC. Therapeutic Potential of Matrix Metalloproteinase Inhibition in Breast Cancer. Journal of Cell Biochemistry. 2017;118(11):3531-48.

69. Radisky ES RD. Matrix metalloproteinase‐induced epithelial‐mesenchymal transition in breast cancer. Journal of Mammary Gland Biology and Neoplasia. 2010;15(2):201-12.

70. Mammoto A ID. Cytoskeletal control of growth and cell fate switching. Current Opinions in Cell Biology. 2009;21(6):864-70.

71. Parri M CP. Rac and Rho GTPases in cancer cell motility control. Cell Communication and Signaling. 2010;8(23).

72. Boguski MS MF. Proteins regulating Ras and its relatives. Nature. 1993;366(6457):643-54.

73. Murrell M GM. F-actin buckling coordinates contractility and severing in a biomimetic actomyosin cortex. Proceedings of the National Academy of Science. 2012;51:20820-5.

74. Murrell M OP, Lenz M, Gardel ML. Forcing cells into shape: the mechanics of actomyosin contractility. Nature Reviews Molecular Cell Biology. 2015;16:486-98.

75. Green RA PE, Oegema K. Cytokinesis in animal cells. Annual Reviews in Cell and Developmental Biology. 2012;28:29-58.

76. Cox EA HA. Regulation of integrin-mediated adhesion during cell migration. Microscopy Research and Technique. 1998;43(5):412-9.

77. le Duc Q SQ, Blonk I, Sonnenberg A, Wang N, Leckband D, de Rooij J. Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner. Journal of Cell Biology. 2010;189(7):1107-15.

78. Mayor R C-FC. Keeping in touch with contact inhibition of locomotion. Trends in Cell Biology. 2010;20(6-3):319-28.

79. Knight B LC, Akhtar N, Hotchin NA, Edlund M, Horwitz AR. Visualizing muscle cell migration in situ. Current Biology. 2000;10:576-85.

80. Lawson CD RA. Rho GTPase signaling complexes in cell migration and invasion. Journal of Cell Biology. 2018;217(2):447.

81. Soule HD MT, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Research. 1990;50(18):6075-80.

82. Debnath J BJ. Modelling glandular epithelial cancers in three-dimensional cultures. Nature Reviews Cancer. 2005;5:675-88.

83. Qu Y HB, Yu Y, Yao W, Bose S, Karlan BY, Giuliano AE, Cui X. Evaluation of MCF10A as a Reliable Model for Normal Human Mammary Epithelial Cells. PLoS One. 2015.

84. Torre LA BF, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global Cancer Statistics, 2012. CA: Cancer Journal for Clinicians. 2015;65:87-108.

85. Barroso-Sousa R M-FO. Differences between invasive lobular and invasive ductal carcinoma of the breast: results and therapeutic implications. Therapeutic Advances in Medical Oncology. 2016;8(4):261-6.

86. McCart RA KJ, Lakhani S, Simpson P. Invasive lobular carcinoma of the breast: morphology, biomarkers and ‘omics. Breast Cancer Research. 2015;17(12).

87. Du T ZL, Levine KM, Tasdemir N, Lee AV, Vignali D, Houten BV, Tseng GC, Oesterreich S. Invasive lobular and ductal breast carcinoma differ in immune response, protein translation efficiency and metabolism. Scientific Reports. 2018;8(7205).

88. Stivalet A LA, Pigneur F, Dao TH, Beaussart P, Merabet Z, Perlbarg J, Meyblum E, Baranes L, Calitchi E, Lepage C, Belkacemi Y, Lagrange JL, Lantieri L, Rahmouni A. Invasive lobular carcinoma of the breast: MRI pathological correlation following bilateral total mastectomy. Acta Radiologica. 2012;53(4):367-75.

89. Chen Z YJ, Li S, Lv M, Shen Y, Wang B, Li P, Yi M, Zhao X, Zhang L, Wang L, Yang J. Invasive lobular carcinoma of the breast: A special histological type compared with invasive ductal carcinoma. PLoS One. 2017;12(9).

90. Inoue M NH, Nakada H, Furuya K, Ikegame K, Watanabe H, Omata M, Oyama T. Specific sites of metastases in invasive lobular carcinoma: a retrospective cohort study of metastatic breast cancer. Breast Cancer. 2017;24(5):667-72.

91. Derksen PW LX, Saridin F, van der Gulden H, Zevenhoven J, Evers B, van Beijnum JR, Griffioen AW, Vink J, Krimpenfort P, Peterse JL, Cardiff RD, Berns A, Jonkers J. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell. 2006;10(5):437-49.

92. Boelens MC NM, Klarenbeek S, de Ruiter JR, Schut E, Bonzanni N, Zeeman AL, Wientjens E, van der Burg E, Wessels L, Wessels L, Amerongen R, Jonkers J. PTEN loss in E-cadherin-deficient mouse mammary epithelial cells rescues apoptosis and results in development of classical invasive lobular carcinoma. Cell Reports. 2016;16:2087-101.

93. Christgen M GR, Kreipe H, Lehmann U. IPH-926 lobular breast cancer cells are triple-negative but their microarray profile uncovers a luminal subtype. Cancer Science. 2013;104(12):1726-30.

94. Schackmann RCJ vAM, Haarhuis JHI, Vlug EJ, Halim VA, Roodhart JML, Vermaat JS, Voest EE, van der Groep P, van Diest PJ, Jonkers J, Derksen PWB. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. Journal of Clinical Investigation. 2011;121(8):3176-88.

95. Schackmann RCJ KS, Vlug EJ, Stelloo S, van Amersfoort M, Tenhagen M, Braumuller TM, Vermeulen JF, van der Groep P, Peeters T, van der Wall E, van Diest PJ, Jonkers J, Derksen PWB. Loss of p120-Catenin Induces Metastatic Progression of Breast Cancer by Inducing Anoikis Resistance and Augmenting Growth Factor Receptor Signaling. Cancer Research. 2013;73(15):4937-49.

Chapter 2.

YAP Regulates Cell Protrusion and Adhesion Through RHO GTPase Activity Independent of TEAD Transcriptional Activity

Natalie M. Hendrick, Duyen Amy Bui, Richard Panayiotou, Marcin Iwanicki, Carman Man-Chung Li, Clarence Yapp, Joan S. Brugge

Statement of Contribution:

N.M.H. and J.S.B. designed the studies, D.A.B. performed initial characterization of YAP knockdown acini and analysis of RHO activity, R.P. assisted with the contractility assays and molecular cloning, M.I. provided support with timelapse microscopy experiments, C.M.L. assisted with wound-healing assays and provided helpful advice, C.Y. designed the algorithm to quantify cellular dissociation from timelapse microscopy, N.M.H. carried out and analyzed all other experiments.

Statement about Supplemental Data

Supplemental Movies accompany this Chapter and are described in Appendix II.

Abstract

YAP is a transcriptional coactivator that is regulated by mechanical forces through its interaction with the Hippo tumor suppressor pathway. Here, we show that YAP knockdown in non-transformed mammary epithelial cells promotes protrusion in three-dimensional culture and lamellipodia formation in monolayer culture. Decreased YAP also impairs maturation of adherens junctions, resulting in weakened cell-cell adhesion. We demonstrate that YAP is required for maintenance of RHO-GTPase activity levels and cellular contractility by promoting RHOA activity and inhibiting RAC activity. Notably, the regulation of contractile behavior and RHO activity by YAP is a cytoplasmic function, independent of its canonical transcriptional function involving co-activation of TEAD. Our findings suggest that cytoplasmic YAP plays an important role in regulating cell-cell adhesion and membrane protrusion when the Hippo pathway is active, and that alterations in cell behavior caused by translocation of YAP to the nucleus following Hippo inactivation can be mediated by both loss of cytoplasmic YAP and gain of YAP nuclear transcriptional co-activation activity.

Introduction

Yes-Associated Protein 1, YAP, is a transcriptional effector of the Hippo tumor suppressor pathway. YAP is canonically viewed as a proto-oncogene due to its regulatory function in organ size, tissue homeostasis, regeneration, and tumorigenesis [1], though in some contexts it has also been implicated as a tumor suppressor in breast cancer progression [2] . When localized to the nucleus, YAP (Yorkie in *Drosophila*) functions as a transcriptional coactivator with TEAD proteins to regulate processes associated with oncogenic transformation [3]. Overexpression of YAP is sufficient to drive a complete epithelial to mesenchymal transition (EMT), and the YAP-TEAD interaction is necessary for YAP mediated metastasis and invasion [4, 5]. Nuclear localization of YAP is associated with an increase in its transcriptional coactivator function.

The localization of YAP is regulated by many factors. The Hippo pathway negatively regulates YAP nuclear localization by sequestering it in the cytoplasm through a protein kinase cascade. Hippo kinases MST1/2 phosphorylate LATS1/2, which phosphorylates YAP; phosphorylated YAP binds to 14-3-3 proteins, promoting YAP retention in the cytoplasm. The Hippo pathway can be activated by multiple membrane proteins, including metabolic signals such as mevalonate, hormonal signals such as estrogen, GPCR signaling such as through lysophosphatidic acid, or adhesion proteins such as E-cadherin [6]. YAP is also directly regulated by the cytoskeleton, intracellular tension, and extracellular matrix rigidity [7]. YAP has a role in cellular mechanotransduction, with its nuclear translocation and transcriptional activity regulated

by RHO GTPase activity and the actomyosin cytoskeleton, independent of the Hippo kinases [8, 9]. Conditions of high cytoskeletal tension and extracellular matrix stiffness are conditions where RHOA activation and cell contractility lead to nuclear translocation of YAP from the cytoplasm and an increase in its transcriptional programs [8, 10].

When Hippo is active, YAP is localized to the cytoplasm. Within the cytoplasm, the fate of YAP diverges: YAP can be polyubiquitinated and degraded, form a complex with □-catenin at the adherens junction, or form a complex with Angiomotin, PATJ, and other Crumbs Polarity Complex Components at the tight junction [11]. Though the function of YAP in the nucleus has been extensively studied, its cytoplasmic functions are less well understood. Previous work from our laboratory demonstrated that YAP is a regulator of cellular mechanical behavior; downregulation of YAP causes dysregulation of mitosis and cytokinesis through alterations in RHO GTPase activation, resulting in aberrant membrane blebbing and protrusions independent of its canonical transcriptional activity with TEAD [12]. These findings motivated us to investigate the role of YAP in interphase cellular morphology and behavior.

In this report, we describe YAP regulation of cellular mechanical behavior in non-transformed epithelial cells and identify a previously unrecognized non-transcriptional function of YAP in the cytoplasm. We find that YAP inhibits protrusive cellular behavior and stabilizes cell-cell junctions through maintenance of RHOA and contractility, and inhibition of RAC activity levels. Interestingly, this activity of YAP is not dependent on nuclear localization or its transcriptional coactivation of TEAD, but rather a novel non-transcriptional function. Our findings reveal a previously

unrecognized cytoplasmic role of mammalian YAP in regulating RHO GTPase activity in non-dividing epithelial cells.

Results

Decreased YAP promotes cellular protrusion and invasive behavior

MCF10A cells are non-transformed breast epithelial cells that retain multiple characteristics of epithelial cells in *in vitro* culture: contact-inhibition of proliferation, collective cell migration, and growth-arrested acinar formation in three-dimensional (3D) culture closely resembling mammary glandular architecture observed *in vivo* [13]. Therefore, MCF10A are a powerful, physiologically relevant *in vitro* model. To examine whether YAP plays a role in epithelial cell behavior, we knocked down its expression using multiple previously characterized short hairpin RNAs (shRNAs) targeting YAP in MCF10A cells (Figure 2.1)[12].

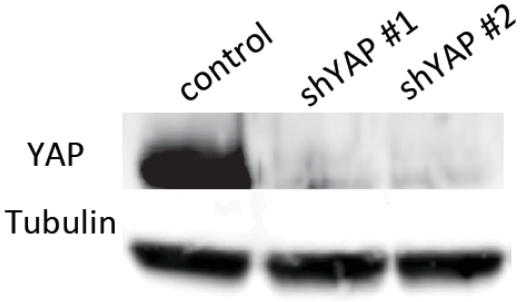

Figure 2.1 YAP knockdown levels in MCF10A.

When grown in 3D Matrigel culture, MCF10A cells form hollow spheroids with multiple characteristics of glandular epithelium, including basement membrane deposition, apicobasal polarity, and strict control of proliferation [13]. Decreased YAP significantly disrupted normal 3D acinar morphogenesis of MCF10A cells (Figure 2.2A). YAP knockdown caused a significant increase in the percentage of acini with stellate membrane structures that protruded from cells at the acinar surface. Whereas only 5.7% of control acini contained these protrusive structures, the penetrance of this phenotype was 45.6% in cells transduced with one YAP hairpin and 40.0% with another (Figure 2.2B). Quantification of the morphological changes via measurement of acinar circularity confirmed that YAP knockdown acini exhibited decreased circularity relative to control MCF10A (Figure 2.2C). This observed protrusive behavior induced by YAP knockdown in MCF10A is particularly noteworthy, as very few manipulations of single genes associated with cancer are capable of overcoming the structural restraints of Matrigel culture [14, 15].

We also assessed whether YAP knockdown affected protrusive behavior in MCF10A cells grown in standard monolayer culture by examining protrusion and retraction cellular dynamics using time lapse microscopy. YAP knockdown cells displayed highly dynamic lamellipodia formation (Movie S1) and highly branched, unpolarized protrusions (Figure 2.2D). We also evaluated the effects of YAP downregulation in fallopian tube nonciliated epithelial (FNE) cells, previously characterized nontransformed cells derived from the epithelium of the fallopian tube [16]. Consistent with our findings in MCF10A cells, we observed significant

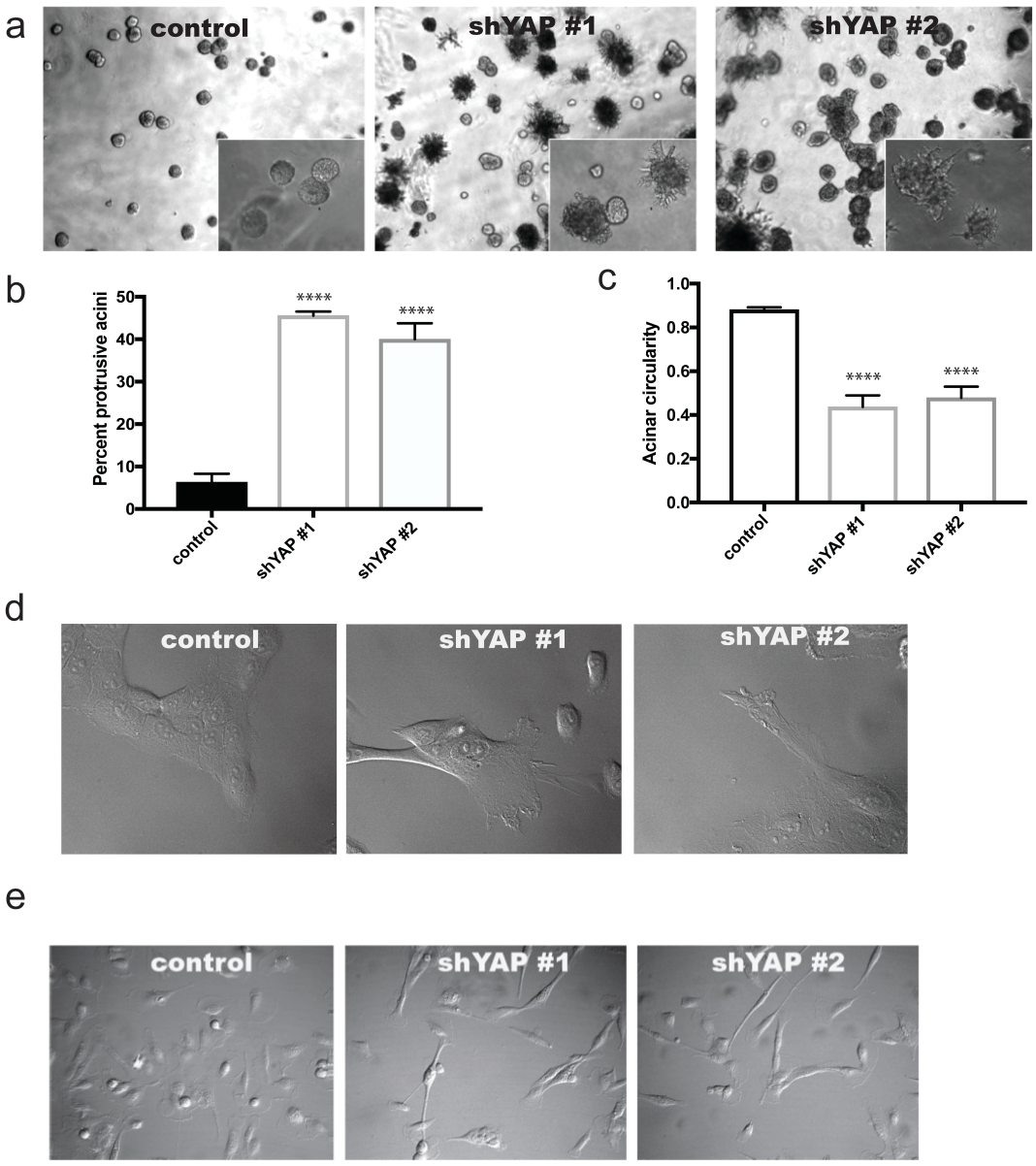

Figure 2.2 Loss of YAP promotes protrusive activity in nontransformed epithelial cells. A) Images from MCF10A 3D Matrigel cultures. MCF10A cells were infected with an empty lentiviral vector (control) or a vector encoding an shRNA against YAP (shYAP). B) Average number of protrusive acini of at least 100 acini from 3 independent experiments. C) Average acinar circularity of at least 50 acini from 3 independent experiments. D) DIC images from vector control and YAP knockdown MCF10A. E) DIC images from vector control and YAP knockdown FNE. **** $p< 0.0001$ by one-way ANOVA.

morphological changes in YAP knockdown FNE cells, which displayed an elongated phenotype relative to control cells (Figure 2.2E). The comparable phenotypic changes caused by YAP downregulation in mammary and fallopian tube epithelial cells suggest that YAP suppresses membrane protrusion.

Decreased YAP impairs cell-cell adhesion

To examine whether these changes in membrane protrusion were associated with changes in cell migration, we performed a timelapse wound-healing assay in MCF10A monolayer culture following YAP knockdown. Knockdown of YAP caused cellular dissociation from the leading edge, with individual cells migrating into the wound space (Figure 2.3A, Movie S2). We quantified cell dissociation by measuring the area of 'closed objects' at each timepoint, in order to capture individual cells that have detached from the leading edge (Figure 2.4A). We found a significant increase in the 'closed-object' area across the timecourse of imaging for both YAP knockdown lines relative to the control cells (Figure 2.3B), suggesting that YAP is required for collective cell migration and that decreased YAP alters cellular adhesion.

The impaired collective cell migration following YAP knockdown could be attributed to weakened cell-cell adhesion or weakened cell-matrix adhesion, both of which could contribute to cell scattering during wound-healing. To distinguish between these two types of adhesion, we examined whether binding to the junctional protein E-cadherin or the matrix protein fibronectin was altered following YAP knockdown [17].

YAP knockdown cells were plated on glass coverslips coated with either Fc-E-cadherin or fibronectin. We found that YAP knockdown did not affect binding to fibronectin (Figure 2.4B). In contrast, we observed a significant decrease in E-cadherin binding following YAP knockdown (Figure 2.3C), indicating that YAP knockdown specifically impairs E-cadherin mediated cell-cell adhesion, but not an integrin-dependent matrix adhesion, in MCF10A cells.

Weakened cell-cell junctions are one characteristic of epithelial-to-mesenchymal transition (EMT). Therefore, we assessed whether YAP knockdown cells expressed known EMT markers by Western blot (Figure 2.3D). We found unaltered expression of the epithelial marker E-cadherin or the mesenchymal markers N-cadherin and Vimentin. Additionally, mRNA expression of mesenchymal transcription factors was not significantly altered following YAP knockdown (Figure 2.4C). This suggests that the impaired cell-cell adhesion in YAP knockdown cells is not due to EMT.

To examine the morphology of adherens junctions in YAP knockdown cells, we performed immunofluorescence of the cell-cell junction component β-catenin. At sites of mature cell-cell contact, β-catenin forms a linear pattern, as seen in control cells (Figure 2.3E)[17]. YAP knockdown cells displayed less organized monolayers, and β-catenin staining displayed a "zipper-like" pattern, closely aligning with the sites of actin interdigitation at sites of nascent cell-cell contact (Figure 2.3E). This "zipper-like" staining is reminiscent of immature cell-cell junctions [18]. To quantify mature cell-cell junction formation, we conducted a line scan analysis of β-catenin staining intensity for control and YAP knockdown monolayers. A single peak of β-catenin fluorescence intensity at a site of cell-cell contact was scored as a mature junction; two or more peaks

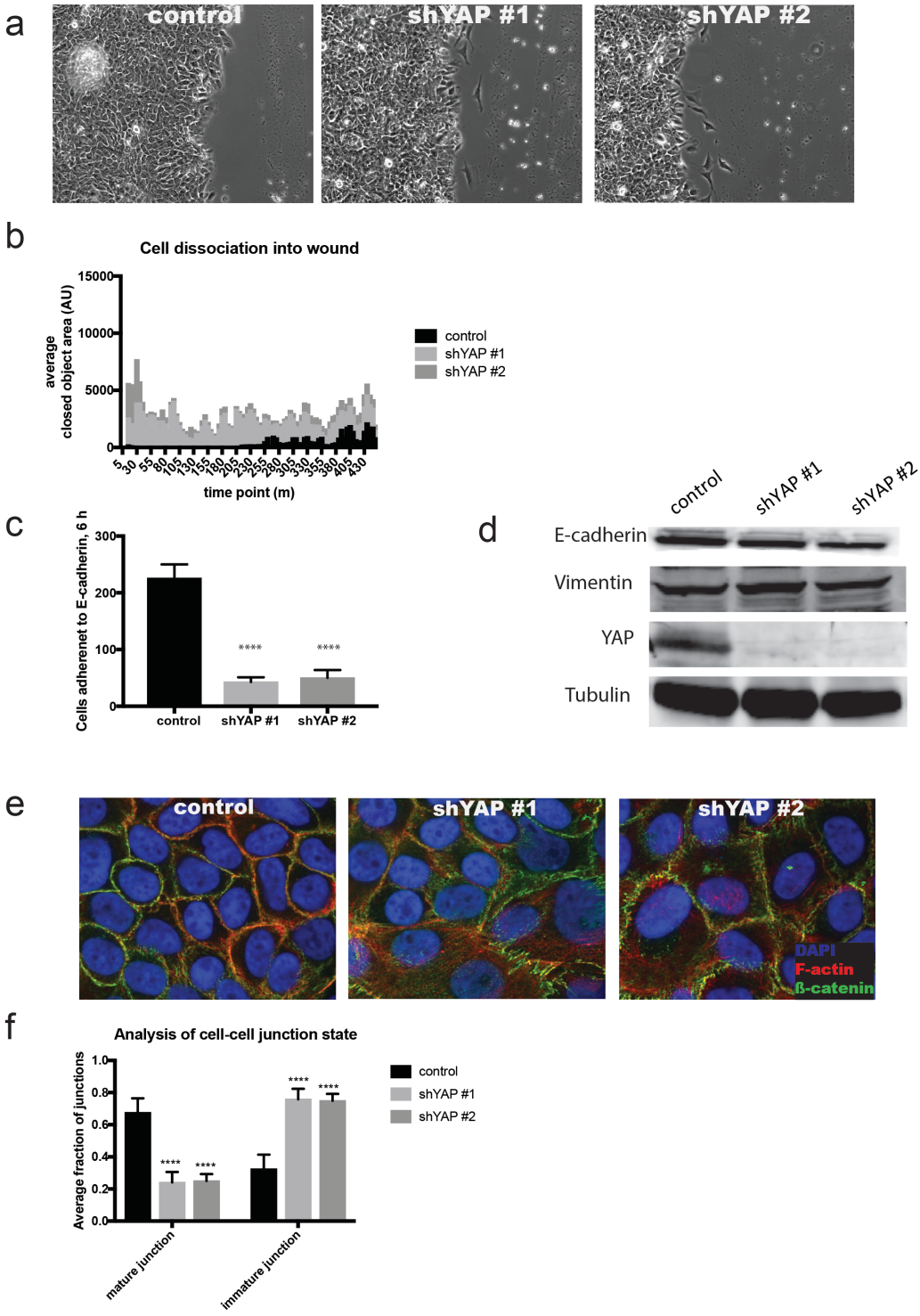

Figure 2.3 YAP is required for collective cell migration and cell-cell adhesion. A) Phase-contrast images from timelapse migration assay of vector control and YAP knockdown MCF10A. B) Quantification of average closed-object area entering wound over timelapse migration assay from (A). Average represents at least 15 fields of view from 3-

independent experiments. C) Number of cells adherent to E-cadherin 6-hours after seeding for vector control and YAP knockdown, from 3 wells, average of 3-independent experiments. D) Western blot for lysates from vector control and YAP knockdown cells for epithelial marker E-cadherin and mesenchymal marker Vimentin. Blots are representative of two independent experiments. E) Confocal images of confluent monolayers of MCF10A vector control and YAP knockdown cells stained with antibody to β-catenin (green), phalloidin for actin (red), and DAPI for nuclei (blue). F) Line-scan analysis of cell-cell junctions for control and YAP knockdown. Over 50 cells were analyzed from 3 independent experiments. **** $p < 0.0001$ by one-way ANOVA.

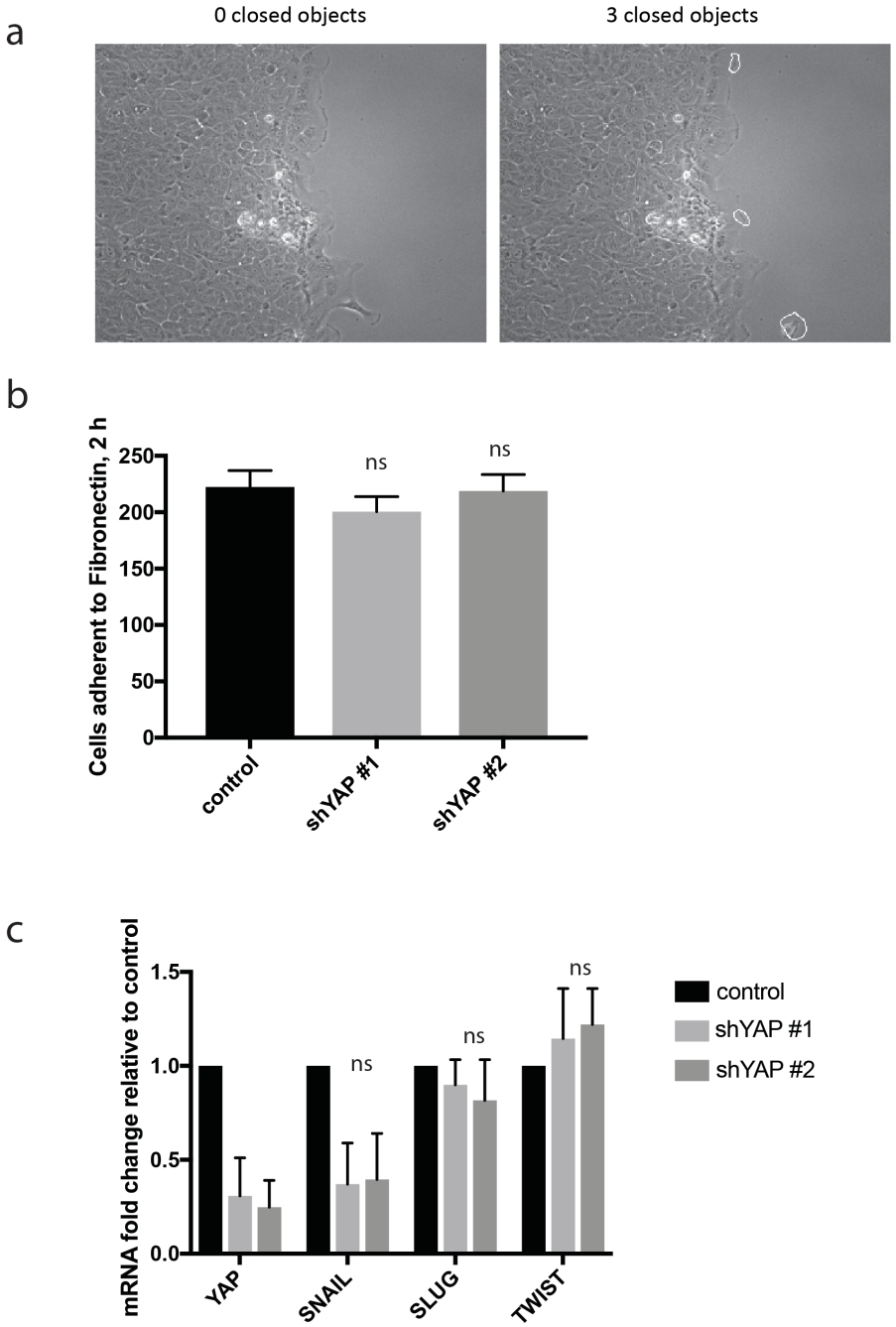

Figure 2.4 YAP regulates cell-cell adhesion independent of EMT. A) Representative detection of closed-objects from wound-healing assay. B) Number of cells adherent to Fibronectin 2-hours after seeding, from 3 wells, average of 3-independent experiments. C) qPCR analysis of mRNA expression levels of EMT transcription factors in YAP knockdown cells.

of fluorescence intensity was scored as a "zipper-like" junction. We observed a significant decrease in cell-cell junctional maturation in YAP knockdown cells relative to control cells (Figure 2.3F). Taken together, these data indicate that YAP is necessary for cell-cell junction maturation, and decreased YAP impairs cell-cell adhesion.

YAP knockdown protrusive phenotype is dependent on YAP regulation of RHO-GTPase activity

The RHO-family GTPases RHO and RAC are well-established regulators of the actin cytoskeleton, contributing to cellular morphogenesis and protrusion, adhesion, and migration [19]. RHOA inhibition decreases cellular contractility and destabilizes cell-cell junctions via altered actin remodeling [20] Several studies have demonstrated that YAP regulates RHOA GTPase activity [21-23]. To examine whether YAP knockdown alters RHOA activity in MCF10A cells, we examined the levels of active, GTP-bound RHOA. We found that RHOA-GTP was significantly decreased in YAP knockdown cells compared to vector control cells (Figure 2.5A). Importantly, total RHOA levels were unaltered, suggesting that YAP regulation of RHOA activity is at the posttranslational level and not directly through regulation of *RHOA* transcription.

Changes in RHOA activity are often associated with reciprocal changes in RAC activity, and indeed, several of our observed YAP knockdown phenotypes, such as increased lamellipodia protrusion and cellular invasion, are consistent with increased RAC activity [24, 25]. To examine if YAP knockdown alters RAC activity, we examined the levels of active, GTP-bound form of RAC. We found that RAC-GTP was

increased in YAP knockdown cells compared to vector control cells (Figure 2.5B) without changes in total RAC levels, suggesting that YAP regulation of RAC activity is not at the transcriptional level. Taken together, these findings indicate that YAP is necessary for RHOA activity and correspondingly decreased YAP is permissive for elevated RAC activity independent of direct transcriptional regulation of these GTPases.

A key downstream effector of RHOA activity is myosin phosphorylation and actomyosin contractility. To assess cellular contractility, we examined whether YAP knockdown affected the ability of the cells to contract collagen gels [9]. Initial characterization of MCF10A cell collagen gel contraction revealed that (1) contraction of the collagen gel increased with increased cell density, (2) treatment with blebbistatin, a myosin II inhibitor, suppressed gel contraction, and (3) induction of myosin contractility with G-Switch RHOA Activator II, which deamidates glutamine-63 and locks RHO in a GTP-bound state, increased gel contraction (Figure 2.6A, 2.6B); these data indicate that gel contraction in MCF10A cells is dependent on actomyosin contractility. Compared to control MCF10A cells, YAP knockdown cells were significantly impaired in their ability to contract the collagen matrix (Figure 2.5C, 2.5D). Taken together, these results demonstrate that YAP knockdown decreases actomyosin contractility.

We next examined whether the phenotypic changes induced by decreased YAP were dependent on altered RHO and RAC activity. To examine this question, we utilized pharmacological activation of RHOA or inhibition of RAC. We utilized the RHO-Activator G-Switch II and the RAC inhibitor EHop-016, which blocks interaction between RAC and the RAC GEF Vav2 [26, 27]. Compounds were added to the Matrigel cultures on Day 1, and replenished with standard media change every 4 days.

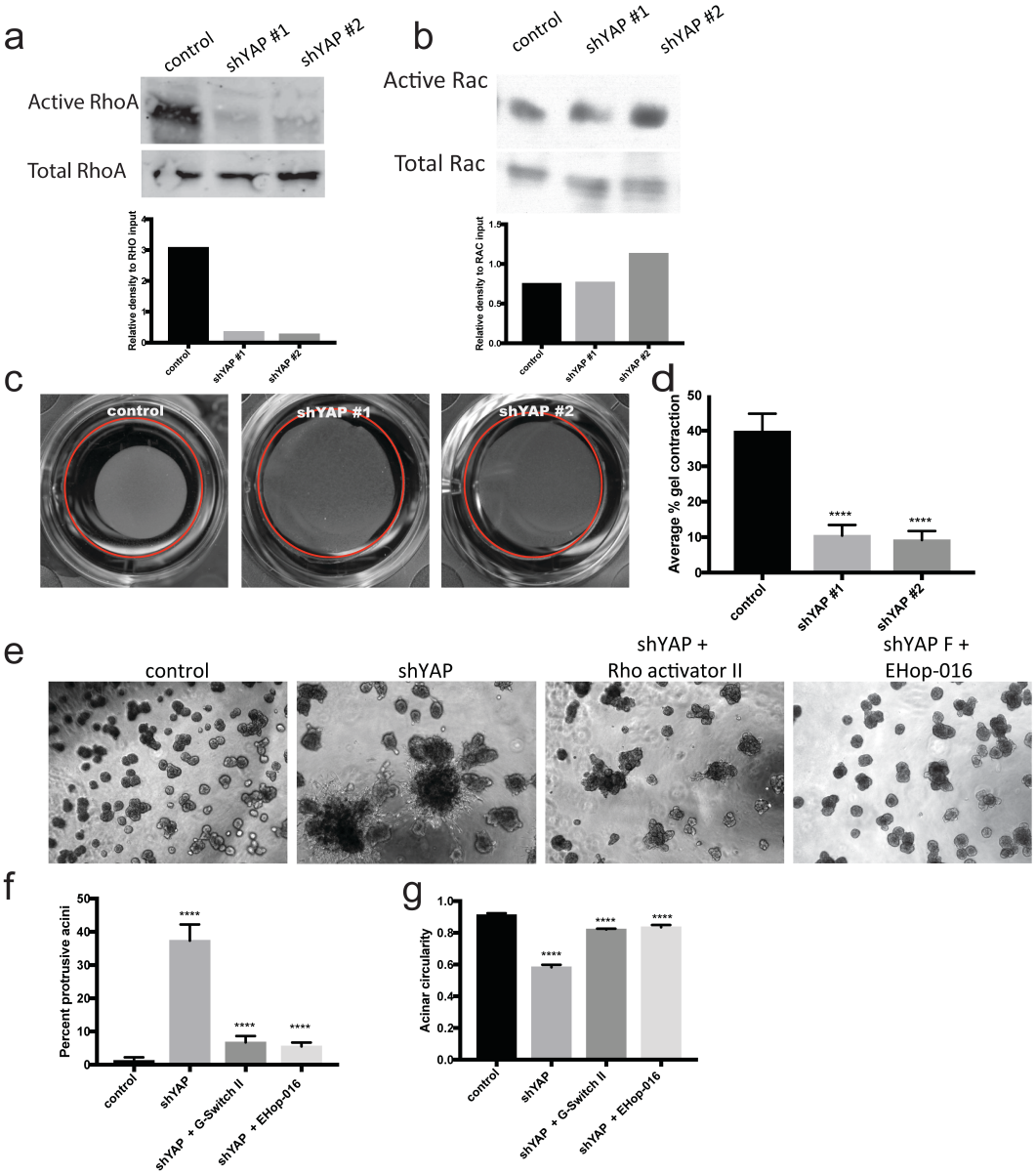

Figure 2.5 YAP regulation of RHO GTPase activity and cell contractility is required for suppression of protrusive behavior. A) Immunoprecipitation of active RHOA from using active RHOA-GTP antibody for vector control and YAP knockdown cells. Immunoblots are representative of 3 independent experiments. B) Immunoprecipitation of active RAC using active RAC-PBD assay for vector control and YAP knockdown cells. Immunoblots are representative of 3 independent experiments. C) Images from collagen gel contraction assay for vector control and YAP knockdown MCF10A. The red circle denotes the original gel size. D) Average percent collagen gel contraction from 2 wells, from 3 independent experiments. E) MCF10A 3D Matrigel culture images for vector control with no drug, YAP knockdown with no drug, YAP knockdown supplemented with RHO Activator G-Switch, and YAP knockdown supplemented with RAC Inhibitor EHop-016. F) Average acinar circularity of at least 50 acini from 3

Figure 2.5 (*continued*)
independent experiments. G) Average number of protrusive acini of at least 100 acini from 3 independent experiments. **** $p < 0.0001$ by one-way ANOVA.

Treatment of YAP knockdown cells with RHO-Activator G-Switch II caused a significant reduction of protrusions into the surrounding matrix. Similarly, inhibition of RAC activity in YAP knockdown cells reduced protrusive activity in Matrigel (Figure 2.5E-G). These data support the conclusion that protrusive activity resulting from decreased YAP is dependent on altered RHO family GTPase activity.

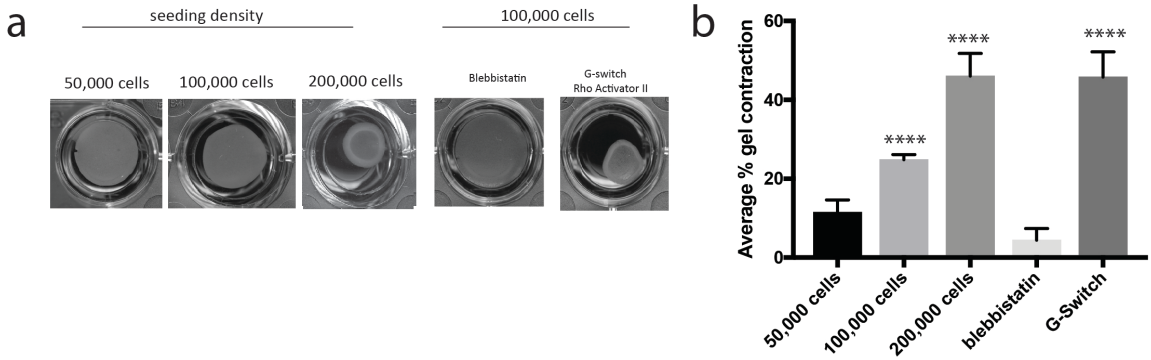

Figure 2.6 The collagen contractility assay can be utilized for MCF10A. A) Representative images from collagen gel contraction assay optimization at 3 seeding densities per well, and with phosphomyosin inhibitor blebbistatin and RHO-activator G-Switch. B) Average percent collagen gel contraction from 2 wells, from 3 independent experiments. **** $p < 0.0001$ by one-way ANOVA.

Inhibition of protrusive behavior is independent of YAP-TEAD transcriptional activity

YAP regulation of RHOA activity in other systems has been attributed to its transcriptional coactivation of TEAD transcription factors and transcriptional regulation

of RHOGAPs (ARHGAP18, ARHGAP29) or RHOGEFs (ARHGEF17) [21-23]. Gene expression analysis of these putative YAP targets did not reveal alterations following YAP knockdown in MCF10A cells, with the exception of a slight but significant decrease in ARHGAP29 expression levels (Figure 2.7). However, as decreased ARHGAP29 levels would not explain the observed phenotypes and decrease in RHO activity observed in MCF10A, we pursued other possible mechanisms of RHO regulation. To further examine if the phenotypes induced by YAP knockdown are due to its function as a transcriptional coactivator, we utilized a mutant YAP containing an alanine substitution of Serine 94 (YAP-S94A), which is unable to interact with TEAD proteins due to mutation of the TEAD-binding domain of YAP [3]. Following depletion of endogenous YAP, we selectively restored the TEAD-binding mutant YAP (shYAP + YAP-S94A) (Figure 2.8A). We confirmed that expression of YAP-S94A was unable to rescue TEAD-dependent transcriptional activity of canonical YAP-TEAD target genes in the YAP knockdown cells (Figure 2.8B). We cultured the shYAP + YAP-S94A cells in the 3D Matrigel assay and found that re-expression of the TEAD-binding mutant YAP was sufficient to restore the percentage of cells displaying protrusions to wild-type levels, and circularity was significantly increased in YAP-S94A rescued cells compared to YAP knockdown (Figure 2.8C-E). These data indicate that YAP inhibition of cellular invasion is primarily independent of its co-transcriptional activity with TEAD.

In addition, we assessed whether the changes in cellular contractility induced by YAP knockdown are dependent on YAP-TEAD transcriptional activity. We found that shYAP + YAP-S94A cells increased contraction of collagen gel to levels comparable to

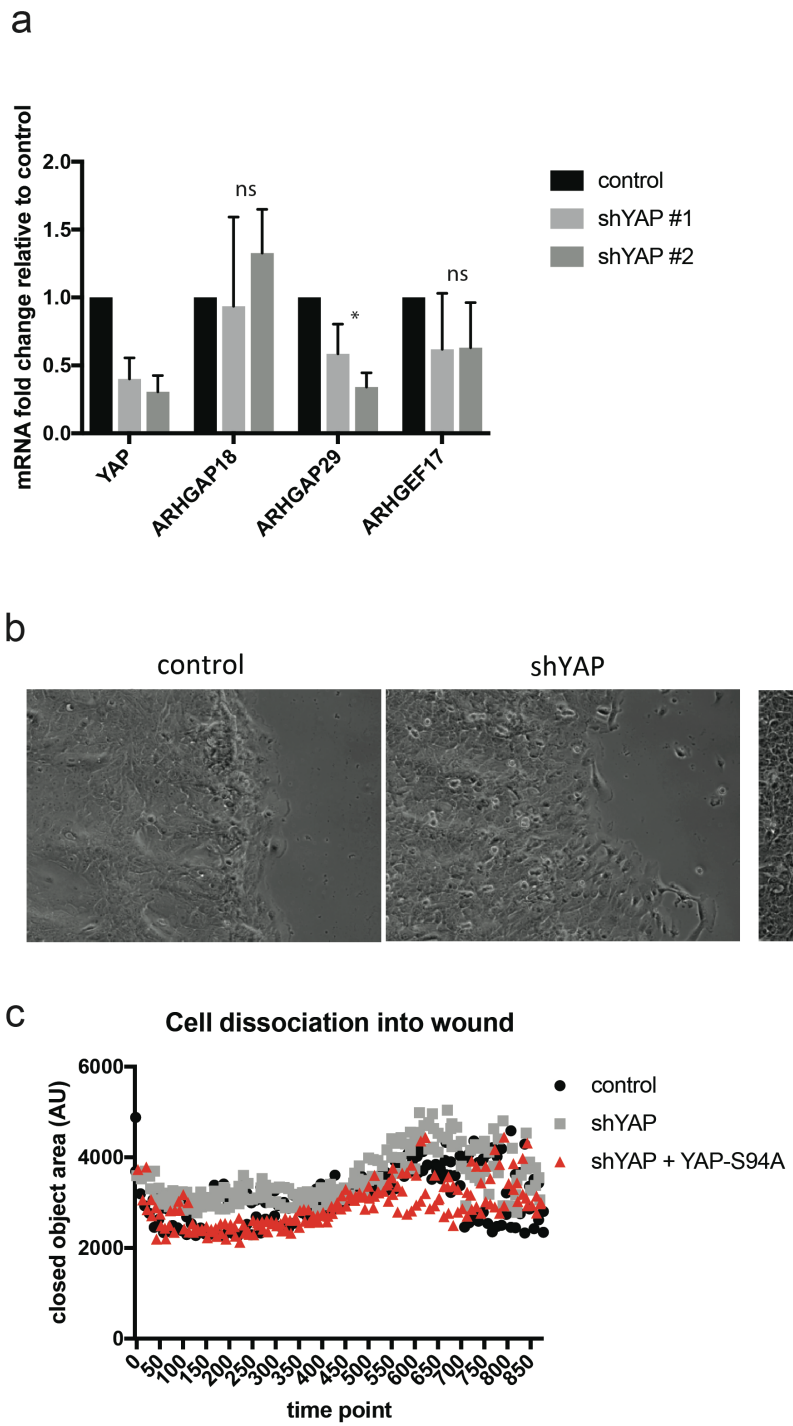

Figure 2.7 Analysis of YAP-TEAD regulation of RHO regulators and cell-cell adhesion. A) qPCR analysis of mRNA expression of published GAPs or GEFs that regulate RHO activity in YAP knockdown cells. B) Representative phase-contrast images from timelapse migration assay of MCF10A C) Quantification of average closed-object area entering wound over timelapse migration assay from (B). Average represents at least 15 fields of view from 2 independent experiments. * $p < 0.01$ by one-way ANOVA.

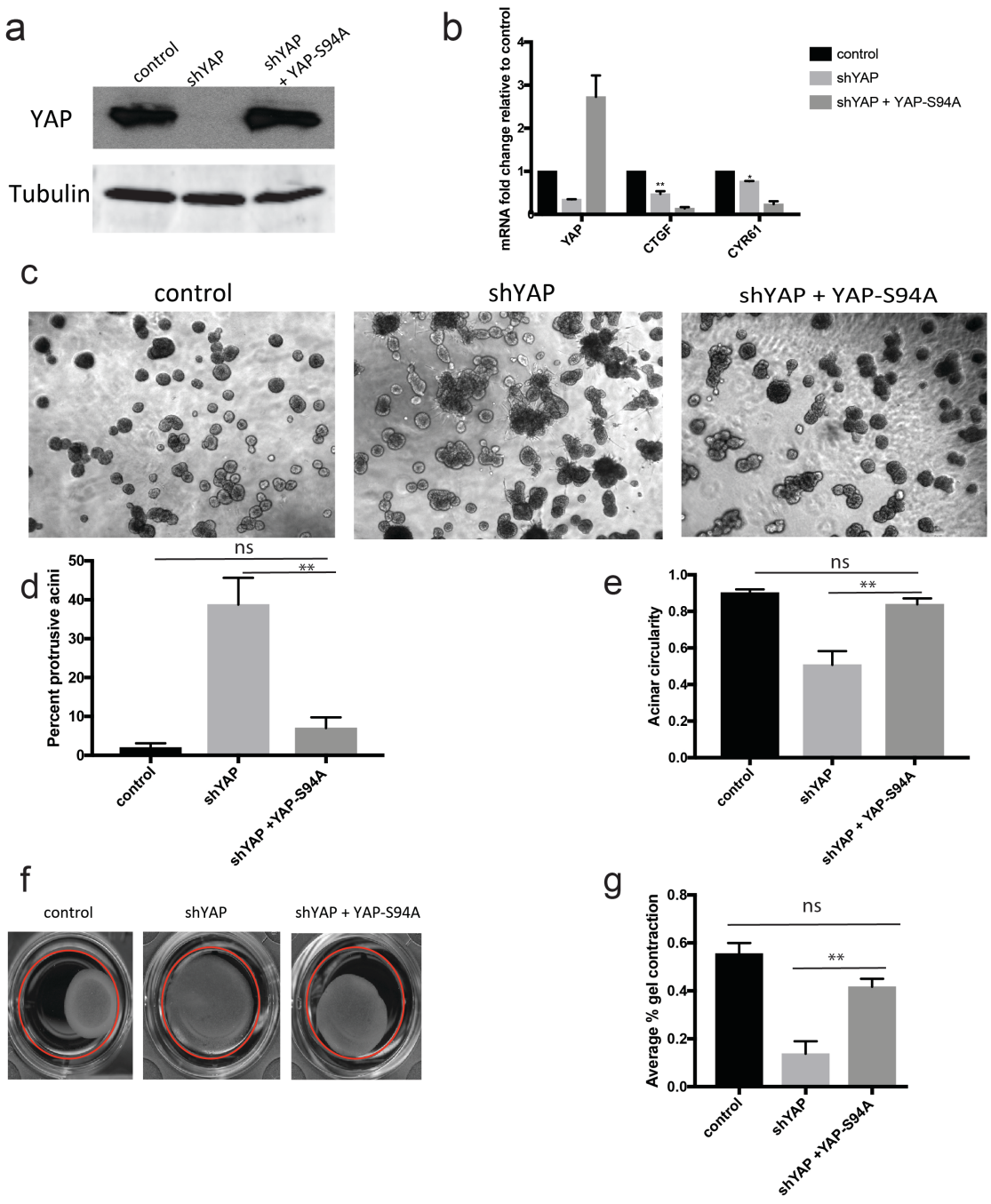

Figure 2.8 YAP suppression of protrusion and promotion of cell contractility is independent of TEAD-transcriptional activity. A) Western blot for vector control, shYAP, and shYAP + YAP-S94A lines. B) qPCR analysis of YAP and YAP-TEAD targets CTGF and CYR61 across lines. C) MCF10A 3D Matrigel culture images for control, shYAP, and shYAP + YAP-S94A lines. D) Average acinar circularity of at 50 least acini from 3 independent experiments. E) Average number of protrusive acini of at least 100 acini from 3 independent experiments. F) Images from collagen gel

Figure 2.8 (*continued*)
contraction assay for control, shYAP, and shYAP + YAP-S94A MCF10A. The red circle denotes the original gel size. G) Average percent collagen gel contraction from 2 wells, from 3 independent experiments. **** $p< 0.0001$, *** $p<0.001$, ** $p<0.01$ by one-way ANOVA.

In addition, we assessed whether the changes in cellular contractility induced by YAP knockdown are dependent on YAP-TEAD transcriptional activity. We found that shYAP + YAP-S94A cells increased contraction of collagen gel to levels comparable to control cells (Figure 2.8F). Additionally, we found that shYAP + YAP-S94A cells were capable of contracting collagen gels similar to wild-type MCF10A (Figure 2.8G-H). To examine if the YAP-TEAD interaction was required for dissociation of collectively migrating cells observed upon YAP knockdown, we assayed cell dissociation in a 'wound-healing' assay with the shYAP + YAP-S94A cells. While YAP-S94A rescued cells did not display complete restoration of collective cell migration, the amount of detachment was decreased relative to YAP knockdown, implying a partial rescue (Figure 2.7B-2.7C). This suggests that YAP regulation of collective cell migration is at least in part dependent on both YAP-TEAD transcriptional activity and on YAP regulation of RHO-GTPase activity.

YAP can serve as a co-activator of other transcription factors; therefore, in order to address whether inhibition of nuclear localization would prevent the rescue of the YAP-depletion phenotypes under investigation in this study, we examined the effects of inhibition of YAP nuclear translocation. Phosphorylated YAP is sequestered in the cytoplasm by 14-3-3 proteins, and upon dephosphorylation following changes in cellular tension or growth factor stimulation, YAP translocates to the nucleus where it functions as a transcriptional coactivator [28]. To directly examine if regulation of protrusion and

contractility by YAP is attributable to cytoplasmic or nuclear YAP, we performed an additional rescue experiment with YAP-5SD (YAP-5SD), a phosphomimetic mutant for the five serine sites of phosphorylation by the Hippo-kinase LATS (Ser^{61}, Ser^{109}, Ser^{127}, Ser^{164}, Ser^{381}) [29]. This YAP mutant displays predominantly cytoplasmic localization (Figure 2.9A, B). We cultured shYAP + YAP-5SD MCF10A cells under 3D Matrigel conditions and found that expression of phosphomimetic YAP is sufficient to inhibit protrusive acinar structures resulting from decreased YAP (Figure 2.9C). The percentage of cells expressing protrusions was significantly reduced and acinar circularity was restored in YAP-5SD rescue cells when compared to YAP knockdown (Figure 2.9D, E). Additionally, YAP-5SD cells were able to contract the collagen gel to wildtype levels (Figure 2.9F, G). These data further indicate that YAP inhibition of cellular protrusion involves a novel cytoplasmic function of YAP sufficient to restore wildtype cellular behavior independent of its transcriptional activity.

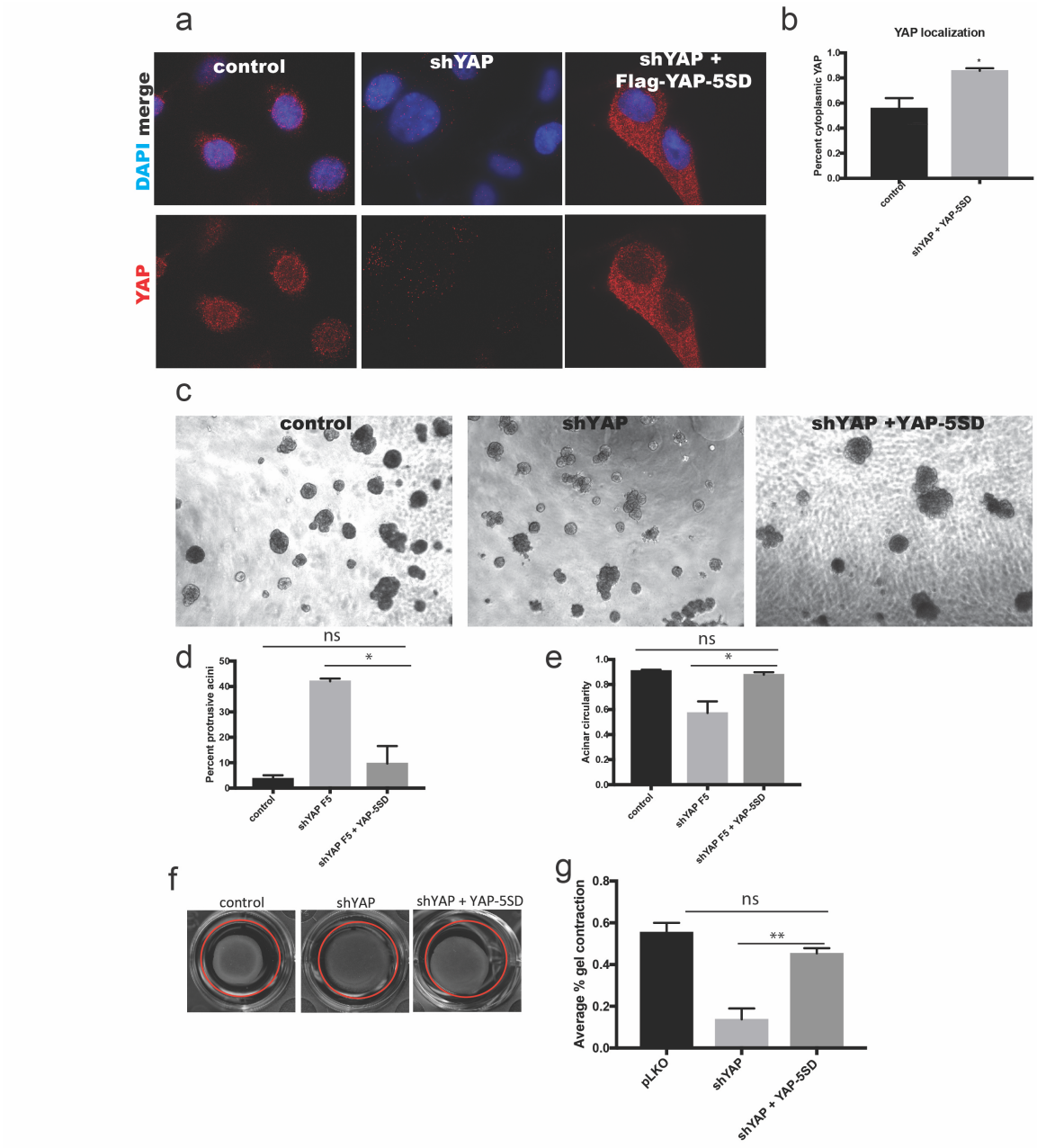

Figure 2.9 Cytoplasmic YAP-5SD suppresses YAP knockdown mediated acinar protrusiveness and promotes contractility. A) Confocal images of vector control, shYAP, and shYAP + YAP-5SD MCF10A cells stained with antibody to YAP (red) and DAPI for nuclei (blue). B) Immunofluorescence microscopy analysis of YAP localization from (A). C) MCF10A 3D Matrigel cultures for control, shYAP, and shYAP + YAP-5SD MCF10A lines. D) Average acinar circularity of at least 50 acini from 3 independent experiments. E) Average number of protrusive acini of at least 100 acini from 3 independent experiments. F) Images from collagen gel contraction assay for control, shYAP, and shYAP + YAP-5SD MCF10A. The red circle denotes the original gel size. G) Average percent collagen gel contraction from 2 wells, from 3 independent experiments. ** $p<0.001$, * $p<0.01$ by one-way ANOVA.

Discussion

To examine the role of YAP in interphase cellular morphology and behavior, we utilized the MCF10A model system and characterized cellular changes observed upon reduction of YAP expression. Here, we show that knockdown of YAP promotes cellular protrusion, impairs collective cell migration, decreases RHOA activity and cellular contractility and increases RAC activity levels (Figure 2.10). Wildtype activities could be rescued by YAP mutants defective for its transcriptional coactivator function, indicating that YAP regulation of these activities is independent of its canonical role as a transcriptional coactivator with TEAD, and rather is mediated by previously unrecognized nontranscriptional functions of mammalian YAP. Our findings suggest that alterations in cell behavior caused by translocation of YAP to the nucleus following Hippo inactivation can be mediated both through loss of cytoplasmic YAP and gain of YAP transcriptional activity.

We have found that knockdown of YAP alters RHOA and RAC activity levels, suggesting YAP maintains RHO GTPase activity levels within the cell. Recent studies have shown that YAP may regulate cellular mechanical behavior in addition to serving as a mechanosensor downstream of RHO. Mutation or downregulation of YAP in the Medaka fish resulted in tissue flattening through impaired cellular contractility; similarly, loss of YAP in the mouse lung led to branching defects resulting from decreased force production [22, 23]. In gastric cancers, YAP was found to regulate actin polymerization and stabilization [21]. All of these studies demonstrated that YAP regulates these cellular processes via transcriptional regulation of RHOA guanine nucleotide exchange factors

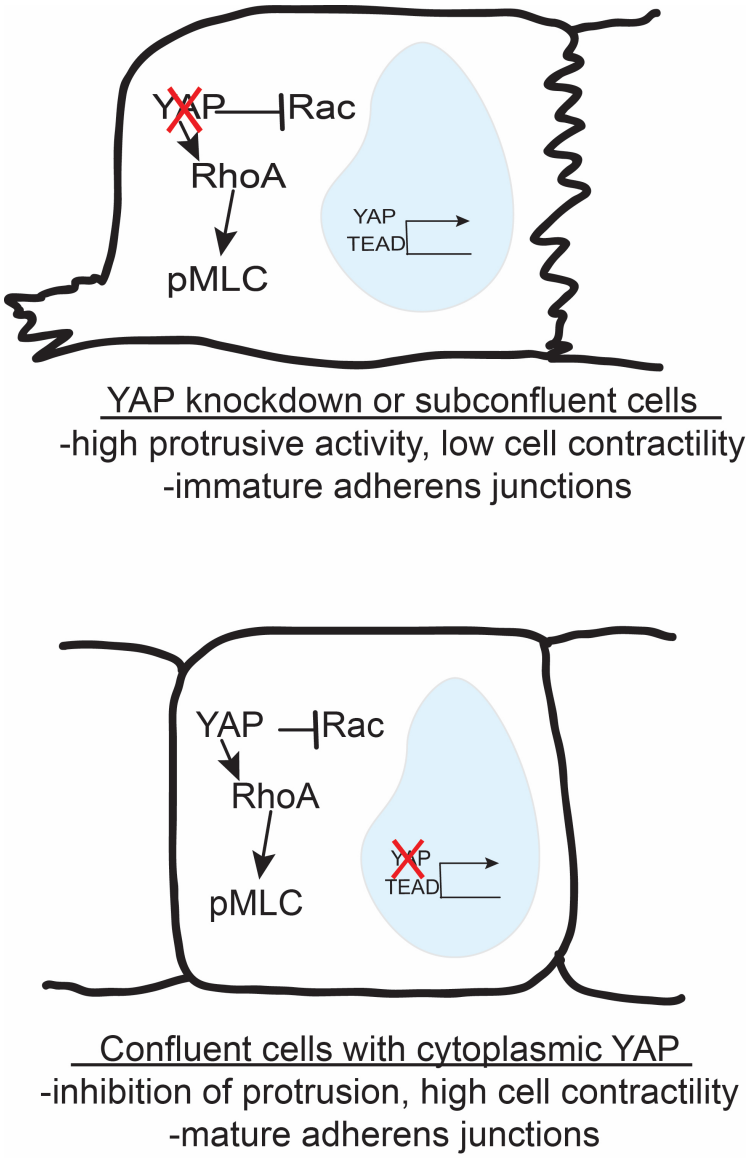

Figure 2.10 Model for YAP suppression of protrusive activity.

(GEFs) or GTPase-activating proteins (GAPs). Each study identified a different transcriptional target of YAP as the relevant RHO regulator, suggesting that the diverse roles and molecular mechanisms of YAP in RHO regulation are yet to be fully understood. Our data indicate that cytoplasmic YAP directly regulates RHOA activation and actomyosin contractility independent of transcriptional activity. Interestingly, Yorkie, the *Drosophila* homolog of YAP, was recently reported to function directly at the cell cortex to control myosin phosphorylation through interaction with myosin light chain kinase [30]. The myosin-interacting domain of Yorkie is not conserved in YAP, but our findings suggest mammalian YAP similarly maintains cellular contractility via regulation of RHO GTPase activity in the cytoplasm. Thus, YAP may have evolved to regulate cellular contractility through RHO through distinct mechanisms.

Additionally, we have identified a critical role for YAP in adherens junction maintenance and cell-cell adhesion. Though previous studies have identified YAP in complex with junctional components, this colocalization was interpreted as sequestration of YAP from its transcriptional function [31]. Our results suggest that YAP is not only responsive to cell-cell junction formation, but it is necessary for proper cell-cell junction maturation. Deregulation of RHO or RAC activities is a known source of cell-cell adhesion impairment. For example, myosin-mediated contractility regulated by RHO secures the adherens junction [32]. It has also been show that suppression of RHO activity associated with Profilin-2 knockdown causes reduction in F-actin bundling and actomyosin contractility, impaired membrane integrity, increased protrusive activity, weakened cell-cell adhesions and enhanced motility [14]. Similarly, downregulation of cell polarity protein PAR3 activates RAC signaling, altering cortical actin organization

and impairing E-cadherin junctional stability [33]. Given the link between junctional maturation, contact inhibition, and activation of Hippo, our findings suggest the existence of a negative feedback loop, where nuclear YAP transcriptionally drives proliferation; resultant contact inhibition and activation of Hippo would then force YAP out of the nucleus. Therefore, we hypothesize that YAP is both regulated by and necessary for contact inhibition of proliferation.

YAP has canonically been considered a transcriptional coactivator, and cytoplasmic YAP has been viewed as sequestered, inactive YAP. Here, we find that cytoplasmically-localized YAP is critical for normal cellular maintenance of cytoplasmic RHO activity. Our previous studies demonstrated that during cytokinesis, YAP and Crumbs Polarity Complex Component PATJ interact at the midbody ring for daughter cell orientation [12]. Proper RHOA localization and activation is required at the cleavage furrow site and is necessary for ingression of the membrane and eventual cleavage of the two new cells [34]. Loss of YAP or PATJ in MCF10A resulted in abnormal membrane blebbing and protrusions from the plane of cell division, consistent with altered RHOA activity. This regulation of RHOA and membrane integrity is similar to our observations; it is possible therefore that YAP and PATJ may interact to regulate cellular behaviors such as invasiveness, protrusiveness and cellular adhesion, prompting questions for future studies. The Crumbs complex (an evolutionarily conserved complex composed of core components Crumbs, PATJ, and PALS1) regulates tight junction formation, apico-basal cell polarity, and directional migration [35, 36]. Intriguingly, our studies suggest that YAP knockdown cells also display impaired directional migration (Movie S2 and data not shown). It has been well established that there are interactions

between YAP and Crumbs-interacting proteins; again, these findings have usually been interpreted as sequestration of YAP from the nucleus [11, 37, 38]. We predict that YAP may also be acting as a scaffold protein at the cell cortex with polarity proteins, possibly tethering a RHO-GEF such as SYX or regulating a RAC-GEF such as TIAM1 [39, 40]. Decreased YAP could perturb the levels of active RHO and RAC at the cortex, resulting in the phenotypic changes we observe. Further studies are needed to fully elucidate the precise RHO GAP or GEF regulated by YAP, to define relevant YAP interacting polarity proteins, and to examine the generalization of this novel regulation across species and tissue types.

While TEAD-independent processes appear to be a primary mechanism of YAP regulation of protrusion and contractility in MCF10A, we have also found decreased transcription of ARHGAP29, a RHOGAP. This GAP is a negative regulator of RHO activity; loss of YAP results in decreased levels of ARHGAP29 and consequently an increase in RHO activity in human gastric cancer cells [21]. It is possible that in transformed, depolarized cancer cells, the active RHO complexes are distinct from those observed in nontransformed cells, such as MCF10A. Additionally, loss of YAP from the cytoplasm following changes in adhesion and stiffening of the microenvironment would further promote dysregulation of RHO-GTPase activities in a transformed setting.

Though it has been well established that YAP can function as a potent oncogene through its nuclear function as a transcriptional coactivator of TEAD, previous findings from our lab and others suggest that decreased expression of YAP can also have tumor promoting consequences. In breast cancer specifically, YAP has been implicated as both an oncogene and tumor suppressor, with decreased YAP in several cancer cell lines

triggering anoikis resistance, taxol resistance, and invasion *in vivo* [2]. Invasive, hormone-negative breast cancers are strongly correlated with decreased YAP expression [41]. A study of breast tumors found that *YAP* mRNA was downregulated in over two-thirds of the cases, and two-thirds of these cases were from advanced stages III and IV [42]. One interesting possibility is that there is temporal selection for varied YAP expression during tumor progression, with a strong selective pressure for upregulation and increased YAP transcriptional activity early in tumorigenesis to drive proliferation, followed by selection for cells with lower YAP as the tumor progresses, with weaker cell-cell adhesion and promotion of invasion into the surrounding stroma in the metastatic cascade. Therefore, decreased total YAP could provide an alternate mechanism for tumor metastasis from the EMT observed in cases of YAP overexpression, resulting in multiple paths for tumor progression [4]. Furthermore, our findings suggest that loss of cytoplasmic YAP during tumor progression as epithelial properties such as cell-cell adhesion are lost would contribute to deregulation of homeostatic RHO GTPase levels; the consequent translocation of YAP to the nucleus would activate YAP-TEAD programming and potentially oncogenic programs. Our previous studies indicated that loss of YAP would also drive genomic instability through alterations in cytokinesis, thus accelerating additional alterations that would drive tumor progression [12]. This possible temporal dependency for YAP expression could explain the apparent dichotomy and conflicting reports of YAP function as an oncogene or tumor suppressor.

In summary, our findings reveal a previously unrecognized role for YAP in maintenance of normal epithelial morphology, and loss of YAP promotes protrusive cellular behavior, impairs cell-cell adhesion, and dysregulates RHO-GTPase activity.

Our studies highlight the importance of studying proteins in their normal cellular contexts to better elucidate how their biological functions can be perturbed in cancer.

Materials and Methods

Cell culture

MCF10A cells were cultured as described (http://brugge.med.harvard.edu/protocols). FTE cells were cultured in WIT-Fo Culture Media (FOMI, http://sylvester.org/shared-resources/live-tumor-culture-core/live-tumor-culture-core-products) supplemented with EGF (0.01 μg/ml; Sigma-Aldrich); insulin (20 μg/ml; Sigma-Aldrich); hydrocortisone (0.5 μg/ml); cholera toxin (25 ng/ml; Calbiochem), and 1% heat-inactivated FBS (Sigma-Aldrich). HEK293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics.

Plasmids

shRNAs in human pLKO.1 lentiviral vectors were used to knockdown YAP with puromycin selection. The RNAi Consortium (TRC) numbers used for YAP shRNAs are TRCN0000107265, TRCN0000107268, and TRCN0000107269. Plasmids containing mutant YAP (YAP-S94A, YAP-5SD) were cloned into pBabe with hygromycin selection and a Flag tag was added at the 5' end using the following primers: Forward CCGGGATCCACCATGGATTACAAGGATGACGACGATAAGATGGACCCCGGG CAGCAGCCGCCGC; Reverse CCGGAATTCCTATAACCATGTAAGAAAGCTTTC. YAP mutants were confirmed by sequencing.

Virus production and infection

For generation of lentiviral particles, HEK293T cells were transfected at 80% confluency with packaging plasmids Pax2, VSVG, and plasmid containing the gene of interest. For generation of retroviral particles, HEK293T cells were transfected at 80% confluency with packaging plasmid pCL-Ampho and plasmid containing the gene of interest. Virus was collected at 48 and 72 hours post-transfection and filtered through 0.45-μm filters. Recipient cells were plated to reach 30% confluency 24 hours after seeding, and virus was added to the cells at a dilution of 1:1 with media containing 8 μg/mL Polybrene. Cells were selected with the appropriate antibiotics 48 hours after infection.

Antibodies

The following antibodies were used in these studies: YAP XP (1:1000 WB, 1:200 IF, Cell Signaling, 14074S), TAZ (1:1000 WB, 1:200 IF, Cell Signaling, 2149), E-cadherin (1:1000 BD, 610182), Tubulin (1:2000 Cell Signaling, 3873), β-catenin (1:1000 BD, 610154), Vimentin (1:500 Cell Signaling, 5741S), N-cadherin (1:500 BD, 610921), RhoA (1:1000 Cell Signaling, 2117S), Rac (1:1000 BD, 610650).

Immunofluorescence staining and protein localization image analysis

Immunofluorescence staining was performed by fixing cells with 3.5% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton-X in PBS for 5 minutes, and blocked in 1% BSA/5% goat serum for 1 hour at room temperature. Samples were incubated with primary antibody at 4 degrees overnight, then washed with PBS. Samples were incubated with Alexa-Fluor conjugated secondary antibodies (Life Technologies) at room temperature for 1 hour then washed with PBS. Nuclei were counterstained with DAPI (Sigma). Actin was stained using Alexa568-phalloidin

(Invitrogen). Samples were mounted in 20 mM Tris, pH 8.0, 0.1% N-propyl gallate, 90% glycerol. Samples were visualized on a Nikon Ti motorized inverted microscope with Perfect Focus System with Yokagawa CSU-X1 spinning disk confocal with Spectral Applied Research Aurora Borealis modification and images were acquired with a Hamamatsu ORCA-ER cooled CCD camera.

To quantify nuclear/cytoplasmic localization of YAP and TAZ, images were analyzed in ImageJ. Confocal Z-stacks of DAPI and YAP/TAZ were projected at maximum intensity, images were thresholded, and a mask of DAPI was made. Using the image calculator, the DAPI mask was subtracted from YAP/TAZ to give cytoplasmic localization. Total YAP/TAZ was determined by analyzing particles to determine area, cytoplasmic YAP/TAZ was determined by analyzing particles for area of the calculated image, and nuclear YAP/TAZ was the difference between these two. Nuclear and cytoplasmic fractions were expressed as percentages of total.

Timelapse microscopy

Cells were plated on #1.5 MatTek dishes and allowed to adhere for at least 24 hours before imaging. Images were acquired at multiple stage positions using a Nikon Ti-E motorized inverted microscope with Perfect Focus, Nikon linear-encoded motorized stage, and a Hamamatsu ORCA-AG cooled CCD camera controlled by NIS elements image acquisition software. The microscope was fitted with a 37 °C Incubation Chamber containing 5% CO_2. Resulting movies were compressed and stitched using Quick Time Pro and Adobe After Effects software.

Lamellipodia protrusion

20,000 cells per condition were seeded in 6-well MatTek dishes. Cells were allowed to adhere overnight, washed three times with PBS, and switched to media devoid of EGF and serum. After 24 hours starvation, plates were moved to the Nikon microscope, and cells were stimulated with 20 ng/ml EGF. Images were acquired every 5 minutes for 24 hours.

3D Matrigel Culture and quantifications

Cells were cultured as previously described (Muthuswamy et al., 2001). For a detailed protocol see also (brugge.med.harvard.edu/protocols).

To quantify acinar circularity, images were analyzed in Fiji [43]. Analysis was set to include circularity, with the range from 0.00 to 1.00. Each acinus was outlined by freehand selection, and then analyzed. At least 50 acini were scored for circularity from each well.

To quantify percent invasiveness, acini with at least one protrusion into the surrounding matrix were counted as positive. Percentage invasiveness was expressed as 100 × (invasive acini/total acini). At least 100 total cells were counted from each well. Each condition was run in duplicate wells, and each experiment was repeated three times.

Adhesion assays

Wound-Healing assay:

50,000 cells were seeded in Ibidi-wound healing inserts (Ibidi). Cells were allowed to adhere for 24 hours before the silicon-insert was removed. Media was changed to

remove any cellular debris. For siRNA 50,000 cells were seeded in 24-well glass-bottom MatTek dishes, allowed to adhere overnight, and scratched with a 200 μL tip. Media was changed to remove any cellular debris. For both migration set-ups, images were acquired either by timelapse microscopy as described above, or phase-contrast images were acquired at timepoints using the Nikon Ti-E microscope.

Quantification of wound-healing timelapse microscopy:

Timelapse videos were saved as .nd2 files and analyzed with a custom script written in Matlab 2017a (Mathworks, USA). First, a median filter with kernel size of 3-by-3 was applied to each timepoint to remove noise. Then, cell movement over time was detected by taking the difference image. This involved subtracting each frame from its predecessor. A standard deviation filter of kernel size 3-by-3 was also applied to render these differences more visible, while suppressing noise in the background. A robust threshold for segmenting moving cells was chosen by sampling a region of the background of the difference image and taking the median intensity value plus 4 standard deviations. Any debris of size 50 pixels or smaller was eliminated. The total area of the binary cell mask was then computed for each timepoint and plotted, and repeated for each dataset as a batch. Masks were manually validated for each video.

Cell-cell adhesion maturation state:

Cell-cell adhesion maturation state was determined as previously described [17]. Briefly, 100,000 cells per well were seeded into 6-well glass-bottom MatTek dishes. After 48 hours, cells were fixed with 4% paraformaldehyde and stained as described above with an antibody to β-catenin. Goat anti-mouse Alexa488 secondary antibody, phalloidin-Alexa568, and DAPI were secondary fluorescent probes used. Images were acquired

with the Nikon spinning disk confocal. Images were analyzed in ImageJ by drawing a line perpendicular to the cell-cell junction, mid-way through the point of adhesion. Fluorescent intensity was plotted for each linescan. A junction was scored as "mature" if there was a single fluorescent peak, and "immature" if there were multiple peaks. Over 50 cells were scored for each condition from three independent experiments.

Quantitative adhesion assays:

12-well glass-bottom MatTek dishes were coated with either 5 μg/ml fibronectin for 30 minutes, or 50 μg/ml protein A for one hour, followed by 120 μg/ml E-cadherin (R&D) for 24 hours. Plates were then incubated for 30 minutes with 0.5 μg/ml BSA in PBS solution to block non-specific binding. Each well was washed with culture media. Cells were trypsinized, quenched with high-serum media, and 5,000 cells were seeded in each well. At indicated times, non-adherent cells were removed from each well by washing three times with media. Cells were fixed using 4% paraformaldehyde and stained with DAPI. Large-stitched images (5x5 fields) were acquired with a 4X objective lens on the Nikon Ti-E. Nuclei were counted in ImageJ by thresholding the image, and analyzing particles. Each condition was carried out in duplicate, and values are the result of three independent experiments.

Western blot analysis

Cells were lysed in ice-cold RIPA buffer supplemented with protease inhibitors, phosphatase inhibitors, and MG-132 (Sigma). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined by BCA assay (Thermo-Fisher Scientific) and protein concentrations were normalized. Lysates were boiled in 1× sample buffer for 5 minutes and resolved 4%–20% SDS-PAGE gradient

gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and then blocked with 5% BSA in TBS for 30 minutes at room temperature. Blots were probed with primary antibody at 4°C overnight. Membranes were probed with secondary antibodies coupled to either HRP or fluorophores, and developed either using Enhanced Chemi-Luminscent detection or imaging on the Odyssey Imaging System.

Analysis of RHO-GTPase activity

Active RHOA pulldown

RHO activity was assessed by pulldown with a RHO-GTP bound specific antibody (NewEast Biosciences, 26904). All steps were conducted at 4°C. Cells were washed with PBS and lysed in mammalian cell lysis buffer (MCLB; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40). Lysates were cleared by centrifugation at 14,000 rpm for 5 minutes and snap-frozen in liquid nitrogen. Protein concentration was determined and normalized by BCA assay as above, and 10% of lysate was reserved as input for western blot analysis. 5 µg of RHO-GTP antibody was added with 30 µl of prewashed protein A/G bead slurry (Santa Cruz) and rocked at 4°C for 1 hour. Beads were washed once, boiled in 2× sample buffer, and levels of active RHOA were resolved by western blot as described above.

RAC-activity assay

RAC activity was assessed by the PAK-PBD pulldown assay. Cells were washed with PBS and lysed in MCLB. Lysates were cleared by centrifugation, and 10 µl PAK-PBD beads were incubated with each lysate at 4°C for 1 hour. Beads were washed once, boiled in 2× sample buffer, and levels of active RAC were resolved by western blot as described above.

RHOA activator and RAC inhibitor culture

3D cultures were maintained with the addition of 1μM RHO activator (RHO Activator II, Cytoskeleton Inc.) or 1μM RAC inhibitor EHop-016 (Selleckchem). New drug was added every 4 days along with the standard media change.

Collagen contraction assay

2×10^5 cells were embedded in 200 μL of Collagen I:Matrigel and seeded on 24-well, glass-bottom MatTek plates. Once the gel was set, it was loosened from the edge of the well using a 200 μLtip. Cells were maintained in MCF10A growth media and imaged daily using an Epson 3000 scanner. Gel contraction was determined after 4 days. To obtain the gel contraction, the area of the well and area of the gel disc were measured in ImageJ. Percentage contraction was calculated as 100 × (gel disc area/well area). Blebbistatin (Sigma) or Rho Activator (Cytoskeleton) was added as negative and positive controls, respectively, at 1μM doses. Media was replaced daily.

Quantitative RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen), and up to 2 μg of RNA were subjected to cDNA synthesis (Quanta). Real-time PCR was carried out using the Power SYBR Green PCR Mix (Applied Biosystems) on a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). Relative gene expression was determined by normalizing to RPLP0 control. Values are the average of three wells, and three independent replicates. qPCR probes used in these studies are available in Table 2.1.

Table 2.1 List of qPCR probes used in this study.

Gene target	Forward Primer	Reverse Primer
RPLP0	5′-ACGGGTACAAACGAGTCCTG-3′	5′-CGACTCTTCCTTGGCTTCAA-3′
YAP	5′-ATCCCAGCACAGCAAATTCT-3′	5′-TGGATTTTGAGTCCCACCAT-3′
CTGF	5′-CCTGCAGGCTAGAGAAGCAG-3′	5′-TGGAGATTTTGGGAGTACGG-3′
CYR61	5’-AAGAAACCCGGATTTGTGAG-3’	5’-GCTGCATTTCTTGCCCTTT-3’
TWIST1	5′-AGCTACGCGTTCTCGGTC-3′	5′-GGCGTTTGGAGTGGTAGAAA-3′
SNAI1	5′-CCTCCCTGTCAGATGAGGAC-3′	5′-CCAGGCTGAGGTATTCCTTG-3′
SNAI2 (Slug)	5′-TGGTTGCTTCAAGGACACAT-3′	5′-GCAATGCTCTGTTGCAGTG-3′
ARHGAP18	5′-GGGAACAGCCATGCAAAG-3′	5′-GTACTGGCCATATCTGCGACT-3′
ARHGAP29	5′-TTAGCACAACTCCGGACACTT-3′	5′-TGCATGTGGAAGAGGTTAACTG-3′
ARHGEF17	5′-GAACCGAGTCCTTGTCCTGA-3′	5′-TGAATCCTGACCCACGTAAAA-3′

References

1. Moroishi T, H.C., Guan KL., *The emerging roles of YAP and TAZ in cancer.* Nature Reviews Cancer, 2015. **15**(2): p. 73-79.

2. Yuan M, T.V., Lara R, Holliday D, Chelala C, Harada T, Gangeswaran R, Manson-Bishop C, Smith P, Danovi SA, Pardo O, Crook T, Mein CA, Lemoine NR, Jones LJ, Basu S, *Yes-associated protein (YAP) functions as a tumor suppressor in breast.* Cell Death and Differentiation, 2008. **15**(11): p. 1752-1759.

3. Zhao B, Y.X., Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL, *TEAD mediates YAP-dependent gene induction and growth control.* Genes and Development, 2008. **22**(14): p. 1962-1971.

4. Overholtzer M, Z.J., Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, Haber DA, *Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon.* PNAS, 2006. **103**(33): p. 12405-12410.

5. Lamar JM, S.P., Liu H, Schindler JW, Jiang ZG, Hynes RO, *The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain.* Proceedings of the National Academy of Science, 2012. **109**(37): p. E2441-2450.

6. Watt KI, H.K., Gregorevic P, *Regulation of Tissue Growth by the Mammalian Hippo Signaling Pathway.* Frontiers in Physiology, 2017. **8**: p. 942.

7. Low BC, P.C., Shivashankar GV, Bershadsky A, Sudol M, Sheetz M, *YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth.* FEBS Letters, 2014. **588**: p. 2663-2670.

8. Dupont S, M.L., Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S., *Role of YAP/TAZ in mechanotransduction.* Nature, 2011. **474**(7350): p. 179-183.

9. Calvo F, E.N., Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, Harrington K, Williamson P, Moeendarbary E, Charras G, Sahai E, *Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts.* Nature Cell Biology, 2013. **15**(6): p. 637-646.

10. Das, A., Fischer R. S., Pan, D., Waterman, C.M., *YAP nuclear localization in the absence of cell-cell contact is mediated by a filamentous actin-dependent, myosin II- and phospho-YAP-independent pathway during extracellular matrix mechanosensing.* Journal of Biological Chemistry, 2016. **291**: p. 6096-6110.

11. Hauri S, W.A., van Drogen A, Varjosalo M, Tapon N, Aebersold R, Gstaiger M, *Interaction proteome of human Hippo signaling: modular control of the co‐activator YAP1.* Molecular Systems Biology, 2013. **9**(1).

12. Bui DA, L.W., White AE, Harper JW, Schackmann RC, Overholtzer M, Selfors LM, Brugge JS., *Cytokinesis involves a nontranscriptional function of the Hippo pathway effector YAP.* Science Signaling, 2016. **9**(417).

13. Carey SP, M.K., Reinhart-King CA, *Three-dimensional collagen matrix induces a mechanosensitive invasive epithelial phenotype.* Scientific Reports, 2017. **7**.

14. Gallegos LL, N.M., Sowa ME, Selfors LM, White A, Zervantonakis IK, Singh P, Dhakal S, Harper JW, Brugge JS, *A protein interaction map for cell-cell adhesion regulators identifies DUSP23 as a novel phosphatase for β-catenin.* Scientific Reports, 2016. **6**.

15. Debnath J, B.J., *Modelling glandular epithelial cancers in three-dimensional cultures.* Nature Reviews Cancer, 2005. **5**: p. 675-688.

16. Mouneinme G, H.S., Selfors LM, Petrak L, Hickey MM, Gallegos LL, Simpson KJ, Lim J, Gertler FB, Hartwig JH, Mullins RD, Brugge JS, *Differential Remodeling of Actin Cytoskeleton Architecture by Profilin Isoforms Leads to Distinct Effects on Cell Migration and Invasion.* Cancer Cell, 2012. **22**(5): p. 615-630.

17. Witt, A., Hines, LM, Collins, NL, Hu, Y, Gunawardane, RN, Moreira, D, Raphael, J, Jepson, D, Koundinya, M, Rolfs, A, Taron, B, Isakoff, SJ, Brugge, JS, LaBaer, J, *Functional Proteomics Approach to Investigate the Biological Activities of cDNAs Implicated in Breast Cancer.* Journal of Proteome Research, 2006. **5**(3): p. 599-610.

18. Iwanicki MP, C.H., Iavarone C, Zervantonakis IK, Muranen T, Novak M, Ince TA, Drapkin R, Brugge JS, *Mutant p53 regulates ovarian cancer transformed phenotypes through autocrine matrix deposition.* JCI Insight, 2016. **1**(10).

19. Vasioukhin V, B.C., Yin M, Fuchs E, *Directed actin polymerization is the driving force for epithelial cell-cell adhesion.* Cell, 2000. **100**: p. 209-219.

20. Begum R, N.-E.-K.M., Zaman MA, *The role of Rho GTPases in the regulation of the rearrangement of actin cytoskeleton and cell movement.* Experimental & Molecular Medicine, 2004. **36**: p. 358-366.

21. Murrell M, O.P., Lenz M, Gardel ML, *Forcing cells into shape: the mechanics of actomyosin contractility.* Nature Reviews Molecular Cell Biology, 2015. **16**: p. 486-498.

22. Qiao Y, C.J., Lim YB, Finch-Edmondson ML, Seshachalam VP, Qin L, Jiang T, Low BC, Singh H, Lim CT, Sudol M, *YAP Regulates Actin Dynamics through ARHGAP29 and Promotes Metastasis.* Cell Reports, 2017. **19**(8): p. 1495-1502.

23. Lin C, Y.E., Zhang K, Jiang X, Croll S, Thompson-Peer K, Chuang PT, *YAP is essential for mechanical force production and epithelial cell proliferation during lung branching morphogenesis.* eLIFE, 2017.

24. Porazinski S, W.H., Asaoka Y, Behrndt M, Miyamoto T, Morita H, Hata S, Sasaki T, Krens SFG, Osada Y, Asaka S, Momoi A, Linton S, Miesfeld JB, Link BA, Senga T, Shimizu N, Nagase H, Matsuura S4, Bagby S, Kondoh H, Nishina H, Heisenberg CP, Furutani-Seiki M, *YAP is essential for tissue tension to ensure vertebrate 3D body shape.* Nature, 2015. **521**(7551): p. 217-221.

25. Bishop AL, H.A., *Rho GTPases and their effector proteins.* Biochemical Journal, 2000. **348**(2): p. 241-255.

26. Gratacap MP, P.B., Nieswandt B, Offermanns S, *Differential Regulation of Rho and Rac through Heterotrimeric G-proteins and Cyclic Nucleotides.* Journal of Biological Chemistry, 2001. **276**: p. 47906.

27. Schmidt G, S.P., Wilm M, Selzer J, Mann M, Aktories K, *Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1.* Nature, 1997. **387**(6634): p. 725-729.

28. Montalvo-Ortiz BL, C.-P.L., Hernández E, Humphries-Bickley T, De la Mota-Peynado A, Cubano LA, Vlaar CP, Dharmawardhane S., *Characterization of EHop-016, Novel Small Molecule Inhibitor of Rac GTPase.* Journal of Biological Chemistry, 2012. **287**: p. 13228-13238.

29. Elosegui-Artola A, A.I., Beedle AEM, Lezamiz A, Uroz M, Kosmalska AJ, Oria R, Kechagia JZ, Rico-Lastres P, Le Roux AL, Shanahan CM, Trepat X, Navajas D, Garcia-Manyes S, Roca-Cusachs P, *Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores.* Cell, 2017. **171**(6): p. 1397-1410.

30. Zhao Y, K.P., Savage P, She YM, Cyr TD, Yang X, *YAP-Induced Resistance of Cancer Cells to Antitubulin Drugs Is Modulated by a Hippo-Independent Pathway.* Cancer Research, 2014. **74**(16): p. 4493-4503.

31. Xu J, V.P., Ludwig M, Sherzod TS, Tokamov A, Fehon RG, *Yorkie Functions at the Cell Cortex to Promote Myosin Activation in a Non-transcriptional Manner.* Developmental Cell, 2018. **46**(3): p. 271-284.

32. Schlegelmilch K, M.M., Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR, Camargo FD, *Yap1 acts downstream of α-catenin to control epidermal proliferation.* Cell, 2011. **144**(5): p. 782-795.

33. le Duc Q, S.Q., Blonk I, Sonnenberg A, Wang N, Leckband D, de Rooij J., *Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner.* Journal of Cell Biology, 2010. **189**(7): p. 1107-1115.

34. Xue B, K.K., Allred DC, Muthuswamy SK, *Loss of Par3 promotes breast cancer metastasis by compromising cell-cell cohesion.* Nature Cell Biology, 2015. **15**(2): p. 189-200.

35. Normand G, K.R., *Understanding Cytokinesis Failure.* Adv Exp Med Biol, 2010. **676**: p. 27-55.

36. Shin K, S.S., Margolis B, *PATJ regulates tight junction formation and polarity in mammalian epithelial cells.* Journal of Cell Biology, 2005. **168**: p. 705-711.

37. Shin K, W.Q., Margolis B, *PATJ regulates directional migration of mammalian epithelial cells.* EMBO Reports, 2007. **8**: p. 158-164.

38. Yi C, S.Z., Stemmer-Rachamimov A, Dawany N, Troutman S, Showe LC, Liu Q, Shimono A, Sudol M, Holmgren L, Stanger BZ, Kissil JL, *The p130 isoform of angiomotin is required for Yap-mediated hepatic epithelial cell proliferation and tumorigenesis.* Science Signaling, 2013. **6**(291).

39. Zhao B, L.L., Lu Q, Wang LH, Liu CY, Lei Q, Guan KL, *Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein.* Genes and Development, 2011. **25**(1): p. 51-63.

40. Diamantopoulou Z, W.G., Fadlullah MZH, Dreger M, Pickering K, Maltas J, Ashton G, MacLeod R, Baillie GS, Kouskoff V, Lacaud G, Murray GI, Sansom OJ, Hurlstone AFL, Malliri A, *TIAM1 Antagonizes TAZ/YAP Both in the Destruction Complex in the Cytoplasm and in the Nucleus to Inhibit Invasion of Intestinal Epithelial Cells.* Cancer Cell, 2017. **31**(5): p. 621-634.

41. Ernkvist M, L.P.N., Audebert S, Lecine P, Sinha I, Liu M, Schlueter M, Horowitz A, Aase K, Weide T, Borg JP, Majumdar A, Holmgren L., *The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells.* Blood, 2009. **113**(1): p. 244-253.

42. Tufail R, J.M., Zhao W, Reis I, Nawaz Z, *Loss of Yes-associated protein (YAP) expression is associated with estrogen and progesterone receptors negativity in invasive breast carcinomas.* Breast Cancer Research Treatment, 2012. **131**(3): p. 743–750.

43. Real SAS, P.F., Rehman AU, Khan MA, Deo SVS, Shukla NK, Husain SA, *Aberrant Promoter Methylation of YAP Gene and its Subsequent Downregulation in Indian Breast Cancer Patients.* BMC Cancer, 2018. **18**(711).

Chapter 3.

Conclusions and Future Directions

My dissertation work uncovers a novel function of YAP in maintenance of breast epithelial biology. In Chapter 2, I have described a novel, TEAD-independent role of YAP in regulation of cellular protrusion, cell-cell adhesion, and cytoskeletal integrity through regulation of RHO GTPases. Our studies highlight the complex biology of YAP and the importance of examining protein function in multiple tissues and cellular contexts.

TEAD-independent YAP regulation of RHO activity

Our studies reveal an inhibitory function of YAP in normal mammary epithelial cell protrusive behavior. Knockdown of YAP promotes protrusive behavior as evidenced by both increased lamellipodia formation in monolayer culture and protrusive spikes extruding from acini grown in 3D culture. Additionally, we find that cell-cell adhesion is impaired in YAP knockdown cells through impeded junctional maturation. These phenotypes are due to aberrant levels of RHO GTPase activity resulting from YAP knockdown, specifically, an increase in RAC activity and a decrease in RHOA activity, triggering a subsequent downregulation of myosin phosphorylation and cellular contractility. We find that YAP regulation of RHO activity levels is independent of its transcriptional function with TEAD, and is instead due to a novel cytoplasmic function of YAP. These observations suggest that YAP acts in both mechanotransduction, as a responder to matrix stiffness and the cytoskeleton, and mechanoregulation, as a regulator of the cytoskeleton.

Mechanism of YAP regulation of RHO

Our findings regarding non-transcriptional YAP regulation of RHO open many avenues for follow-up studies. The most apparent unanswered question is what is the precise molecular mechanism through which YAP regulates RHO in the cytoplasm? We have conducted preliminary studies that implicate polarity proteins, particularly the Crumbs polarity complex component PATJ, as relevant YAP interactors for this cytoskeletal maintenance.

Potential interaction of YAP and polarity proteins to inhibit protrusiveness

Our lab previously identified an interaction between YAP and the Crumbs polarity complex component PATJ that is necessary for proper cytokinesis in mitosis. Interestingly, the two proteins co-immunoprecipitate, and knockdown of PATJ produces a comparable hyperdynamic mitosis phenotype to that observed upon YAP knockdown. We therefore hypothesized that PATJ and/or other polarity proteins may play a role in the interphase cellular processes we described in Chapter 2.

To identify a relevant cytoplasmic interactor for YAP, we analyzed previously published mass spectrometry data of proteins that co-immunoprecipitate with YAP in MCF10A, and selected several of the cytoplasmic proteins with highest interaction score [1]. Utilizing siRNA technology, we sought to examine if a decrease in any of the cytoplasmic YAP interactors phenocopied the impaired collective cell migration observed following knockdown of YAP. We assessed collective migration of cells

transduced with siRNAs targeting the angiomotin-like tight junction protein AMOTL2, the cytoskeletal protein NF2, the non-receptor protein phosphatase PTPN14, or the Crumbs cell polarity complex component PATJ. Knockdown was verified by mRNA expression (Figure 3.1A). siCTNNB1 (β-catenin) was used as a positive control for dissociation, and siYAP was utilized as a reference.

As expected, we observed significant cell dissociation with siCTNNB1 and, as we previously characterized, with siYAP. We also observed cell dissociation with siNF2, siAMOTL2, and siPATJ. As NF2 functions upstream of YAP, we did not follow-up on this hit, as we reasoned that knockdown of NF2 would promote YAP transcriptional activity and cause EMT [2]. Instead, we focused on the dissociation caused by reduction of PATJ (Figure 3.1B, C). siPATJ did not fully recapitulate the extent of cell dissociation induced by knockdown of YAP. This result is expected, given that the TEAD-binding mutant of YAP only partially rescued the dissociator phenotype induced by YAP knockdown.

Interestingly, knockdown of PATJ has previously been shown to be sufficient to induce invasiveness in MCF10A acini grown in Matrigel [3]. To examine if YAP and PATJ are functioning in the same pathway to inhibit protrusiveness, we created a YAP-PATJ double knockdown and examined acinar morphogenesis in 3D culture (Figure 3.2D). Decrease of PATJ alone resulted in significantly lower levels of protrusion than that observed when YAP alone is decreased. We found that combinatorial knockdown of YAP and PATJ did not result in significantly increased protrusive activity relative to knockdown of YAP alone (Figure 3.2E, F). These data suggest that YAP functions with

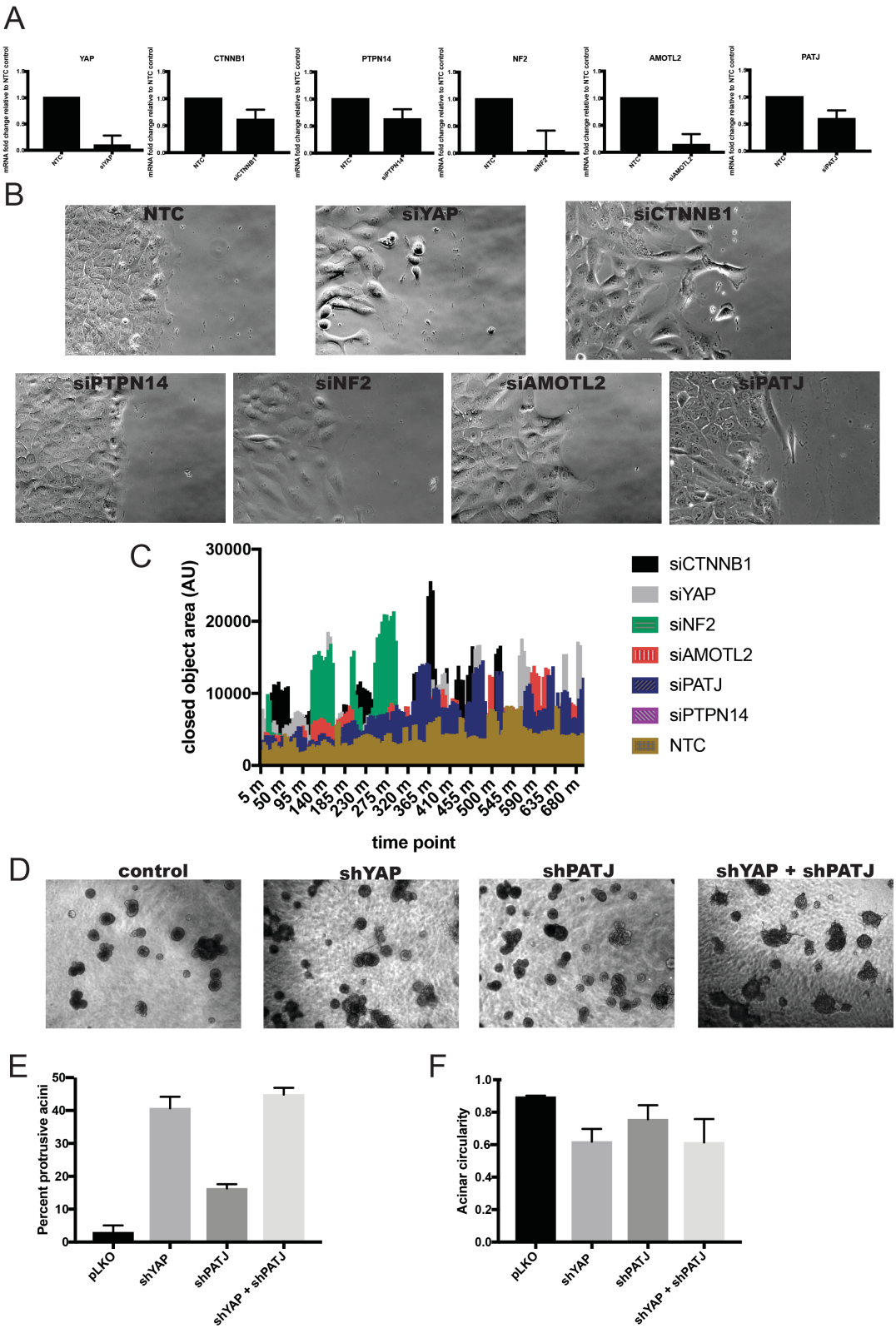

Figure 3.1 YAP and polarity proteins act together to inhibit protrusion. A) Knockdown validation by qPCR analysis of mRNA expression for known cytoplasmic YAP-

Figure 3.1 (*continued)*
interacting genes. NTC is non-targetting control siRNA. B) siRNA-mediated knockdown of known cytoplasmic YAP interactors in wound-healing assay. C) Quantification of average closed-object area entering wound over timelapse migration assay from (B). Average represents at least 15 fields of view from 2 independent experiments. D) MCF10A 3D Matrigel cultures for control, shYAP, shPATJ, and shYAP + shPATJ lines. E) Average acinar circularity of at least 50 acini from 1 independent experiment. F) Average number of protrusive acini of at least 100 acini from 1 independent experiment.

the Crumbs polarity complex component PATJ to inhibit protrusive cellular behavior independent of the canonical co-transcriptional activity of YAP.

Identifying a YAP-interacting GAP or GEF

Previous studies from our lab demonstrated that during cytokinesis, YAP and PATJ interact at the midbody ring for daughter cell orientation [1]. Our preliminary findings that YAP and PATJ may function in one pathway to regulate the cellular behaviors I have characterized in interphase cells–invasiveness, protrusiveness, and cellular adhesion—raise interesting questions for future studies. The Crumbs complex (an evolutionarily conserved complex composed of core components Crumbs, PATJ, and PALS1) regulates tight junction formation, apico-basal cell polarity, and directional migration [4, 5]. Intriguingly, our studies suggest that YAP knockdown cells also display impaired directional migration. These observations lead us to hypothesize that loss of YAP impairs epithelial cell polarity. We hypothesize that YAP acts at the cell cortex to stabilize polarity complexes; future studies can test this by examining stability of tight

junction complex interactions in the absence of YAP, or in the presence of overexpressed cytoplasmic YAP.

Interactions between YAP and Crumbs have been well established, but have been largely described as a sequestration mechanism that prevents YAP nuclear translocation [6-8]. Our findings suggest that YAP may also be acting as a scaffold protein at the cell cortex with the Crumbs complex, possibly tethering a RHO-GEF such as SYX or regulating a RAC-GEF such as TIAM1[9, 10]. Therefore, decreased YAP or PATJ perturbs the levels of active RHO and RAC at the cell cortex, resulting in the phenotypic changes we observe. Further studies are needed to fully elucidate the precise RHO GAP or GEF regulated by YAP, to characterize the dynamics of the YAP-Crumbs interaction, and to examine the generalization of this novel regulation across species and tissue types.

Examination of YAP loss *in vivo*

We have used the non-transformed mammary epithelial line MCF10A as a model system for the physiological biology of the mammary duct; however, studies *in vivo* will provide greater insight into the role of YAP in mammary gland biology. Organoid culture, which allows for study of tissue architecture (such as budding or branching) and function through isolation of source tissue and culture in a rich 3D matrix and growth media *in vitro*, allows for more physiological study [11]. Preliminary studies using inducible YAP-knockout mammary organoids (a gift from the laboratory of Dr. Fernando Camargo) revealed high levels of cell death once YAP knockout was induced, complicating phenotypic studies (Figure 3.2). In the MCF10A system, we have observed

cell death/growth arrest on occasions where YAP knockdown has been extremely efficient. Given the pleiotropic effects of YAP knockdown—impaired proliferative gene expression, impaired cytokinesis, and aberrant morphology—studies involving complete loss of YAP pose biological and technical complications that will require optimization and precise regulation to circumvent.

An inducible, mammary-specific YAP knockout system could provide a means of temporally controlling YAP knockout in a manner optimized for the tissue. A similar loss-of-function study conducted in murine developing lung found impaired branching and cyst development; these defects resulted in embryonic lethality [12]. Based on our studies in the MCF10A model system, we hypothesize that loss of YAP in mammary tissue will result in aberrant structural development, perhaps via branching defects similar to those seen in lung, although precisely how the protrusiveness, multiacinar structures, and cell-cell adhesion defects we observe *in vitro* will manifest in the intact breast tissue remains to be seen. One study examining dependencies on YAP (both knockout and overexpression) and the Hippo protein Salvador in the mouse mammary gland concluded that Hippo is dispensable in virgin mammary tissue but required in pregnancy, where loss of YAP results in hypoplasia, apparent defects in ductal structure, and a denser epithelial network than control tissue [13]. Examination of ductal regeneration in a cleared fat pad of shYAP/shTAZ also demonstrates that development of the ductal tissue is possible in the absence of YAP. However, examination of the tissue at higher resolution may reveal defects in branching or other structural alterations. Mammary gland development

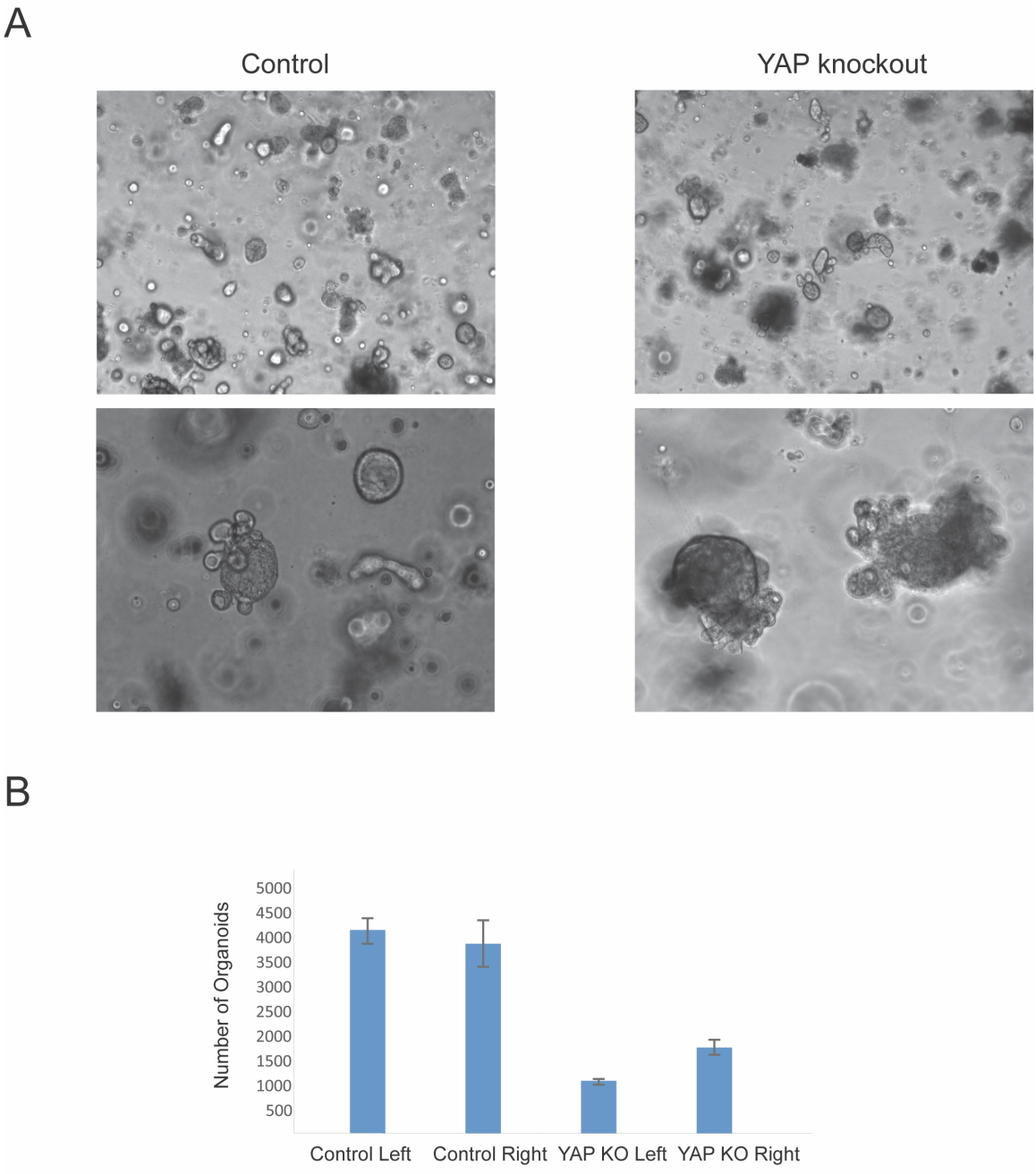

Figure 3.2 Preliminary YAP knockout organoid studies suggest biological complications in studying complete loss of YAP *ex vivo*. A) Phase contrast images of control and induced YAP knockout mammary organoids grown in organoid culture. B) Number of organoids in left and right mammary glands uninduced (control) and induced (YAP knockout) after two weeks in culture, as determined by Trypan Blue exclusion. *With Jennifer Rosenbluth.*

proceeds, as the authors discuss, though density of mammary tissue appears to be increased. Perhaps, therefore, in the absence of YAP, the increase in RAC activity leads to protrusive budding and aberrant branching. Alternatively, YAP loss may result in the absence of contact inhibition, promoting abnormal architecture. Further study is required to conclusively define physiological YAP function in the mammary gland.

YAP stoichiometry, localization, and RHO activities

In our studies, we have utilized shRNA-mediated YAP knockdown to demonstrate altered cellular contractility and cell-cell adhesion resulting from changes in YAP levels. Our rescue with transcriptionally defective YAP was sufficient to rescue wildtype behaviors, but one can imagine normal cellular conditions where YAP would be predominantly localized to the nucleus, such as in instances of sparse cells. Interestingly, we have found that lamellipodia formation increases in shYAP cells even in these conditions. Therefore, one open question is: what is the pool of YAP that maintains cytoplasmic RHO activity, and what is the nature of its interaction with the transcriptionally active pool? What is the stoichiometry of these YAP pools?

Similarly, our findings lead to hypotheses about changes in YAP localization associating with changes in RHO activities. Our data suggest that loss of YAP from the cytoplasm is permissive for increased RAC activity and decreased RHO activity. Interestingly, published studies have demonstrated in some contexts nuclear YAP transcriptionally activates RHO regulators, such as the RHOGAP ARHGAP29. In such contexts, loss of YAP from the nucleus would be predicted to activate RHO, perhaps

synergizing with YAP cytoplasmic activation of RHO and resulting in enhanced cellular/tissue stiffness. Further examination of the relationship between YAP localization and RHO and RAC activity levels will better elucidate this complex, bidirectional regulation.

YAP: both a tumor suppressor and an oncogene

The functionality of YAP as both an oncogenic transcription factor and a tumor suppressive protein is confounding. The studies described herein illustrate this point, and abundant literature suggests this dual function of YAP as well. One possible reason for this apparent dichotomy could be due to temporal regulation of YAP during tumor progression.

This hypothesis that YAP levels vary during tumor progression could help explain the diverse phenotypes observed upon YAP knockdown. The role of YAP as a driver of proliferation through transcriptional regulation of pro-proliferative genes, including Cyclin E, is well-defined [14]. In our studies, we have also found strong selection against cells with loss of YAP. Therefore, early in tumorigenesis, when there exists strong pressure for the tumor to proliferate and grow in mass, one hypothesizes that there would be a selection for cells with high levels of transcriptionally active YAP. Either through loss of Hippo regulation or via upregulation/amplification of YAP, the tumor would grow in size and acquire properties such as anchorage-independent survival [15, 16]. Given that many studies focus on early events in tumorigenesis, YAP would appear as an oncogene in this context.

As the tumor progresses towards metastasis, the selective pressures on a cancer cell change. Cells must weaken cell-cell adhesions, protrude and invade into the surrounding microenvironment, intravasate into the bloodstream and migrate to a secondary site where they extravasate, lay new adhesions, and seed a new tumor site [17]. As cells progress to this point, one intriguing possibility is that upregulation of YAP promotes an EMT that initiates the metastatic cascade [16]. In tumors that have lost epithelial characteristics such as strong cell-cell adhesion and cell polarity, YAP would be predicted to localize to the nucleus; our findings from Chapter 2 suggest that loss of YAP from the cytoplasm further deregulates RHO GTPase activities, promoting protrusive behavior and weakening cell-cell adhesion. Our studies raise an additional possibility that in tumors that have maintained more epithelial characteristics, total downregulation of YAP, which promotes protrusive behavior, weakens cell-cell adhesions, and promotes invasiveness independent of EMT, could also play a role in tumor progression towards metastasis. Studies looking at this phase of tumor progression (e.g. examining circulating tumor cells) may therefore find little or no YAP expression, and therefore, YAP would be considered a tumor suppressor (Figure 3.3).

Several avenues to test this hypothesis could be undertaken. One simple first step could be to examine YAP levels in CTCs, where decreased YAP expression would be predicted. This hypothesis could also be tested *in vivo* using inducible YAP expression. Using mouse genetics, one could introduce inducible CRISPR-mediated YAP knockout in a breast cancer model. Once tumors progressed to a benchmark size, knockout could be induced and metastasis tracked. If YAP knockout tumors displayed enhanced metastasis, follow-up studies could be conducted. YAP knockout could be induced early

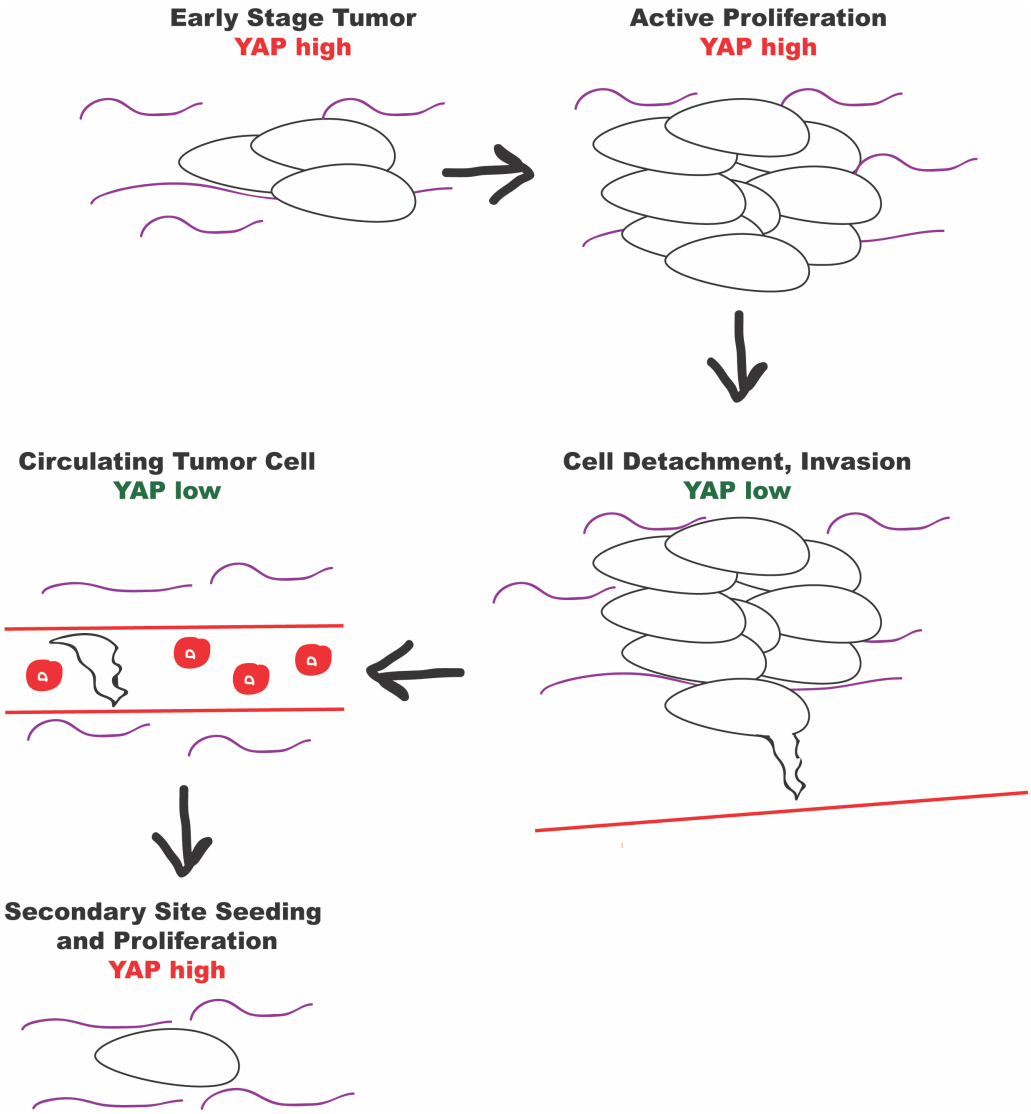

Figure 3.3 Hypothesis regarding YAP expression changes during tumor progression. YAP levels are high when there is selective pressure on the tumor to proliferate and grow in size. YAP downregulation decreases cell-cell adhesion, thus enhancing motility of cells with migratory phenotype and facilitating metastasis. Once a secondary site is established, cells with higher YAP and proliferative potential are selected for and expand.

on, and YAP could be re-expressed via another inducible system, to have precise control over YAP expression in the tumor. These studies would offer greater insight into the varied roles of YAP in tumor progression and help unravel the complex biology of this protein.

Further probing YAP and invasiveness

While we have demonstrated that restoration of cytoplasmic YAP is sufficient to inhibit protrusive activity of MCF10A cells resulting from knockdown of YAP, we have also observed that knockdown of PATJ alone induces protrusive activity at less penetrance than that observed with decreased YAP alone. While this could in part be explained by lower efficiency of the PATJ knockdown in comparison to YAP knockdown, PATJ invasiveness has previously been reported at a penetrance of approximately 20% [3]. Therefore, we hypothesize that YAP may be contributing to this phenotype in part due to its transcriptional activity. Previous studies have demonstrated that loss of polarity proteins synergizes with expression of pro-inflammatory cytokines to promote invasion [3]; if decreased YAP expression results in upregulation of such a cytokine or other matrix remodeling factor, it could help account for the differential invasiveness observed between shYAP and shPATJ. In support of this hypothesis, we observe that re-expression of a non-transcriptional YAP only partially rescues the collective cell migration deficit of YAP knockdown cells, raising the possibility that both transcriptional YAP and cytoplasmic YAP contribute to the severity of this phenotype.

YAP is known to regulate several growth factors and cytokines—canonical YAP-TEAD targets CTGF and CYR61 are both extracellular signaling proteins. Given the ability of YAP knockdown to perturb the spherical properties of MCF10A acini, one might hypothesize that YAP inhibits matrix-remodeling proteins, such as MMPs. Interestingly, AP-1 is an established repressor of MMP9 [18]. In multiple studies in breast and other cancer cell lines, YAP and AP-1 interact [19, 20]; therefore, it may be that in certain biological contexts YAP and AP-1 regulate MMP9 expression. This hypothesis would suggest that in some cases YAP and AP-1 inhibit invasiveness, such as in normal tissue.

Therapeutic applications of targeting YAP

The complex biology of YAP raises questions regarding the suitability of YAP as a therapeutic target in cancer. Our findings demonstrate that decreasing YAP expression can produce diverse phenotypes, such as impaired cell-cell adhesion, enhanced protrusive behavior, and decreased cell contractility. Notably, our studies suggest that the tumor suppressive function of YAP is independent of transcriptional function with TEAD, whereas other findings support that YAP-TEAD signaling is oncogenic. Therefore, we propose that specifically targeting the YAP-TEAD interaction (such as with the inhibitor Verteporfin, which binds the TEAD-binding domain of YAP) rather than aiming to decrease overall YAP levels, may represent a potential therapeutic avenue for treatment of human cancer.

References

1. Bui DA LW, White AE, Harper JW, Schackmann RC, Overholtzer M, Selfors LM, Brugge JS. Cytokinesis involves a nontranscriptional function of the Hippo pathway effector YAP. Science Signaling. 2016;9(417). doi: 10.1126/scisignal.aaa9227.

2. Zhang N BH, David KK, Dong J, Zheng Y, Cai J, Giovannini M, Liu P, Anders RA, Pan D. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Developmental Cell. 2010;19(1):27-38.

3. Chatterjee S SL, Feigin ME, Gibbons DL, Scuoppo C, Lin W, Rizvi ZH, Lind E, Dissanayake D, Kurie J, Ohasha P, Muthuswamy SK. Dysregulation of Cell Polarity Proteins Synergize with Oncogenes or the Microenvironment to Induce Invasive Behavior in Epithelial Cells. PLoS One. 2012;7(4):e34343.

4. Shin K SS, Margolis B. PATJ regulates tight junction formation and polarity in mammalian epithelial cells. Journal of Cell Biology. 2005;168:705-11.

5. Shin K WQ, Margolis B. PATJ regulates directional migration of mammalian epithelial cells. EMBO Reports. 2007;8:158-64.

6. Hauri S WA, van Drogen A, Varjosalo M, Tapon N, Aebersold R, Gstaiger M. Interaction proteome of human Hippo signaling: modular control of the co‐activator YAP1. Molecular Systems Biology. 2013;9(1).

7. Yi C SZ, Stemmer-Rachamimov A, Dawany N, Troutman S, Showe LC, Liu Q, Shimono A, Sudol M, Holmgren L, Stanger BZ, Kissil JL. The p130 isoform of angiomotin is required for Yap-mediated hepatic epithelial cell proliferation and tumorigenesis. Science Signaling. 2013;6(291).

8. Zhao B LL, Lu Q, Wang LH, Liu CY, Lei Q, Guan KL. Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. Genes and Development. 2011;25(1):51-63.

9. Diamantopoulou Z WG, Fadlullah MZH, Dreger M, Pickering K, Maltas J, Ashton G, MacLeod R, Baillie GS, Kouskoff V, Lacaud G, Murray GI, Sansom OJ, Hurlstone AFL, Malliri A. TIAM1 Antagonizes TAZ/YAP Both in the Destruction Complex in the Cytoplasm and in the Nucleus to Inhibit Invasion of Intestinal Epithelial Cells. Cancer Cell. 2017;31(5):621-34. doi: 10.1016/j.ccell.2017.03.007.

10. Ernkvist M LPN, Audebert S, Lecine P, Sinha I, Liu M, Schlueter M, Horowitz A, Aase K, Weide T, Borg JP, Majumdar A, Holmgren L. The Amot/Patj/Syx signaling complex spatially controls RhoA GTPase activity in migrating endothelial cells. Blood. 2009;113(1):244-53.

11. Jamieson PR DJ, Rios AC, Fu NY, Lindeman GJ, Visvader JE. Derivation of a robust mouse mammary organoid system for studying tissue dynamics. Development. 2017;144(6):1065-71.

12. Lin C YE, Zhang K, Jiang X, Croll S, Thompson-Peer K, Chuang PT. YAP is essential for mechanical force production and epithelial cell proliferation during lung branching morphogenesis. eLIFE. 2017. doi: 10.7554/eLife.21130.

13. Chen Q ZN, Gray RS, Li H, Ewald AJ, Zahnow CA. A temporal requirement for Hippo signaling in mammary gland differentiation, growth, and tumorigenesis. Genes and Development. 2014;28(5):432-7.

14. Huang J WS, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell. 2005;122(3):421-34.

15. Zhao B LL, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes and Development. 2012;26(1):54-68.

16. Overholtzer M ZJ, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, Haber DA. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. PNAS. 2006;103(33):12405-10. doi: 10.1073/pnas.0605579103.

17. Melzer C vdOJ, Hass R. Breast Carcinoma: From Initial Tumor Cell Detachment to Settlement at Secondary Sites. BioMed Research International. 2017;2017:8534371.

18. Mittelstadt ML PR. AP-1 Mediated Transcriptional Repression of Matrix Metalloproteinase-9 by Recruitment of Histone Deacetylase 1 in Response to Interferon β. PLoS One. 2012;7(8):e42152.

19. Shao DD XW, Krall EB, Bhutkar A, Piccioni F, Wang X, Schinzel AC, Sood S, Rosenbluh J, Kim JW, Zwang Y, Roberts TM, Root DE, Jacks T, Hahn WC. KRAS and YAP1 converge to regulate EMT and tumor survival. Cell. 2014;158(1):171-84.

20. Zanconato F FM, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Bicciato S, Cordenonsi M, Piccolo S. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. Nature Cell Biology. 2015;17(9):1218-27.

Appendix I.

YAP regulates immediate early gene expression in invasive lobular carcinoma

Natalie M. Hendrick, Daniel G. Stover, Lisa L. Gallegos, Laura M. Selfors, Joan S. Brugge

Statement of Contribution*:* N.M.H. and J.S.B. designed the studies and interpreted data. D.G.S. performed bioinformatics analysis of the CTGF-CYR61 signature correlation with PAM50, ILC, and CCLE. L.L.G. conceptualized the initial hypothesis of YAP activation in ILC. L.M.S. performed initial bioinformatics analysis of CTGF-CYR61 coexpressed genes. N.M.H. carried out and analyzed all other experiments.

Abstract

Loss of E-cadherin is a driving event in Invasive Lobular Carcinoma (ILC), the second most common histological breast cancer subtype. Loss of E-cadherin is predicted to suppress the Hippo pathway leading to transcriptional activation of YAP. To examine whether YAP transcriptional activity contribute to ILC and thus represents a target for this breast cancer subtype with limited therapeutic options once resistance to hormone therapy arises, we used a tissue microarray to confirm that nuclear YAP staining is associated with ILC. We found evidence for YAP transcriptional activity in ILC through analysis of expression levels of the YAP target genes CTGF and CYR61 in The Cancer Genome Atlas breast cancer dataset. Interestingly, we found that CTGF and CYR61 coexpressed genes in these cancers included many immediate early response genes (IEG). Using the ILC line SUM44PE, we found expression of multiple IEG to be dependent on YAP-TEAD activity *in vitro*. Our findings suggest high levels of YAP-TEAD transcriptional activity in ILC and reveal potential YAP regulation of the IEG program in ILC.

Introduction

Invasive Lobular Carcinoma (ILC) is the second most common breast cancer histological subtype, accounting for approximately 15% of diagnoses. Loss of E-cadherin, the adherens junction protein, is a causal, initiating event in ILC, and a hallmark of these tumors. Loss of E-cadherin in ILC results in early initiation of anchorage independence, loss of cell-cell adhesion, and pro-inflammatory signals through altered localization of p120 catenin, distinguishing ILC from other breast cancers, where such changes occur later in the metastatic process [1, 2]. ILC tumors are classified as luminal A subtype and the majority respond to hormone therapies; however, once resistance arises, treatment options are limited, particularly given the low proliferative index of these tumors. As such, identification of oncogenic drivers downstream of E-cadherin can reveal novel therapeutic targets in this breast cancer.

YAP, the canonical transcriptional effector of the Hippo tumor suppressor pathway, is responsive to changes in cell-cell contact, and is negatively regulated by contact inhibition and cellular confluency when adherens junctions are matured [3, 4]. YAP localization is predominantly nuclear in both human and mouse ILC, suggesting YAP transcriptional programming is active in these tumors [5, 6]. Genes regulated by YAP and its coactivator TEAD regulate a diverse array of genes, such as growth factors (CTGF), cytokines (AREG, CYR61, IL6) and the cytoskeleton (ITGB2) [7]. Other studies in cancer cells have demonstrated that YAP-TEAD drive genes directly involved in S-phase entry and epithelial-to-mesenchymal transition (EMT) via interaction with the c-FOS/JUN heterodimer AP-1 [8, 9]. Oncogenic functions of YAP have been described

in multiple cancer types, rendering YAP a putative target for therapeutic intervention [10-12].

Here, we present preliminary evidence that YAP transcriptional activity in ILC and other E-cadherin negative cancers regulates an immediate early gene (IEG) signature. We describe bioinformatics analysis which shows that YAP transcription and expression of an immediate early gene (IEG) signature correlate, independent of proliferation in ILC tumors. In addition, we provide initial evidence that expression of YAP is necessary for expression of a subset of IEG in the ILC line SUM44PE and in E-cadherin negative ovarian cancer cell lines, and that pharmacological disruption of YAP-TEAD is sufficient to decrease a subset of IEGs. Our data suggest that nuclear YAP may be a characteristic of ILC and IEG expression, suggesting YAP transcriptional activity may be active in ILC and other E-cadherin negative tumors.

Results

Co-expression analysis of YAP regulated genes identifies an immediate early gene program in ILC

To assess YAP transcriptional activity in ILC, we examined expression levels of two canonical YAP target genes, CTGF and CYR61 using RNAseq data from the Cancer Genome Atlas (TCGA) breast cancer dataset. The transcriptional coactivator function of YAP is primarily regulated by its nuclear localization following posttranslational phosphorylation; therefore, in order to probe YAP transcriptional activation using a

bioinformatics approach, rather than looking at YAP expression levels, we examined expression levels of YAP targets CTGF and CYR61 [13]. Furthermore, we sought to identify potential YAP targets through analysis of genes co-expressed with these known YAP targets. We observed strong expression of CTGF and CYR61, and found a pattern of CTGF-CYR61coexpressed genes in a majority of ILC tumors, distinct from non-lobular tumors (Figure S1A). Interestingly, we found this CTGF-CYR61 coexpressed gene signature is enriched for immediate early genes (IEG) (Figure S1B). Analysis of IEG expression signature score revealed that ILC display significantly higher IEG levels compared to non-lobular breast tumors, with a p-value of p = 1.23e-14**,** suggesting this may be a distinguishing feature of ILC (Figure S1C) [14].

As previously stated, ILC are less proliferative than other breast tumor types. To examine if the observed IEG signature was a consequence of differences in proliferation across tumor types, we performed analysis of the PAM50 proliferation signature and the CTGF-CYR61 coexpressed gene signature to examine correlation [15]. The R^2 value of these two variables is 0.001, strongly suggesting that the two factors are independent of each other (Figure S1D). This significant lack of correlation between IEG mRNA expression and proliferative rate suggests that an alternate factor other than proliferative rate is driving this transcriptional expression pattern in ILC.

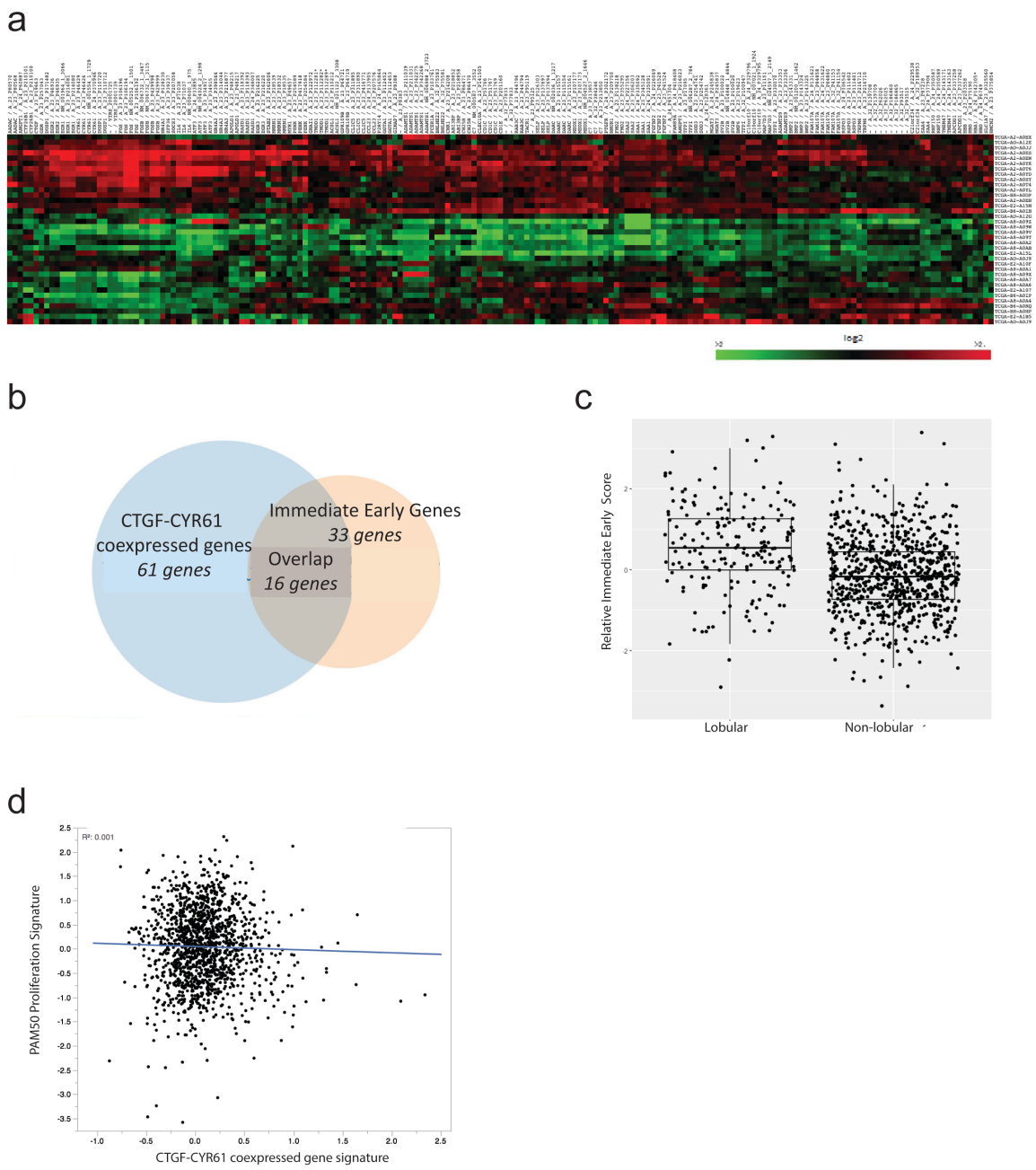

Figure S1 Analysis of coexpressed YAP target genes in patient data reveals enrichment for immediate early genes in invasive lobular breast cancer. a) Heatmap showing RNA expression from the TCGA breast cancer patient dataset of CTGF and CYR61 coexpressed genes. Relative mRNA levels of the identified genes are shown; data are median centered Log2. b) Venn Diagram of the CTGF-CYR61 coexpressed gene signature and immediate early genes. c) Relative expression analysis of all 40-immediate early gene across TCGA breast cancer patients (Student's t test, p = 1.23e-14) d) Correlation analysis of the PAM50 proliferative signature with the CTGF-CYR61 coexpressed gene signature; line indicates best fit.

YAP transcriptional coactivator is nuclear in ILC

To directly confirm nuclear localization of YAP in invasive breast cancers, we stained a human tissue microarray (TMA) containing a mix of invasive ductal and invasive lobular carcinomas by immunohistochemistry (IHC). Clinicopathological characteristics of the tumors are described in Table S1. As YAP nuclear localization is associated with its transcriptional activity, we used YAP positive nuclear staining for scoring, as well as cytoplasmic YAP staining, using an IHC-validated antibody (Figure S2A). Representative YAP staining from the TMA is also shown in Figure S2B, and analysis of the YAP expression patterns with breast cancer characteristics is shown in Table S2. ILC tumors show a slight but significantly higher percentage of cells with primarily nuclear YAP ($p<0.05$) compared to ductal carcinomas; all other classes of YAP staining were not statistically different across the tumor types. This confirms that increased nuclear YAP localization is associated with ILC.

Table S1 Clinicopathological features of 160 breast cancer patients examined for YAP expression

Feature	Grouping	N or value	%
Age (years)	Mean	49	
	Range	30 to 77	
Histologic type	IDC	80	50
	ILC	80	50
Histologic grade	1	4	2.5
	2	60	25
	3	14	8.8
	undetermined	82	51.3
Receptor Positivity	ER	32	20
	PR	22	13.8
	HER2	29	18.1

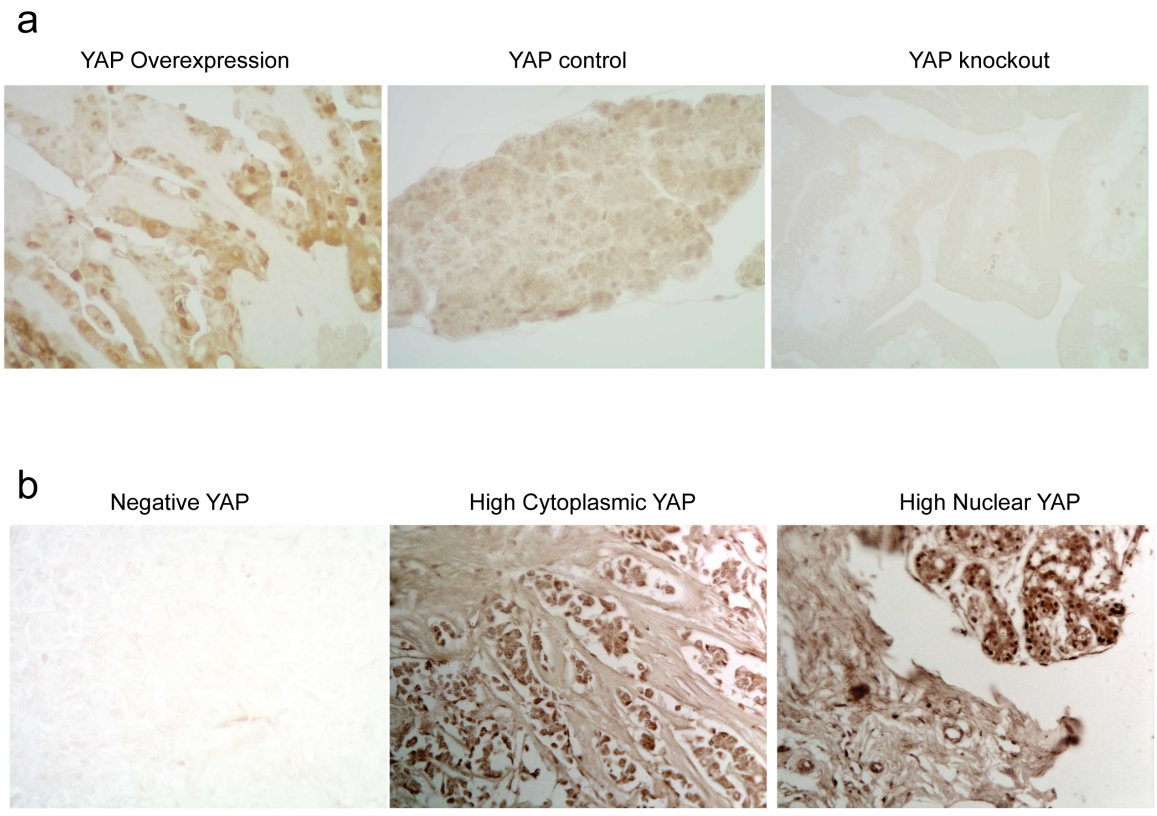

Figure S2 Representative YAP IHC staining. a) Validation of YAP antibody for IHC using YAP knockout and YAP overexpression mouse intestinal tissue. b) Representative example IHC staining for Human Tissue Microarray.

Table S2 Scoring of Tissue Microarray.

	Nuclear YAP (%)	Cytoplasmic YAP (%)	Nuclear and Cytoplasmic (%)	Negative YAP (%)
Ductal	26 (32.5)	2 (2.5)	24 (30)	28 (35)
Lobular	41 (51.3)	2 (2.5)	20 (25)	25 (31.3)

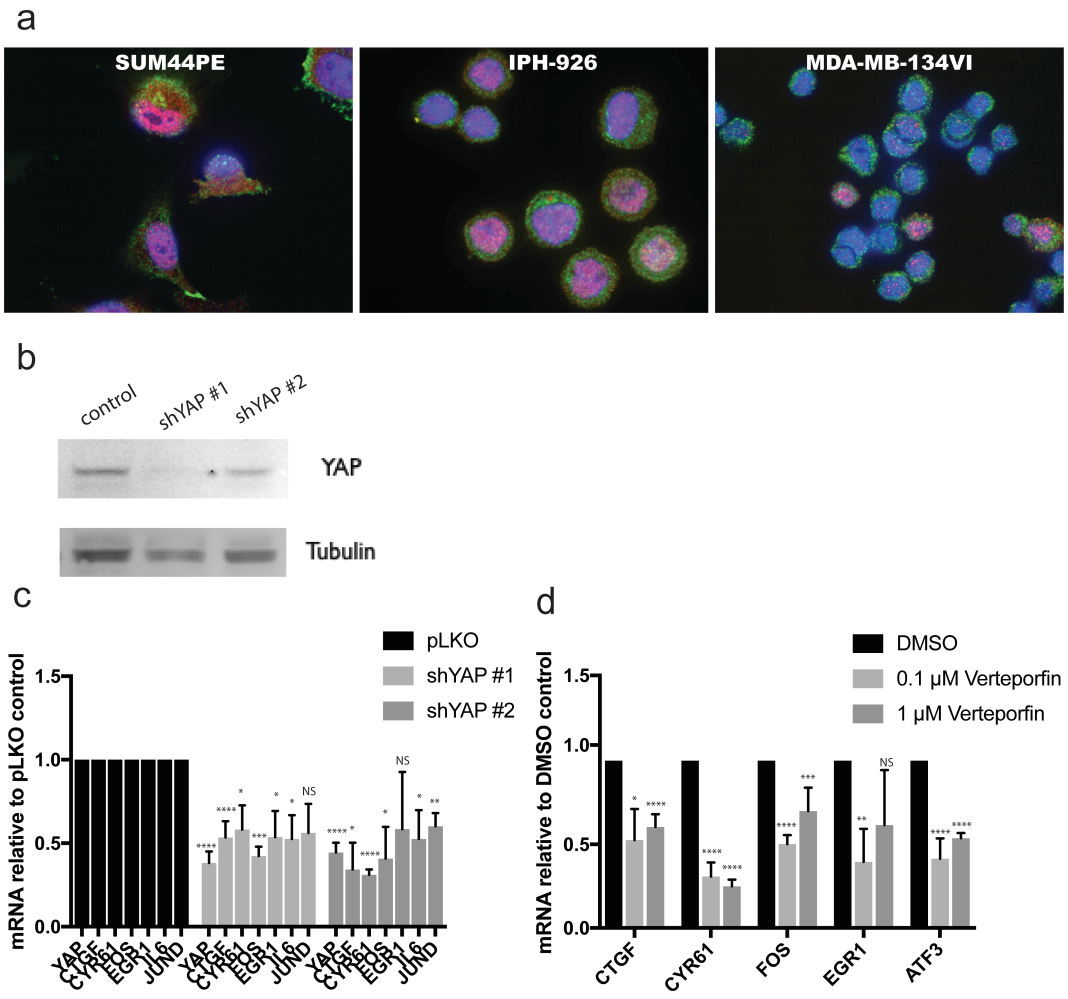

Figure S3 YAP-TEAD transcriptional activity is necessary for expression of IEG in ILC line SUM44PE. a) ILC lines stained for YAP (Red) and cell-cell adhesion protein -catenin (Green). b) Stable knockdown of YAP in SUM44PE by lentiviral infection. c) YAP knockdown SUM44PE analyzed for CTGF-CYR61 coexpressed signature genes by RT-qPCR. Data are an average of three independent experiments. d) SUM44PE treated with YAP-TEAD inhibitor Verteporfin analyzed for CTGF-CYR61 coexpressed signature genes by RT-qPCR. Data are an average of two independent experiments. *p<0.05, **p<0.01***p<0.001, ****p<0.0001 by Students t-test

There are only few cell line models of ILC, limiting *in vitro* studies. We examined YAP localization in the three established human ILC lines, MDA-MB-134VI, SUM44-PE, and IPH-926 [16] and observed expression of nuclear YAP in all three, though only a small fraction of the MDA-MB-134VI cells displayed detectable YAP staining (Figure S3A). Taken together, these findings suggest that nuclear YAP is a feature of E-cadherin deficient ILC tumors, consistent with previous studies demonstrating inverse correlation between YAP activity and E-cadherin [3, 5].

YAP-TEAD transcription is necessary for immediate early gene expression in ILC

To determine whether expression of the CTGF-CYR61 coexpressed gene signature is dependent on YAP activity, we attempted to knock down YAP in the three aforementioned established ILC cell lines using the lentiviral vectors that have been shown to cause effective knockdown of YAP [17]. SUM44PE was the single ILC line with successful YAP knockdown (Figure S3B); MDA-MB-134VI and IPH-926 both underwent senescence after transduction of the pLKO vectors (data not shown).

To probe the CTGF-CYR61 coexpressed gene signature, we collected RNA from SUM44PE cells transduced with the control vector or YAP shRNA vectors and examined expression YAP targets CTGF and CYR61, as well as multiple IEGs (FOS, EGR1, IL6, and JUND). We observed decreased mRNA expression of multiple IEG following YAP knockdown, suggesting that YAP is necessary for expression of a subset of IEG (Figure S3C). However, since knockdown of YAP is predicted to decrease proliferative rate, it

must also be considered that the impaired proliferation may decrease IEG levels. Further study is needed to eliminate this alternate hypothesis.

To assess if canonical YAP transcriptional activity is required for IEG expression, we utilized the YAP-TEAD inhibitor Verteporfin to examine if such inhibition could phenocopy YAP knockdown effect on IEG expression [8, 9, 18]. Verteporfin is used clinically for photodynamic treatment of macular degeneration but has recently been identified as a direct inhibitor of YAP-TEAD interaction, as it binds directly to the TEAD-interacting domain of YAP [18, 19]. Verteporfin treatment did not result in a dose-response as anticipated, suggesting the possibility of off-target effects; cellular toxicity with this compound has been observed in other cell lines [20]. At the lowest dose, inhibition of YAP-TEAD interaction was sufficient to reduce expression of IEGs c-FOS and ATF3 in SUM44PE cells, similar to our observations with YAP knockdown (Figure S3D). These findings suggest that YAP-TEAD signaling is necessary for expression of a subset of IEG in ILC lines; follow-up studies with alternatives to the Verterporfin inhibitor will be needed to further validate this.

YAP is required for IEG expression in ovarian cancer lines

Given the limited number of ILC lines permissible for *in vitro* studies, we next considered other cancer types that may reveal high levels of YAP activity. We therefore assessed the CTGF-CYR61 coexpressed gene signature across ovarian cancer lines, a cancer type that frequently displays loss of E-cadherin expression. We found strong expression of the signature across multiple cell lines from the CCLE panel (Figure S4A).

We selected four E-cadherin-negative lines for *in vitro* validation: DOV13, ES2, OV207, and OVCA432. We detected high levels of nuclear YAP in ES2, OV207, and OVCA432, significantly high total YAP in ES2, but overall low YAP staining in DOV13 (Figure S4B). Though OV207 display apparent cell-cell junctions, they have lost many epithelial characteristics; they are characterized as a mesoepithelial line and do not display contact inhibition of proliferation [21]. In line with the immunofluorescence data, we found that *YAP* is amplified in the ES2 cell line upon further analysis of the CCLE genomic database. Additionally, both DOV13 and ES2 are epithelial lines that have undergone an epithelial-mesenchymal transition, offering an explanation for their E-cadherin loss and explaining their *in vitro* morphologies [22].

We generated stable knockdown of YAP in multiple ovarian cancer lines. Upon examination by RT-qPCR, we found that decreased YAP expression correlated with decreased CTGF and CYR61 expression, though OVCA432 did not show dependency of CYR61 on YAP and CYR61 was only minimally reduced in OV207 cells (Figure S4C). We detected several additional IEG transcripts in ovarian line ES2, and we found YAP is necessary for the expression of ATF3, FOS, EGR1, EGR2, and IL6.

Taken together, these studies support the working model shown in Figure S4D where loss of E-cadherin results in increased nuclear YAP expression, which activates a subset of IEG expression.

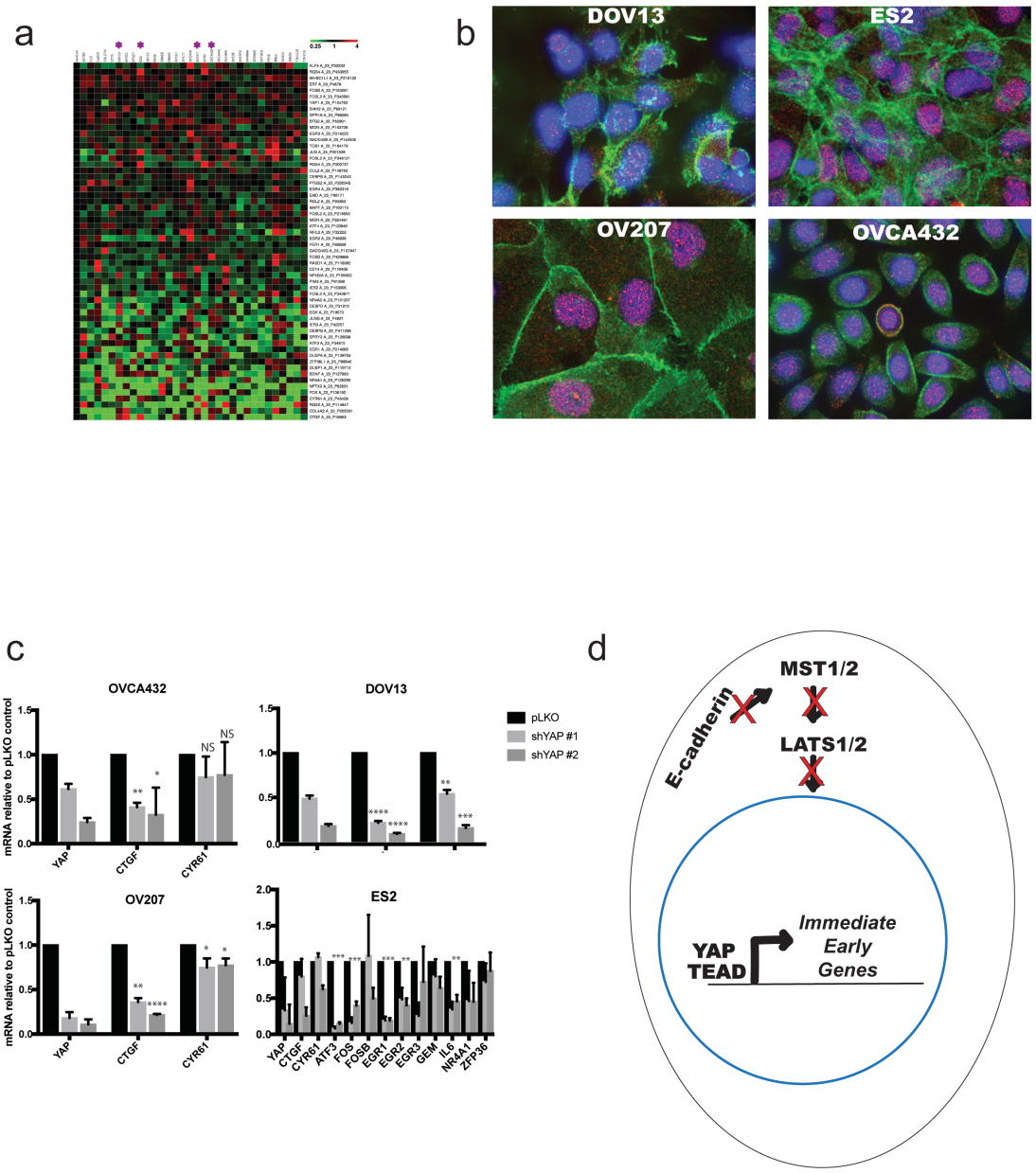

Figure S4 Ovarian cancer lines require YAP for IEG expression. a) Gene expression analysis of RNAseq data from the Cancer Cell Line Encyclopedia for ovarian cancer cell lines. Starred lines are E-cadherin negative and were selected for *in vitro* study. b) Ovarian cancer cell lines stained for YAP (red) and -catenin (green) c) YAP knockdown lines analyzed for CTGF-CYR61 coexpressed signature genes by RT-qPCR. Data are an average of two independent experiments. d) Model for YAP-TEAD driven IEG activation in E-cadherin negative cancers. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Students t test.

Discussion

The studies in this appendix represent initial analyses to investigate YAP transcriptional activity in tumor cells lacking E-cadherin. Our preliminary studies suggest high YAP transcriptional activity in invasive lobular carcinoma, using bioinformatics, *ex vivo*, and *in vitro* methods. We identify a candidate YAP-driven immediate early gene signature in ILC and present preliminary evidence that YAP is necessary for expression of this signature in an E-cadherin deficient ILC line and ovarian cancer lines *in vitro*. Our preliminary studies with the YAP-TEAD inhibitor Verteporfin suggest this inhibitor decreases IEG expression, though additional studies are required to confirm the specificity of the effect. Additional studies regarding YAP and regulation of the IEG can provide insight into the underlying biology of the cancer and a novel function of YAP transcriptional activity.

Further studies are needed to confirm the role of YAP-TEAD signaling in regulation of IEG. Use of Verteporfin may have off-target effects, as observed through the lack of a dose response to the inhibitor. Therefore, a genetic approach through rescuing YAP expression in knockdown with the TEAD-mutant YAP S94A would better demonstrate a requirement for IEG on YAP-TEAD activity. Once this interaction is confirmed as the relevant function of YAP for this gene regulation, we can aim to identify the precise mechanism of YAP-TEAD regulation of the immediate early response. As stated previously, interaction between the YAP-TEAD complex and AP-1, the Fos/Jun heterodimer composed of two IEG products, was observed in both lung cancer and breast cancer lines [8, 9]. Additionally, Shao et al. noted an overlap in YAP

regulated genes and KRas regulated genes, with IEG composing one of the largest groups [8]. Indeed, analysis of our IEG signature in the ChIP-Seq data in MDA-MB-231 cells from Zanconato et al. revealed that 65% of the genes demonstrate YAP binding in the regulatory region [9]. It is possible that the YAP-TEAD-AP-1 complex is prolonging the expression of the IEG signature, which should be repressed after the initial transcriptional wave, in E-cadherin deficient tumors. Interestingly, Shao et al. links this complex to an EMT; therefore, examination of the activity of this transcriptional complex in ILC (where EMT is not triggered) would shed light on ILC biology and could reveal novel functions of YAP.

It must also be considered that YAP-TEAD regulation of IEG may not be direct. Another link between YAP-TEAD and IEG expression comes from the mechanotransduction properties of YAP [23]. SRF, the canonical master regulator of IEG, is activated by serum, growth factors, and mechanobiology. MRTF, the mechanoregulated cofactor of SRF, shows activational dependency on YAP-TEAD regulated TGFβ signaling in fibroblasts; conversely, YAP-TEAD transcriptional activity was dependent on MRTF-SRF mediated cytoskeletal maintenance [24]. Therefore, it is possible that the cytoskeletal changes resulting from loss of E-cadherin trigger both altered MRTF and altered YAP activity, and that decreasing YAP expression decreases IEG by altering MRTF transcriptional activity. Furthermore, our own studies from Chapter 2 implicate YAP as a regulator of the cytoskeleton; YAP knockdown would alter the F/G-actin ratio in the cell, and as MRTF is sequestered by G-actin, this would affect its activation of SRF [25]. Therefore, YAP signaling may respond to cytoskeletal

changes resulting from MRTF-SRF function, and MRTF-SRF signaling may respond to cytoskeletal changes resulting from YAP function.

One avenue for further study is elucidating contribution(s) of IEG products to YAP oncogenicity in ILC and eventually in other E-cadherin deficient cancers. It is important to understand the biological implications of YAP-dependent IEG expression in ILC. One way to approach this would be to examine the contribution of YAP to growth in soft agar of an anchorage-independent cell line. As YAP transcriptional function has previously been implicated in anchorage-independent survival, we hypothesize it may promote this function in ILC as well [26]. Such a dependency on YAP in ILC would be particularly interesting given that anchorage independent survival occurs early in ILC [2]. This experiment would ideally be done using CRISPR and observing severity of the phenotype of YAP knockout. One could then screen via CRISPR knockout of IEG for genes that phenocopy the YAP knockout phenotype, followed by re-expression of the candidate genes in sgYAP ILC lines. Ideally, this would first be performed *in vitro*, then *in vivo* in the mouse, possibly using one of the murine models and allograft methods previously discussed [6, 27]. Such studies would better elucidate the complex oncogenic function of YAP and may offer insight into ILC therapeutic targets.

While future studies are required to elucidate the precise mechanism of YAP regulation of the CTGF-CYR61 coexpressed gene signature and to articulate any function it may have in ILC, we have potentially found another candidate oncogenic function of YAP transcription in its regulation of IEG. Many IEG products function as oncogenes themselves, such as c-MYC, c-FOS, and IL-6. Additional investigations to assess contributions of the IEG to ILC biology are warranted. Our studies provide preliminary

evidence of transcriptional activity of YAP in ILC and provide the foundation for future studies to identify vulnerabilities of this cancer subtype.

Materials and Methods

Bioinformatics analysis

Raw data were processed with RMA normalization and $\log_2$ transformation. To determine a single value for IEG expression, we used a published list of IEG, we and calculated the mean of the gene expression values identified as "up" [28].

Cell Culture

SUM44PE were cultured in M171 medium (Gibco) supplemented with HUMEC supplement (Gibco). MDA-MB-134IV were cultured in McCoy's 5A medium (VWR) supplemented with 20% FBS. IPH-926 were cultured in DMEM/F-12 (Gibco) with 10% FBS (Gibco), penicillin-streptomycin, and 2mM L-glutamine (Gibco). OVCA432, OV207, DOV13, and ES2 were cultured in a 1:1 mixture of MCDB105 (Cell Applications Inc.) and medium 199 (Gibco) media, supplemented with 10% inactivated calf serum, 2mM L-glutamine and antibiotics [29]. HEK293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics.

Plasmids

shRNAs in human pLKO.1 lentiviral vectors were used to knockdown YAP with puromycin selection.

Virus Production and Infection

For production of lentiviral particles, HEK293T cells were transfected at 80% confluency with packaging plasmids Pax2, VSVG, and pLKO-plasmid containing the gene of interest. Virus was collected at 48 and 72 hours post-transfection and filtered through 0.45-μm filters. Recipient cancer cells were plated in order to reach 30% confluency at 24 hours after seeding, and lentivirus was added to the cells at a 1:1 dilution with fresh media. Cells were selected with puromycin 48 hours after infection.

Drug Treatment

Verteporfin (Sigma) was added to cells in normal growth media at dosage of 0.1 μg/ml or 1 μg/ml and treated for 24 hours before lysis for RNA analysis.

Antibodies

The following antibodies were used in these studies: YAP XP (1:1000 WB, 1:200 IF, Cell Signaling, 14074S), YAP (1:1000 WB, 1:200 IF, 1:50 IHC, Santa Cruz, sc-15407), Tubulin (1:2000 Cell Signaling, 3873), β-catenin (1:1000 BD, 610154).

Immunohistochemistry

IHC was carried out on high-density human tissue microarrays with both ductal and lobular carcinomas (Biomax). After deparaffinization and rehydration in decreasing percentages of ethanol solutions, endogenous peroxidase activity was blocked for 15 minutes in 0.3% hydrogen peroxide. Sections were then boiled for 20 minutes in antigen retrieval buffer pH 9 (Dako) for antigen retrieval. After blocking with 10% goat serum in TBST for one hour, sections were stained using standard immunohistochemistry

protocols. Signal was amplified using the Vectastain Elite ABC Kit (Vector Laboratories Inc.). Images were acquired on a Nikon Ti inverted microscope, with Perfect Focus System, using a Plan Apo 10x 0.75 NA lens and an Orca-ER cooled CCD camera (Hamamatsu). Human samples used in these studies are exempt from informed consent.

Tissue Microarray Scoring

Blinded subjects were given images of the tissue microarray for YAP IHC and asked to evaluate the staining as follows: overall (positive or negative), cytoplasmic (positive or negative), nuclear (positive or negative).

Immunofluorescence

Cells were seeded in glass-bottom coverslip dishes (MaTek). Immunofluorescence staining was performed by fixing cells with 3.5% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton-X in PBS for 5 minutes, and blocked in 1% BSA/5% goat serum for 1 hour at room temperature. Samples were incubated with primary antibody at 4 degrees overnight, then washed with PBS. Samples were incubated with Alexa-Fluor conjugated secondary antibodies (Life Technologies) at room temperature for 1 hour then washed with PBS. Nuclei were counterstained with DAPI (Sigma). Samples were mounted in 20 mM Tris, pH 8.0, 0.1% N-propyl gallate, 90% glycerol. Samples were visualized on a Nikon Ti motorized inverted microscope with Perfect Focus System with Yokagawa CSU-X1 spinning disk confocal with Spectral Applied Research Aurora Borealis modification and images were acquired with a Hamamatsu ORCA-ER cooled CCD camera.

Western Blot Analysis

Cells were lysed in ice-cold RIPA buffer supplemented with protease inhibitors, phosphatase inhibitors, and MG-132 (Sigma). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined by BCA assay (Thermo-Fisher Scientific) and protein concentrations were normalized. Lysates were boiled in 1× sample buffer for 5 minutes and resolved 4%–20% SDS-PAGE gradient gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and then blocked with 5% BSA in TBS for 30 minutes at room temperature. Blots were probed with primary antibody at 4°C overnight. Membranes were probed with secondary antibodies coupled to fluorophores and developed by imaging on the Odyssey Imaging System (Licor).

Quantitative RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen), and up to 2 μg of RNA were subjected to cDNA synthesis (Quanta). Real-time PCR was carried out using the Power SYBR Green PCR Mix (Applied Biosystems) on a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). Relative gene expression was determined by normalization to RPLP0 control. Values are the average of three wells, and three independent replicates. qPCR probes used in these studies are available in Supplemental Table 3.

Supplemental Table 3 List of qPCR probes used in this study.

Gene	Forward Primer	Reverse Primer
RPLPO	5′-ACGGGTACAAACGAGTCCTG-3′	5′-CGACTCTTCCTTGGCTTCAA-3′
YAP	5′-ATCCCAGCACAGCAAATTCT-3′	5′-TGGATTTTGAGTCCCACCAT-3′
CTGF	5′-CCTGCAGGCTAGAGAAGCAG-3′	5′-TGGAGATTTTGGGAGTACGG-3′
CYR61	5’-AAGAAACCCGGATTTGTGAG-3’	5’-GCTGCATTTCTTGCCCTTT-3’
EGR1	5’-CAGTTCCTCTACCCCAAGGTG-3’	5’-TTCTGCTTGTGTAAATCCTCCA-3’
JUND	5’-AGCCCTACGAGCACCTGAC-3’	5’-GGTTTGGCTGGGGTAACTG-3’
FOS	5’-ACTACCACTCACCCGCAGAC-3’	5’-CCAGGTCCGTGCAGAAGT-3’
IL6	5’-CTGCAAGTTCCCACAGTTCA-3’	5’-CCCACCTTCTTCAAAATCCA-3’
ATF3	5’-AAGGATTTTCAGCACCTTGC-3’	5’-GATGGCAGAAGCACTCACTTC-3’
FOSB	5’-AGCAGCTAAATGCAGGAACC-3’	5’-TCCTCCAACTGATCTGTCTCC-3’
EGR2	5’-TTGACCAGATGAACGGAGTG-3’	5’-TGGTTTCTAGGTGCAGAGACG-3’
EGR3	5’-CAATCTGTACCCCGAGGAGA-3’	5’-CCGATGTCCATTACATTCTCTG-3’
GEM	5’-GACAGCATGGACAGCGACT-3’	5’-AACCATCAGGGTTCGTTCAT-3’
NR4A1	5’-CCACTGCCTCCTTCAACC-3’	5’-GGCTTGGATACAGGGCATC-3’
ZFP36	5’-CCCAAGTGTGCAAGCTCAG-3’	5’-CCCCAAGAACCTCGGAAG-3’

References

1. Schackmann RCJ KS, Vlug EJ, Stelloo S, van Amersfoort M, Tenhagen M, Braumuller TM, Vermeulen JF, van der Groep P, Peeters T, van der Wall E, van Diest PJ, Jonkers J, Derksen PWB. Loss of p120-Catenin Induces Metastatic Progression of Breast Cancer by Inducing Anoikis Resistance and Augmenting Growth Factor Receptor Signaling. Cancer Research. 2013;73(15):4937-49.

2. Schackmann RCJ vAM, Haarhuis JHI, Vlug EJ, Halim VA, Roodhart JML, Vermaat JS, Voest EE, van der Groep P, van Diest PJ, Jonkers J, Derksen PWB. Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance. Journal of Clinical Investigation. 2011;121(8):3176-88.

3. Kim NG KE, Chen X, Gumbiner BM. E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. Proceedings of the National Academy of Science. 2011;108(29):11930-5.

4. Schlegelmilch K MM, Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR, Camargo FD. Yap1 acts downstream of α-catenin to control epidermal proliferation. Cell. 2011;144(5):782-95.

5. Vlug EJ vdVR, Vermeulen JF, Bult P, van Diest PJ, Derksen PW. Nuclear localization of the transcriptional coactivator YAP is associated with invasive lobular breast cancer. Cellular Oncology. 2013;36(5):375-84.

6. An Y AJ, Hollern DP, Zhao A, Chang SG, Gams MS, Chung PED, He X, Jangra R, Shah JS, Yang J, Beck LA, Raghuram N, Kozma KJ, Loch AJ, Wang W, Fan C, Done SJ, Zacksenhaus E, Guidos CJ, Perou CM, Egan SE. Cdh1 and Pik3ca Mutations Cooperate to Induce Immune-Related Invasive Lobular Carcinoma of the Breast. Cell Reports. 2018;25:702-14.

7. Zhao B YX, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL. TEAD mediates YAP-dependent gene induction and growth control. Genes and Development. 2008;22(14):1962-71.

8. Shao DD XW, Krall EB, Bhutkar A, Piccioni F, Wang X, Schinzel AC, Sood S, Rosenbluh J, Kim JW, Zwang Y, Roberts TM, Root DE, Jacks T, Hahn WC. KRAS and YAP1 converge to regulate EMT and tumor survival. Cell. 2014;158(1):171-84.

9. Zanconato F FM, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Bicciato S, Cordenonsi M, Piccolo S. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. Nature Cell Biology. 2015;17(9):1218-27.

10. Kang MH JG, Smoot DT, Ashktorab H, Hwang CM, Kim BS, Kim HS, Park YY. Verteporfin inhibits gastric cancer cell growth by suppressing adhesion molecule FAT1. Oncotarget. 2017;8(58):98887-97.

11. Zhang H RS, Triner D, Centofanti B, Maitra D, Győrffy B, Sebolt-Leopold JS, Dame MK, Varani J, Brenner DE, Fearon ER, Omary MB, Shah YM. Tumor-selective proteotoxicity of verteporfin inhibits colon cancer progression independently of YAP1. Science Signaling. 2015;8(397).

12. Eales KL WE, Cruickshank G, Tucker JHR, Tennant DA. Verteporfin selectively kills hypoxic glioma cells through iron-binding and increased production of reactive oxygen species. Scientific Reports. 2018;8(1):14358.

13. Anonymous. Cancer Genome Atlas Network Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:61-70.

14. Weigelt B GF, Natrajan R, Lopez-Garcia MA, Ahmad AS, Savage K, Kreike B, Reis-Filho JS. The molecular underpinning of lobular histological growth pattern: a genome-wide transcriptomic analysis of invasive lobular carcinomas and grade- and molecular subtype-matched invasive ductal carcinomas of no special type. Journal of Pathology. 2010;220(1):45-57.

15. Stover DG CJ, Barry WT, Brugge JS, Winer EP, Selfors LM. The Role of Proliferation in Determining Response to Neoadjuvant Chemotherapy in Breast Cancer: A Gene Expression-Based Meta-Analysis. Clinical Cancer Research. 2016;22(24):6039-50.

16. Christgen M BH, Hadamitzky C, Rudolph C, Steinemann D, Gadzicki D, Hasemeier B, Römermann D, Focken T, Krech T, Ballmaier M, Schlegelberger B, Kreipe H, Lehmann U. Comprehensive genetic and functional characterization of IPH-926: a novel CDH1-null tumour cell line from human lobular breast cancer. Journal of Pathology. 2009;217(5):620-32.

17. Bui DA LW, White AE, Harper JW, Schackmann RC, Overholtzer M, Selfors LM, Brugge JS. Cytokinesis involves a nontranscriptional function of the Hippo pathway effector YAP. Science Signaling. 2016;9(417). doi: 10.1126/scisignal.aaa9227.

18. Liu-Chittenden Y HB, Shim JS, Chen Q, Lee SJ, Anders RA, Liu JO, Pan D. Genetic and pharmacological disruption of the TEAD–YAP complex suppresses the oncogenic activity of YAP. Genes and Development. 2012;26(12):1300-5.

19. Lui JW XS, Ogomori K, Hammarstedt JE, Little EC, Lang D. The Efficiency of Verteporfin as a Therapeutic Option in Pre-Clinical Models of Melanoma. Journal of Cancer. 2019;10(1):1-10.

20. Konstantinou EK NS, Kosmidou C, Brodowska K, Al-Moujahed A, Nicolaou F, Tsoka P, Gragoudas E, Miller JW, Young LH, Vavvas DG. Verteporfin-induced formation of protein cross-linked oligomers and high molecular weight complexes is mediated by light and leads to cell toxicity. Scientific Reports. 2017;7:46581.

21. Conover CA HL, Bradley S, Stalboerger P, Klee GG, Kalli KR, Jenkins RB. Biological Characterization of Human Epithelial Ovarian Carcinoma Cells in Primary Culture: The Insulin-like Growth Factor System. Experimental Cell Research. 1998;238:439-49.

22. Davidowitz RA SL, Iwanicki MP, Elias KM, Karst A, Piao H, Ince TA, Drage MG, Dering J, Konecny GE, Matulonis U, Mills GB, Slamon DJ, Drapkin R, Brugge JS. Mesenchymal gene program–expressing ovarian cancer spheroids exhibit enhanced mesothelial clearance. Journal of Clinical Investigation. 2014;124(6):2611-25.

23. Dupont S ML, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S. Role of YAP/TAZ in mechanotransduction. Nature. 2011;474(7350):179-83.

24. Foster CT GF, Treisman R. Mutual dependence of the MRTF-SRF and YAP-TEAD pathways in cancer-associated fibroblasts is indirect and mediated by cytoskeletal dynamics. Genes and Development. 2017;31(23-24):2361-75.

25. Olson EN NA. Linking actin dynamics and gene transcription to drive cellular motile functions. Nature Reviews Molecular Cell Biology. 2010;11(5):353-65.

26. Zhao B LL, Wang L, Wang CY, Yu J, Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes and Development. 2012;26(1):54-68.

27. Derksen PW LX, Saridin F, van der Gulden H, Zevenhoven J, Evers B, van Beijnum JR, Griffioen AW, Vink J, Krimpenfort P, Peterse JL, Cardiff RD, Berns A,

Jonkers J. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell. 2006;10(5):437-49.

28. Tullai JW SM, Mullenbrock S, Sholder G, Kasif S, Cooper GM. Immediate-early and delayed primary response genes are distinct in function and genomic architecture. Journal of Biological Chemistry. 2007;282(33):23981-95.

29. Muranen T SL, Worster DT, Iwanicki MP, Song L, Morales FC, Gao S, Mills GB, Brugge JS. Inhibition of PI3K/mTOR Leads to Adaptive Resistance in Matrix-Attached Cancer Cells. Cancer Cell. 2012;21(2):227-39.

Appendix II.

Supplemental Material for Chapter 2.

Supplementary Movie 1 Representative DIC movies of control or shYAP MCF10A cells stimulated with EGF

Supplementary Movie 2 Representative phase contrast movies of control or shYAP MCF10A cells in a wound-healing assay