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Role of TRIP6 and Angiomotins in the Regulation of the Hippo Signaling Pathway

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**ROLE OF TRIP6 AND ANGIOMOTINS IN THE REGULATION OF
THE HIPPO SIGNALING PATHWAY**

A Dissertation Presented

By

SHUBHAM DUTTA

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March, 16th 2018

INTERDISCIPLINARY GRADUATE PROGRAM

REVIEWER PAGE

ROLE OF TRIP6 AND ANGIOMOTINS IN THE REGULATION OF
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SHUBHAM DUTTA

This work was undertaken in the Graduate School of Biomedical Sciences

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APPROVAL PAGE

*Dedicated to
my grandparents*

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I would like to thank my parents for providing me with good education and teaching me values like honesty, truth, and compassion. They never stopped me from following my dreams and have been my source of inspiration and courage. I would like to thank my beautiful sister for her constant support. She means the world to me and I wish all her dreams come true.

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ABSTRACT

Mechanical tension is an important regulator of cell proliferation, differentiation, migration and cell death. It is involved in the control of tissue architecture and wound repair and its improper sensing can contribute to cancer. The Hippo tumor suppressor pathway was recently shown to be involved in regulating cell proliferation in response to mechanical tension. The core of the pathway consists of the kinases MST1/2 and LATS1/2, which regulate the target of the pathway, the transcription co-activator YAP/TAZ (hereafter referred to as YAP). When the Hippo pathway is inactive, YAP remains in the nucleus and promotes cell proliferation and stem cell maintenance. When the Hippo signaling pathway is turned on, MST1/2 phosphorylate and activates LATS1/2. LATS1/2 phosphorylates and inactivates YAP in the cytoplasm which is sequestered and degraded, stopping cell proliferation and promoting differentiation of stem cells. Mechanical forces are transmitted across cells and tissues through the cell-cell junctions and the actin cytoskeleton. However, the factors that connect cell-cell junctions to the Hippo signaling pathway were not clearly known. We identified a LIM domain protein called TRIP6 that functions at the adherens junctions to regulate the Hippo signaling pathway in a tension-dependent manner. TRIP6 responds to mechanical tension at adherens junctions and regulates LATS1/2 activity. Under high mechanical tension, TRIP6 sequesters and inhibits LATS1/2 at adherens junctions to promote YAP activity. Conditions that reduce tension at adherens junctions by inhibition of actin stress fibers or disruption of cell-cell junctions reduce TRIP6-LATS1/2 binding, which activates LATS1/2 to inhibit YAP. Vinculin has been shown to act as part of a mechanosensory complex at adherens junctions. We show that vinculin promotes TRIP6 inhibition of LATS1/2 in response to mechanical tension. Furthermore, we show

that TRIP6 competitively inhibits MOB1 (a known LATS1/2 activator) from binding and activating LATS1/2. Together these findings reveal TRIP6 responds to mechanical signals at adherens junctions to regulate the Hippo signaling pathway in mammalian cells.

TABLE OF CONTENTS

TITLE PAGE	i
REVIEWER PAGE	ii
APPROVAL PAGE	iii
ACKNOWLEDGEMENTS	v
ABSTRACT	vii
LIST OF TABLES	xi
LIST OF FIGURES.....	xii
PREFACE	xiv
CHAPTER 1: INTRODUCTION	1
1.1 The Hippo signaling pathway	1
1.2 Regulators of the Hippo signaling pathway	6
1.3 Regulation of YAP/TAZ by the Hippo signaling pathway.....	10
1.4 Mechanosensory regulation of the Hippo signaling pathway	12
1.5 Role of LIM domain proteins in the Hippo pathway	16
CHAPTER 2: TRIP6 INHIBITS THE HIPPO SIGNALING PATHWAY IN RESPONSE TO TENSION AT ADHERENS JUNCTIONS.....	19
2.1 Abstract.....	19
2.2 Introduction	19
2.3 Results	21
2.4 Discussion.....	57

CHAPTER 3: DISCUSSION	61
APPENDIX.....	68
4.1 ROLE OF ANGIOMOTINS IN REGULATION OF THE HIPPO SIGNALING PATHWAY.....	68
4.1.1 Introduction.....	68
4.1.2 Results.....	70
4.1.3 Discussion	75
4.2 MATERIALS AND METHODS	77
4.3 TABLES	84
BIBLIOGRAPHY.....	88

LIST OF TABLES

Table 1: RT-qPCR primers	84
Table 2: TRIP6 CRISPR primers	84
Table 3: Reagent and resource table.....	84

LIST OF FIGURES

Figure 1.1 The Hippo signaling pathway in <i>Drosophila</i> and in the mammalian system are conserved.	3
Figure 1.2 Structural basis for MOB1 dependent activation of LATS1/2.....	5
Figure 2.1 TRIP6 promotes YAP activity by inhibiting LATS1/2.	25
Figure 2.2 TRIP6 knockdown in MCF10A cells activates hippo signaling and TRIP6-KO knockout cells are rescued by FLAG-TRIP6 expression.	28
Figure 2.3 TRIP6 competes with MOB1 for binding to LATS2.....	32
Figure 2.4 Coomassie-stained gel showing the amounts of BSA and highest amount of HIS-MOB1A used in Figure 2.3B.	34
Figure 2.5 TRIP6-LATS binding and localization to cell-cell junctions is regulated by tension.....	39
Figure 2.6 Regulation of TRIP6 and LATS1 localization and binding.	42
Figure 2.7 Perturbations of junctions and f-actin reduce TRIP6-LATS1 binding and localization to cell-cell junctions.	43
Figure 2.8 Regulation of TRIP6 and LATS1 localization in HEK293A cells and E-cadherin staining in MCF10A cells after various treatments, TRIP6, LATS1 and 2, and vinculin knockdown.	45
Figure 2.9 Vinculin interacts with TRIP6 and regulates LATS and YAP activity.	49
Figure 2.10 FAK and vinculin co-staining in MCF10A cells, vinculin knockdown efficacy, LATS1 and vinculin co-staining in MCF10A cells, the effect of TRIP6 knockdown on vinculin, the effect of vinculin knockdown by single siRNAs on YAP activity and localization and rescue of siRNA knockdown.....	51

Figure 2.11 Vinculin regulates TRIP6-LATS1 interaction and localization.....	56
Figure 2.12 TRIP6 inhibits the Hippo signaling pathway in response to tension at adherens junctions.	60
Figure 4.1 A diagram of AMOT80, AMOT130, AMOTL1, and AMOTL2.....	69
Figure 4.2 Determining conserved domains in angiomotins and testing their role in LATS2 activity	71
Figure 4.3 Mapping actin binding sites on angiomotins	72
Figure 4.4 LATS2 regulates AMOT130 function	74
Figure 4.5 AMOT130 regulates YAP localization.	76

PREFACE

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CHAPTER 1

INTRODUCTION

The growth of multicellular organisms is regulated by intricate cellular processes during their development, which controls cell proliferation, stem cell maintenance and differentiation, and cell death. Dysfunction of these processes can lead to uncontrolled cell division and cancer. Inherent errors in DNA replication machinery, exposure to carcinogens and other environmental factors induce mutations in DNA. Such mutations can lead to hyperactivation of oncogenes and/or inactivation of tumor suppressor genes, which leads to uncontrolled cell proliferation causing malignancy and tumor formation. These tumors can evade apoptosis and spread throughout the body via metastasis, which can result in lethal interference with normal body functions. Various signaling pathways such as the TOR/TSC, the MAPK, and the Hippo pathway have been shown to regulate cell proliferation, organ growth, and development. Genes involved in these pathways are found to be mutated/misregulated in tumorigenic cells. The focus of this work is to understand the signals that control Hippo pathway signaling.

1.1 The Hippo signaling pathway

The Hippo signaling pathway (also known as the Salvador/Warts/Hippo pathway in *Drosophila*) is a highly conserved pathway in metazoans, such as *Drosophila* and mammals, which regulates cell proliferation, stem cell differentiation and cell death (Figure 1.1). This pathway was first discovered in a mutagenic screen in *Drosophila* to identify tumor suppressor genes (Kango-Singh and Singh, 2009; Pan, 2007; Saucedo and Edgar, 2007). It was seen that mutation in a protein kinase gene leads to hippopotamus-like overgrowth phenotype in the head, eyes and imaginal discs (Harvey

et al., 2003; Wu et al., 2003). This protein kinase gene, later named as Hippo (Hpo), is a core kinase of the Hippo signaling cascade. Hpo belongs to the serine/threonine Ste-20 kinase family of proteins (Harvey et al., 2003; Jia et al., 2003). Hpo binds to its adaptor protein called Salvador (Sav) to phosphorylate and activate another kinase called Warts (Wts), which belongs to the Dbf-2-related (NDR) family of kinases (Tapon et al., 2002; Kango-Singh, 2002; Xu et al., 1995; Justice et al., 1995). Wts forms a complex with Mats (MOB as a tumor suppressor), which is crucial for Wts to be activated by Hpo (Lai et al., 2005). Active Wts phosphorylates and restricts the function of a transcription coactivator called Yorkie (Yki) (Huang et al., 2005). Phosphorylated Yki translocates into the cytoplasm where it is sequestered and/or degraded, which stops tissue growth. Active Yki stays in the nucleus where it binds to various transcription factors such as Scalloped (Sd), Homothorax (Hth), and Teashirt (Tsh) (Oh and Irvine, 2011). This regulation promotes the expression of a plethora of genes that stimulate cell cycle progression (cyclin A, cyclin E, E2F1), cell survival (a micro RNA called bantam, myc), and apoptosis (diap1) that regulate cell proliferation, stem cell differentiation and tissue growth. Interestingly, Yki can upregulate certain genes within the Hippo signaling network (such as myc, crumbs, ex, merlin, four-jointed, and kibra) to eventually inactivate itself through a negative feedback loop (Pan, 2010; Neto-Silva et al., 2010; Zhu et al., 2015).

The mammalian Hippo signaling pathway is similar to the *Drosophila* pathway and is mostly studied in mice and mammalian cell lines (see Figure 1.1 for comparison of the two hippo signaling pathways). MST1/2 (homolog of Hpo) in association with WW45 (a Sav homolog) phosphorylates and activates LATS1/2 (a homolog of Wts), which forms

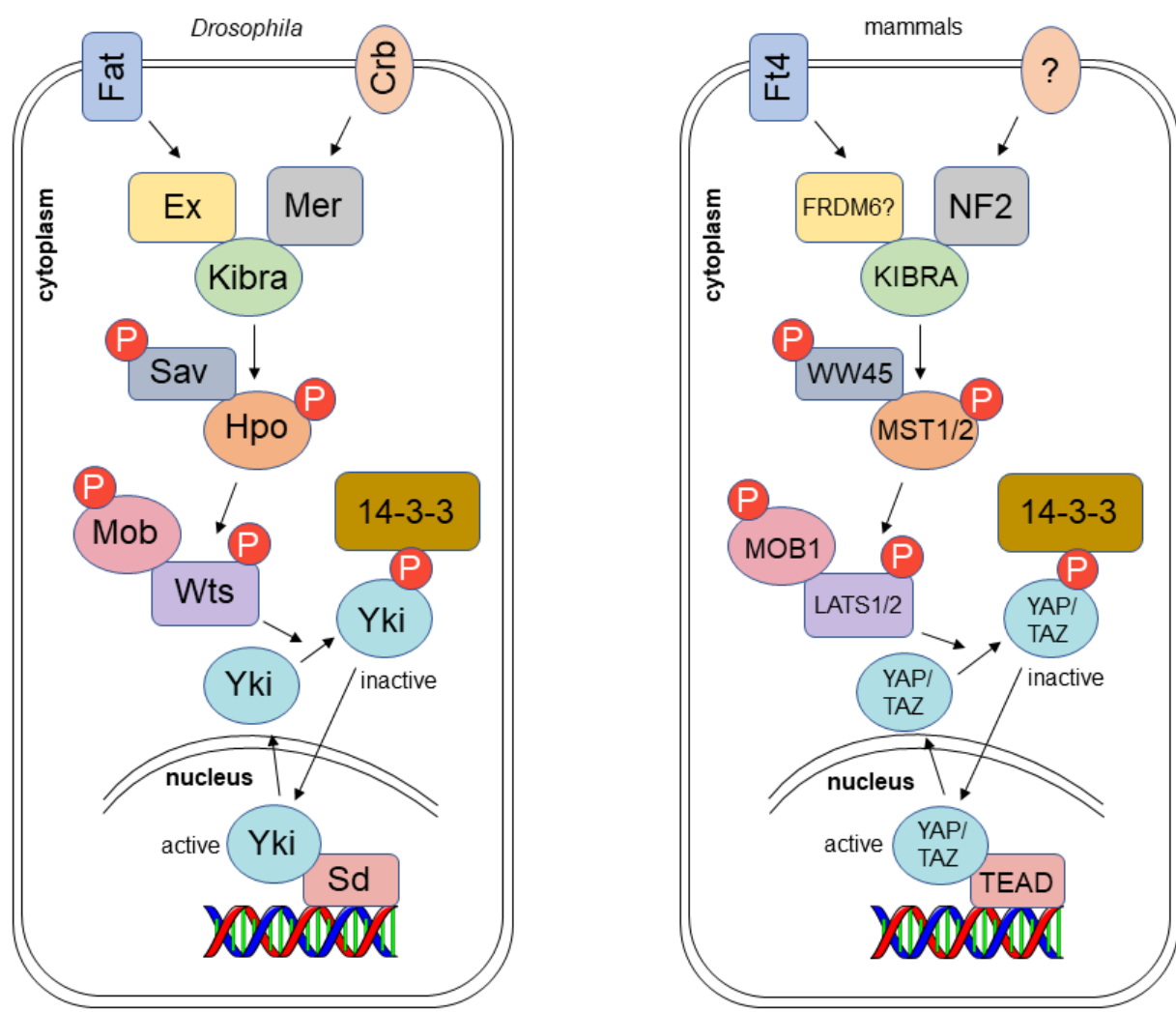


Figure 1.1 The Hippo signaling pathway in *Drosophila* and in the mammalian system are conserved.

Homologous proteins in *Drosophila* and mammals are indicated by matching colors. Proximity indicates direct biochemical interaction and direct/ indirect activation. Phosphorylation is indicated by P.

a complex with MOB1 (a homolog of Mats). Structural studies have shed light on how MST1/2 and MOB1 collaborate to activate LATS1/2 (Ni et al., 2015). The catalytic

kinase domain of MST1/2 autophosphorylates itself to allow the docking of MOB1. Under normal circumstances, MOB1 remains in an autoinhibited state. Once MOB1 binds the phospho-docking site on MST1/2, it is relieved of its autoinhibition. Thereafter LATS1/2 binds MOB1 and subsequently MST1/2 phosphorylates both MOB1 and LATS1/2 on its hydrophobic domain (HD). MOB1 phosphorylation by MST1/2 releases MOB-LATS complex which triggers LATS1/2 to autophosphorylate on its activation loop (AL) (Ni et al., 2015) (See Figure 1.2). These phosphorylations activates LATS1/2 which then phosphorylates and inactivates two transcription co-activators YAP and TAZ in the cytoplasm (homologs of Yki). Subsequently phosphorylated cytoplasmic YAP and TAZ are sequestered by various proteins (14-3-3 and Angiomotins) or degraded by the proteasomal degradation machinery (Mana-Capelli et al., 2014; Paramasivam et al., 2011; Chan et al., 2011a; Zhao et al., 2011; Lei et al., 2008; Hao et al., 2008; Liu et al., 2010b; Ren et al., 2010; Zhao et al., 2010b). Active YAP remains inside the nucleus, where it binds primarily to the TEAD family of transcription factors (homolog of Sd) and promotes cell survival, stem cell maintenance and organ growth (Zhao et al., 2008; Zhang et al., 2009). Similar to *Drosophila*, YAP promotes its own inactivation through negative feedback by upregulating certain upstream activators of the Hippo signaling pathway such as LATS1/2 and AMOTL2 (Dai et al., 2015; Moroishi et al., 2015). In mesothelial cells, LATS1/2, MST1, Sav1 and Merlin have shown to be nuclear (Li et al., 2014). However, whether they can regulate YAP activity while inside the nucleus is not known and requires further investigation.

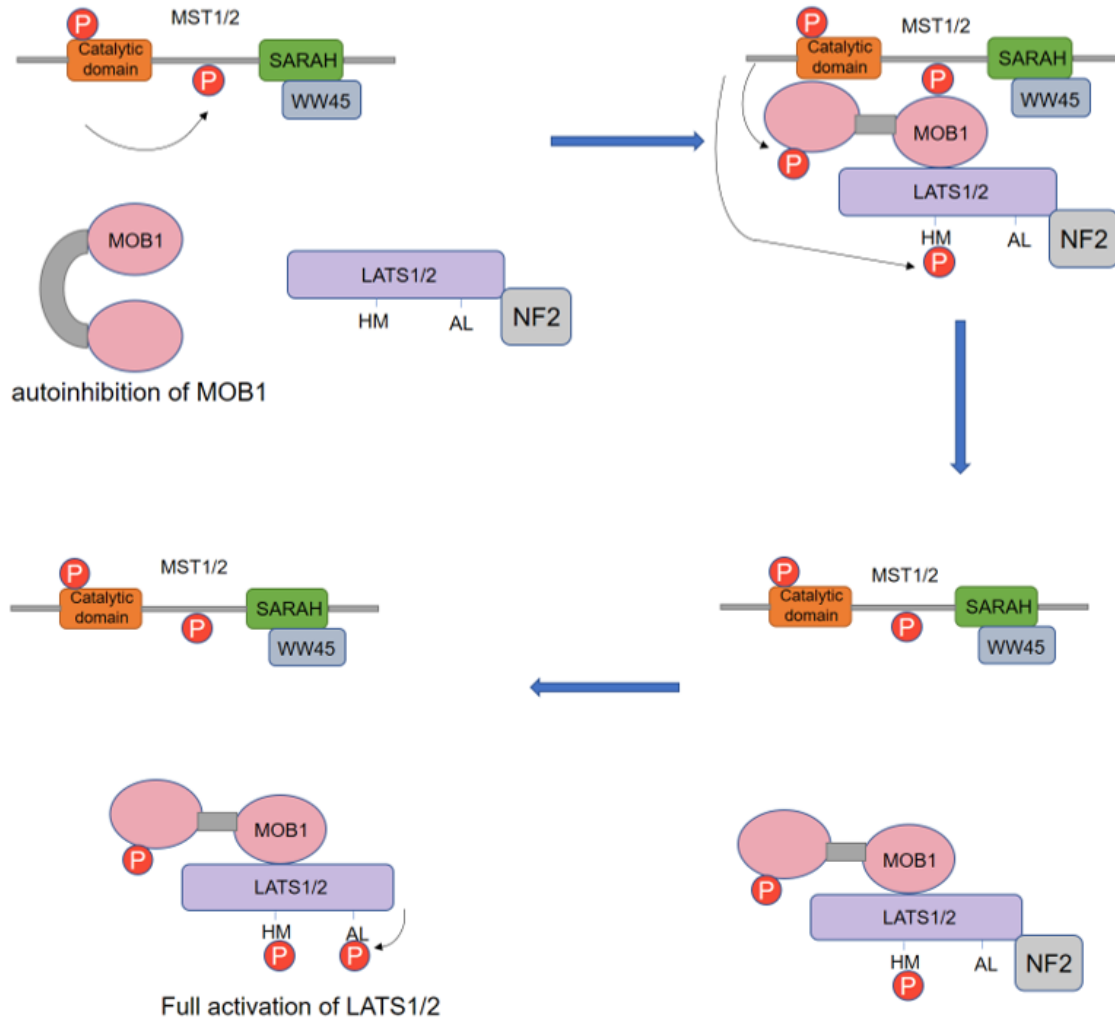


Figure 1.2 Structural basis for MOB1 dependent activation of LATS1/2.

Autophosphorylation of MST1/2 creates a MOB1 docking site. MOB1 which remains in its autoinhibited state then binds MST1/2 and is relieved of autoinhibition. This promotes LATS1/2 to bind MOB1. MST1/2 then phosphorylates MOB1 and the hydrophobic motif (HM) on LATS1/2. The MOB1-LATS1/2 complex then dissociates from MST1/2 and phosphor-MOB1 triggers autophosphorylation of LATS1/2 on its activation loop (AL) to activate LATS1/2.

1.2 Regulators of the Hippo signaling pathway

Studies have identified a complex and diverse upstream signaling system that regulates the Hippo signaling pathway and YAP activity. These signals can be both external and internal. They primarily affect the phosphorylation of the core Hippo pathway components such as LATS1/2 and YAP, although some of them directly affect YAP activity without influencing LATS1/2 activity (Yu and Guan, 2013).

Soluble factors: Extracellular soluble factors are one of the major regulators of the Hippo signaling pathway. Soluble growth factors also regulate the Hippo signaling pathway through G-protein coupled receptors (GPCRs). Studies have shown that two serum components namely LPA and S1P activate YAP by binding to GPCRs and activating Rho kinase. Rho activation leads to actin stress fiber formation and stabilization. This inactivates LATS1/2 and promotes YAP activity. Another study shows that estrogen can activate its specific GPCRs to inhibit LATS1/2 and hyperactivate YAP which could signify a novel role for estrogen in breast cancer (Zhou et al., 2015). The mechanism by which Rho inactivates LATS1/2 is not known. On the other hand, glucagon and epinephrine activate other GPCRs to promote PKA mediated LATS1/2 activation and YAP inhibition (Yu et al., 2012; Miller et al., 2012). Protein kinase A (PKA) promotes LATS1/2 activation through an unknown mechanism. Knockdown on MST1/2 has no effect on LATS1/2 activity suggesting that MST1/2 might not be the upstream kinase in this scenario. Therefore, it would be interesting to see whether PKA could activate other kinases which then could be responsible for LATS1/2 activation. Alternatively, PKA could also inhibit LATS1/2 inhibitory phosphatases. Wnt ligands (specifically Wnt5a/b) also that act through GPCRs bind to Frizzled receptors and

activate YAP by inactivating LATS1/2 through an unknown mechanism (Park et al., 2015). It would be interesting to unravel the mechanism by which soluble factors could regulate the Hippo signaling pathway.

Cell polarity: Studies in both *Drosophila* and mammals indicate a role of apical-basal cell polarity proteins in modulating Yki/YAP activity. These cell polarity proteins are found in complexes at the cell-cell junctions such as adherens junctions (AJ) and tight junctions (TJ). One important complex found in the apical regions in polarized epithelial cells in *Drosophila* is the Merlin/NF2-Expanded-Kibra complex that activates Hippo signaling. Mutations in Merlin and Expanded promote an overgrowth phenotype like that of Hpo mutants in *Drosophila* (Hamaratoglu et al., 2006). Further studies showed that Merlin associates with Hpo, Warts, and Kibra (Yu et al., 2010; Genevet et al., 2010) suggesting a possible localization of core hippo kinases to the apical region for activation. It has been shown that artificial myristoylation of MST1 promotes plasma membrane localization which enhances its activity (Hamaratoglu et al., 2006).

There are two other membrane components, Fat/ Dachshous and Crumbs, which regulate the Hippo signaling pathway. *Drosophila* Crumbs (Crb), which is an important polarity factor, regulates the Hippo signaling pathway by interacting with Mer to modulate the Hippo signaling kinases to regulate Yki function (Robinson et al., 2010; Ling et al., 2010; Chen et al., 2010a). In mammals, Crb3 (one of the most abundant Crumbs isoform in epithelial cells) is shown to inhibit YAP by promoting its interaction with LATS1/2 or by recruiting other upstream Hippo signaling components such as Kibra or FRDM6 (Szymaniak et al., 2015; Mao et al., 2017). The Fat/ Dachshous (Ft/Ds) system in *Drosophila*, which is also involved in maintaining cell polarity in epithelial and

mesenchymal cells has been shown to regulate the Hippo signaling pathway. Loss of Ft activates Ex or hippo signaling kinase Wts resulting in Yki inactivation and the stopping of tissue growth. The orthologs of Fat in mammalian cells are Fat 1-4 (with Fat 4 being the closest homolog to *Drosophila* Ft) and orthologs of Dachs are Dchs 1-2. Mouse knockout of neither Fat4 nor Dchs1 had a significant effect on the Hippo signaling pathway; thus further studies are needed to understand their role (Cho et al., 2006; Reddy et al., 2008; Rogulja et al., 2008; Grusche et al., 2011; Rodrigues-Campos and Thompson, 2014; Vrabioiu and Struhl, 2015; Misra and Irvine, 2016). Together, the Fat/Dachsous and Crumbs components act in parallel at the membranes to regulate Hippo pathway signaling.

Recent studies have implicated the role of the mammalian tight junction protein NF2 (Merlin in *Drosophila*) as an important regulator of the Hippo signaling pathway. In *Drosophila*, NF2 is responsible for taking LATS1/2 to junctions where it is activated by MST1/2 (Sav mediates MST1/2 junctional localization) (Yin et al., 2013). In mammalian cells, NF2 is also shown to be crucial for LATS1/2 activity and MST1/2 mediated LATS1/2 phosphorylation (Plouffe et al., 2016). Overexpression of NF2 promotes LATS1/2 activation by inhibiting its ubiquitination-mediated degradation to promote YAP inhibition (Zhao et al., 2007; Zhang et al., 2010; Li et al., 2014). In NF2 knockout cells, YAP remains dephosphorylated and hyperactive (Plouffe et al., 2016). NF2 knockout mice develop hepatocarcinoma and cataracts and could be rescued by deletion of YAP (Zhang et al., 2010). The angiomin family of proteins (AMOTL1, AMOTL2 and AMOT p130) which maintains epithelial cell polarity has been shown to activate LATS1/2 kinase and inhibit YAP in an F-actin-dependent manner (Chan et al., 2011a; Zhao et al.,

2011; Paramasivam et al., 2011; Mana-Capelli et al., 2014). Angiomotins also bind NF2/Merlin at tight junctions to regulate the Hippo signaling pathway and are required for NF2 mediated tumorigenesis (Yi et al., 2011).

Cell cycle: Hippo pathway signaling components have been shown to be involved in cell cycle regulation. LATS1/2 can be phosphorylated by CDK1 and Aurora A kinase during mitosis (Toji et al., 2004; Morisaki et al., 2002; Yabuta et al., 2007). CDK1 also phosphorylates YAP during the G2/M checkpoint. LATS1/2 has also been shown to inhibit CDK2 mediated phosphorylation of BRCA2 (Yang et al., 2013; Zhao et al., 2014; Yang et al., 2015b; a). Abnormalities during cytokinesis failure lead to extra chromosomes that activate LATS1/2 and promotes p53 stabilization and YAP inactivation (Ganem et al., 2014). The role of the Hippo signaling pathway with respect to cell cycle needs further investigation to understand its physiological implication.

Contact inhibition: One important controller of cell proliferation and tissue growth is contact inhibition (McClatchey and Yap, 2012; Fagotto and Gumbiner, 1996; Perrais et al., 2007). When cells growing in monolayer or tissues reach high density it is thought they undergo contact inhibition. This could happen because of various factors such as constraint of space, limited supply of growth factors and changes in mechanical tension. Studies have implicated the Hippo signaling pathway in contact inhibition of cell proliferation (Nishioka et al., 2009; McClatchey and Yap, 2012; Halder et al., 2012; Dupont et al., 2011; Aragona et al., 2013). It was seen that YAP and high cells density regulated gene expression in an opposite manner and overexpression of YAP-5SA (a constitutively active variant of YAP) inhibited contact inhibition and promoted overgrowth in cultured epithelial cells and fibroblasts (Zhao et al., 2007). Tumorigenic

tissues also have been shown to have elevated levels of YAP compared to normal ones (Zhao et al., 2007). YAP mediated contact inhibition has also been shown to be important in embryo development (Nishioka et al., 2009). It is hypothesized that as cells spread out they experience more tension by pulling on the ECM through their integrin mediated focal adhesions. This phenomenon also depends on how stiff the ECM is. The stiffer the ECM the more tension is experienced by the individual cells. As cells touch each other they generate more adherens junctions and tight junctions which leads to LATS1/2 activation and YAP inhibition (Zhao et al., 2007; Silvis et al., 2011). Space limitation restricts cell spreading and decrease in cell size which leads to change in cell geometry and F-actin regulation YAP localization and activity (Dupont et al., 2011; Driscoll et al., 2015). One report shows that attachment of cells to the ECM activate Rho-GTPases to promote YAP function (Zhao et al., 2012). Another report implicates the role of FAK-Src-PI3K signaling pathway in regulating YAP activity (Kim and Gumbiner, 2015). The physiological relevance of cell density and tension sensing has been shown in *Drosophila* where it is shown that younger tissues having low number of cells experience greater tension which trigger growth. LIM domain protein Jub was shown to sense tension at adherens junctions and regulate Wts and Yki activity (Rauskolb et al., 2014). The role of MST1/2 in contact inhibition is not known. The mechanism by which cells sense density and space around them is also not clearly understood and needs further investigation.

1.3 Regulation of YAP/TAZ by the Hippo signaling pathway

YAP is a transcription coactivator that associates with TEAD and other TFs to regulate genes that control cell proliferation, differentiation, and apoptosis. Activated LATS1/2

phosphorylate YAP on multiple sites (YAP on Ser61, Ser109, Ser127, Ser164, Ser381; TAZ on Ser66, Ser89, Ser117, Ser311) and inhibit its activity (Zhao et al., 2007; Hao et al., 2008; Dong et al., 2007; Oka et al., 2008; Zhao et al., 2010a; Lei et al., 2008; Kanai et al., 2000; Varelas et al., 2008; Liu et al., 2010b). Once YAP is phosphorylated on Ser127 (S89 on TAZ) 14-3-3 can bind and promote YAP nuclear exclusion and inactivation (Varelas et al., 2008; Kanai et al., 2000; Lei et al., 2008; Oka et al., 2008; Zhao et al., 2007; Dong et al., 2007). Angiomotins can also sequester and inactivate YAP in the cytoplasm (Zhao et al., 2011; Chan et al., 2011a; Mana-Capelli et al., 2014). LATS1/2 phosphorylation on S381 of YAP (S311 on TAZ) primes it for proteasomal degradation. S381 phosphorylation of YAP induces casein kinase 1 to phosphorylate YAP. This phosphorylation leads to recruitment of β -Transducin (β -TRCP; a subunit of the SCF ubiquitin E3 ligase) and leads to degradation of YAP (Zhao et al., 2010b; Liu et al., 2010b).

YAP is implicated in maintaining stemness of cells and promoting stem cell renewal (Totaro et al., 2017; Hu et al., 2017; Sun et al., 2017; Noto et al., 2017; Henle and Link, 2017; Escoll et al., 2017; Tang and Weiss, 2017). YAP is also important in tissue repair and wound healing in differentiated cells. The Hippo pathway is inactivated during tissue injury and YAP is activated leading to stem cell self-renewal, cell migration, and proliferation that leads to wound healing (Lee et al., 2014; Schlegelmilch et al., 2011; Cai et al., 2010). The function of YAP in suppressing apoptosis and promoting cell proliferation is often co-opted in cancer, raising the possibility that it could be used as a therapeutic target for different cancers (Zhao et al., 2014; Han et al., 2014; Yan et al.,

2014; Jeong et al., 2013; Huang et al., 2013; Yang et al., 2012; Zhao et al., 2010b; Liu et al., 2010a; Chan et al., 2009).

YAP has no DNA binding domain and therefore it acts through transcription factors such as TEAD, RUNX, Smads, p73, ERBB4, EGR-1, and TBX5 (Marzia et al., 2000; Murakami et al., 2005; Schuchardt et al., 2014; Vassilev et al., 2001; Ferrigno et al., 2002; Yagi et al., 1999; Strano et al., 2001; Zagurovskaya et al., 2009; Kim et al., 2018). However, the TEAD proteins (TEAD1-4 in mammals) seem to be one of the most important factors through which YAP mediates its function (Santucci et al., 2015; Zanconato et al., 2015; Han et al., 2015; Hiemer et al., 2014; Diepenbruck et al., 2014; Shimomura et al., 2014; Hau et al., 2013; Pobbati and Hong, 2013; Chen et al., 2010b; Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b; Chan et al., 2009). YAP functions both as a transcription co-activator and a co-repressor along with TEADs. YAP recruits the SWI/SNF (a chromatin remodeling protein complex) and/ or NCOA6 (a histone methyltransferase protein) to stimulate TEAD activity to act as co-activators of gene expression (Qing et al., 2014; Skibinski et al., 2014). On the other hand, the YAP-TEAD can act as repressors by engaging deacetylase complexes (Valencia-Sama et al., 2015; Kim et al., 2015). Additional studies are required to understand the role of YAP and associated transcription factors in transcription regulation to control growth and development.

1.4 Mechanosensory regulation of the Hippo signaling pathway

Mechanical forces regulate important physiological events in organisms. These include pumping of the heart, dilation, and constriction of blood vessels, cell migration during development, stem cell differentiation, and wound healing. On a microscopic level,

metazoan cells organize themselves to form tissues and complex organs by adhering to both the proteinaceous extracellular matrix (ECM) through specialized junctions called focal adhesions (FA) and to other cells through cell-cell junctions called adherens junctions (AJ). It is through FAs and AJs that the cells perceive external forces to regulate their behavior. Abnormalities in sensing the environment can lead to various diseases.

Focal adhesions: The ECM is composed of proteins (collagen and elastin), proteoglycans (perlecan and hyaluronan), and glycoproteins (fibronectins and laminins). Together these components confer tensile strength and elasticity and act as a basal layer for cell adhesion. Cells, on the other hand, have surface receptors such as integrins, syndecans, and CD44, which interact with the ECM for attachment. Integrin-based adhesions are known to transmit actomyosin-generated forces to the ECM. Integrins are heterodimeric transmembrane proteins that connect ECM to the actomyosin filaments. On attachment to the ECM, the extracellular domain of integrins undergoes conformational changes to stably bind ECM components (Luo et al., 2007; Su et al., 2016). Then the cytoplasmic domains bind an important mechanosensory protein called talin, which connects them to actin filaments and leads to integrin activation (Calderwood et al., 2013; Hemmings et al., 1996). Integrin-bound talin undergoes mechanical strain induced conformational changes exposing binding sites for another key mechano-responsive protein called vinculin (Ziegler et al., 2008; del Rio et al., 2009). Subsequently, additional proteins are assembled at these focal adhesions that regulate important cellular behaviors such as cell migration, cell proliferation, and cell differentiation ((Ingber, 1997a; Wang et al., 1993; Ingber, 1997b; Shyy and Chien,

1997). These mechano-responsive integrin-actomyosin components in the focal adhesions can influence YAP activity. It has been shown that the rigidity of ECM leads to changes in F-actin/G-actin (F=filamentous, G=globular) ratio and alters actomyosin stress fiber formation (Das et al., 2016). Space restraint and soft ECM lowers the F/G actin ratio, which could be responsible for YAP inhibition. It has been shown reducing tension, either by inhibiting actomyosin formation (cytoskeletal inhibitory drugs) or by restricting space or by decreasing substrate stiffness, inhibit YAP activity (Cui et al., 2015; Aragona et al., 2013; Dupont et al., 2011). Actin capping proteins such as cofilin, capZ, and the actin-severing protein gelsolin are also shown to regulate YAP activity (Aragona et al., 2013). One report suggests that this regulation of YAP through substrate rigidity and cell shape is independent of the Hippo signaling pathway (Aragona et al., 2013) whereas other reports implicate the role of Hippo pathway (Wada et al., 2011; Mana-Capelli et al., 2014). ECM components and soluble factors also regulate the Hippo signaling pathway by activation of Src kinase. It has been shown that ECM protein fibronectin activates focal adhesion kinase (FAK) to promote Src kinase activity which activates PI3K and PDK1 to inhibit LATS1/2 and promote YAP activity (Kim and Gumbiner, 2015). Active Src can directly phosphorylate LATS1/2 and decrease MOB binding to inactivate LATS1/2 (Si et al., 2017). Interestingly, Src can also phosphorylate and activate YAP (Y341, Y357, and Y394) in a non-canonical LATS1/2 independent manner (Li et al., 2016). Soluble components such as EGF and insulin also regulate LATS1/2 and YAP activity by activating Src and PI3K respectively (Fan et al., 2013; Straßburger et al., 2012). Serum components LPA and S1P also inhibit LATS1/2 and promote YAP activity by promoting by binding to GPCRs and

activating Rho kinase. Rho activation leads to actin stress fiber formation and stabilization. Rho activation inactivates LATS1/2 and promotes YAP activity. On the other hand, glucagon and epinephrine activate other GPCRs and acts as an antagonist of YAP (Miller et al., 2012; Yu et al., 2012). Another report implicates the role of an ECM proteoglycan called Agrin that transduces ECM stiffness through Lrp4-MuSK complex and regulate YAP activity (Chakraborty et al., 2017).

Adherens junctions: Cell-cell contacts through adherens junctions are mediated by cadherins, which like integrins are transmembrane proteins consisting of an extracellular domain that interacts with the cadherins of adjacent cells and an intracellular domain that can directly bind actin and other actin-binding proteins such as α -catenin, β -catenin and vinculin (Niessen et al., 2011). Actomyosin filaments function together with the other components at adherens junctions to sense and transduce mechanical tension to regulate cell behavior (Hoffman and Yap, 2015). One well-studied protein complex that senses mechanical tension at the adherens junctions is the α -catenin/vinculin complex. High mechanical tension promotes conformational changes in α -catenin that exposes vinculin binding sites thereby recruiting vinculin to adherens junctions (Hoffman and Yap, 2015). This conformational change in α -catenin is then stabilized by vinculin binding (Hoffman and Yap, 2015). Another α -catenin interacting protein at the zonula adherens called EPLIN has been shown to be important for mammalian cell reshaping by remodeling the zonula adherens in a tension-dependent manner (Taguchi et al., 2011). Similar to α -catenin, β -catenin can recruit vinculin to the adherens junctions and tension might be important in this process (Ray et al., 2013; Peng et al., 2010). LIM domain protein Zyxin has been studied with respect to force

transduction at adherens junctions. Under high mechanical tension Zyxin localizes to the adherens junctions and along with Ena/VASP it promotes F-actin bundling thus emerging as one of the central proteins in mechanotransduction (Hirata et al., 2008b; Oldenburg et al., 2015). Interestingly, Zyxin is implicated in regulation of the Hippo signaling kinase LATS1/2 (see below). Another LIM domain protein Jub also regulates LATS1/2 by sensing mechanical tension at adherens junctions (see below).

Although these studies implicate YAP in responding to mechanical tension and regulating cell behavior, further investigation is required to understand the molecular mechanisms and the protein complexes involved in signal transduction.

1.5 Role of LIM domain proteins in the Hippo pathway

LIM domain proteins are a large family of proteins which have one or more LIM domains. Each LIM domain is cysteine-rich, and forms two zinc-fingers, and often functions as a protein-protein binding interface. These proteins modulate diverse cellular functions such as regulation of actin dynamics, integrin-dependent cell adhesion, determination of cell fate, regulation of gene expression and development. They have been shown to be overexpressed in breast, lung, blood and prostate cancer (Sang et al., 2014; Matthews et al., 2013; Kadrmaz and Beckerle, 2004).

Certain LIM domain proteins have been shown to be regulators of the Hippo signaling pathway. The Zyxin and Ajuba family of LIM domain proteins have been shown to be regulators of the Hippo signaling kinase LATS1/2. *Drosophila* has one member of each family (Zyxin and Jub) whereas mammals have three members of each family (Zyxin family: Zyxin, LPP, and TRIP6; Ajuba family: Ajuba, LIMD1, and WTIP). Interestingly, various reports show that these proteins sense the mechanical tension in cells and

regulate the Hippo signaling pathway. The role of Zyxin, in particular, has been well studied with respect to the actin cytoskeleton and mechanotransduction. The N-terminus of zyxin binds to FA proteins such as Ena/VASP and α -actinin (Niebuhr et al., 1997; Golsteyn et al., 1997; Drees et al., 2000, 1999; Li and Trueb, 2001; Nix et al., 2001). The C-terminus of Zyxin has 3 LIM domains which are important for its localization to FAs under high mechanical tension (Nix et al., 2001; Uemura et al., 2011). Zyxin is shown to dissociate from FAs and relocate into the nucleus under cyclic stretch (Cattaruzza et al., 2004). It has also been shown that Zyxin promotes actin stress fiber formation in a tension dependent manner and induces thickening of actin stress fibers (Furman et al., 2007; Yoshigi et al., 2005; Hoffman et al., 2006; Hirata et al., 2008a). Zyxin is also a component of the adherens junctions and is responsible for linking actin stress fibers to the adherens junctions to regulate epithelial-mesenchymal transition (Sperry et al., 2010; Crawford and Beckerle, 1991). These findings implicate its role as a key mechanosensory protein in cells. Genetic screens in *Drosophila* identified zyxin as a negative regulator of the Hippo pathway (Rauskolb et al., 2011). Membrane protein Dach5 stimulates Zyxin to bind to Warts and promote its degradation keeping Yorkie active (Rauskolb et al., 2011). Zyxin also antagonizes Expanded to promote Yki activity and tissue growth (Gaspar et al., 2015). Another report shows that Zyxin acts as a scaffold protein to recruit LATS2 and SIAH2 under the influence of TGF- β and hypoxia. SIAH2 then promotes LATS2 ubiquitination and degradation which activates YAP (Gaspar et al., 2015). Another LIM domain protein Jub binds and inhibits Warts kinase when phosphorylated by the EGFR-Ras-MAPK signaling pathway activating Yki and promoting tissue growth in *Drosophila*. Similarly, in mammalian cells,

the EGFR-Ras-MAPK signaling pathway phosphorylates LIM domain protein WTIP to enhance its binding to LATS2 and WW45 (Reddy and Irvine, 2013). In *Drosophila*, the Ajuba family of LIM domain proteins called Jub inhibits Warts at adherens junctions in a tension-dependent manner to regulate the Hippo pathway and tissue growth (Rauskolb et al., 2014). Another report shows that cyclic stretch in mammalian cells activates JNK signaling pathways which results in LIMD1 phosphorylation and promotes its binding to and inhibition of LATS1/2 (Codelia et al., 2014). These findings raise key questions such as: (a) How do these LIM domain proteins sense mechanical tension and (b) What is the mechanism of LATS1/2 inhibition by LIM domain proteins.

In my thesis, we wanted to understand how tension is sensed by mammalian cells to regulate the Hippo signaling pathway. Although it was known how tension regulates Hippo signaling in *Drosophila*, the factors important in the mammalian system were not well-studied. We identified LIM domain protein TRIP6 which acts as a sensor of mechanical tension at adherens junctions through vinculin and regulate LATS1/2 activity. Our studies unravel a novel tension dependent regulation at the adherens junctions in mammalian cells that regulate the Hippo signaling pathway.

CHAPTER 2

TRIP6 INHIBITS THE HIPPO SIGNALING PATHWAY IN RESPONSE TO TENSION AT ADHERENS JUNCTIONS

2.1 Abstract

The transcriptional co-activator YAP controls cell proliferation, survival, and tissue regeneration in response to changes in the mechanical environment. It is not known how mechanical stimuli such as tension are sensed and how the signal is transduced to control YAP activity. Here we show that the LIM domain protein TRIP6 acts as part of a mechanotransduction pathway at adherens junctions to promote YAP activity by inhibiting the LATS1/2 kinases. Previous studies showed that vinculin at adherens junctions becomes activated by mechanical tension. We show that vinculin inhibits Hippo signaling by recruiting TRIP6 to adherens junctions and stimulating its binding to and inhibition of LATS1/2 in response to tension. TRIP6 competes with MOB1 for binding to LATS1/2 thereby blocking MOB1 from recruiting the LATS1/2 activating kinases MST1/2. Together these findings reveal a novel pathway that responds to tension at adherens junctions to control Hippo pathway signaling.

2.2 Introduction

Tissue architecture and mechanical forces are major regulators of cell proliferation, and they play important roles during development, organ growth, and tissue regeneration (Heller and Fuchs, 2015; Mammoto et al., 2013; Huang and Ingber, 1999). The cytoskeleton, extracellular matrix, and cell-cell adhesion are critical for transmitting force between cells and across tissues (Vogel and Sheetz, 2006). The Hippo signaling pathway is a major regulator of cellular responses to mechanical inputs (Halder et al., 2012; Sun and Irvine, 2016). The core Hippo pathway (Meng et al., 2016) consists of

two kinase modules: the first includes several Ste20-superfamily kinases (MST1/2 are the best characterized), which phosphorylate and activate the LATS1/2 kinases. MST1/2 phosphorylation of LATS1/2 is mediated by MOB1, which promotes association of MST1/2 with LATS1/2. LATS1/2 then phosphorylate and inhibit the transcriptional co-activator YAP (and its homolog TAZ) by causing it to be sequestered in the cytoplasm or degraded. When in the nucleus, YAP associates with transcription factor TEAD to upregulate genes responsible for survival, proliferation, and stem cell maintenance. The growth promoting properties of YAP are frequently co-opted by cancer cells, in which YAP is often activated and overexpressed (Yu et al., 2015). Although the activity of both LATS1/2 and YAP are clearly regulated by mechanical inputs, how those inputs are sensed and the signals are transduced remain obscure.

Experiments in *Drosophila* and mammalian cells revealed that Hippo pathway regulation of YAP is controlled by mechanical tension (Benham-Pyle et al., 2015; Codelia et al., 2014; Rauskolb et al., 2014; Aragona et al., 2013). When cells experience high mechanical tension, YAP localizes to the nucleus and promotes cell proliferation. Conversely, low tension causes YAP to exit the nucleus and cells to arrest growth. Transmission of tension across tissues requires cell-cell adhesion such as that provided by cadherins (Mui et al., 2016). Tension experienced by cells can be generated by the cells themselves through actomyosin stress fibers or by externally imposed stretch or force (Halder et al., 2012). Studies in *Drosophila* indicate that tension within tissues decreases as cell density increases, and hence tension sensing could contribute to the density-dependent inhibition of cell growth, a property that is typically lost in cancer cells (Rauskolb et al., 2014). Perturbation of stress fibers, externally applied stretch, and cell

density all modulate LATS1/2 and YAP activity; however, the sensors and transduction pathways are not known. In *Drosophila*, the LIM domain protein Ajuba inhibits Warts (the LATS1/2 homolog) and recruits it to adherens junctions in a tension-dependent manner (Rauskolb et al., 2014). The mechanism by which Ajuba regulates Warts activity is not clearly understood. Although Zyxin and Ajuba LIM domain proteins have been shown to interact with LATS1/2 in mammalian cells (Das Thakur et al., 2010; Abe et al., 2006; Hirota et al., 2000), it is unclear whether Ajuba/Zyxin-related proteins function similarly in mammals (Sun and Irvine, 2013; Ma et al., 2016; Jagannathan et al., 2016; Codelia et al., 2014). Here we show that the human LIM domain protein TRIP6 acts as part of a mechanotransduction cascade at adherens junctions to regulate LATS1/2 in response to mechanical tension at cell-cell junctions.

2.3 Results

TRIP6 activates YAP through inhibition of LATS1/2

Although TRIP6 is overexpressed in various cancers where it promotes proliferation and invasion (Chastre et al., 2009; Grunewald et al., 2013; Fei et al., 2013), prior studies had not connected TRIP6 to the Hippo signaling pathway. We previously identified TRIP6 along with several other LIM domain proteins as LATS2 binding partners using tandem affinity purification and mass spectrometry (Paramasivam et al., 2011). To determine which of these LIM domain proteins (TRIP6, FHL2 and WTIP) could regulate the YAP phosphorylation, we co-expressed MST2, LATS2 and YAP2 with or without TRIP6, FHL2 and WTIP and looked at LATS2 mediated YAP2 phosphorylation. We saw that TRIP6 expression reduced YAP phosphorylation compared to the control (Figure 2.1A). To validate the LATS2-TRIP6 interaction, we performed co-immunoprecipitation

experiments. LATS2 was pulled down in TRIP6 immunoprecipitates when both proteins were overexpressed (Figure 2.1B). In addition, endogenous LATS1 was present in TRIP6 immune complexes isolated from MCF10A cells (Figure 2.1C). Like its related family members (Zyxin, LPP, Ajuba, WTIP, and LIMD1), the carboxy-terminal half of TRIP6 consists of 3 conserved LIM domains (Figure 2.1B). Truncation experiments showed that LATS2 binding maps to the C-terminal LIM domain half of TRIP6 (Figure 2.1B). We next tested which parts of LATS2 interacted with TRIP6. TRIP6 bound to the N-terminal region of LATS2 and specifically interacted with two segments (amino acids 376-397 and 625-644) (Figure 2.1D) previously identified to interact with Ajuba and Zyxin (Abe et al., 2006; Hirota et al., 2000).

To determine whether TRIP6 regulates LATS1/2 activity, we examined the effects of TRIP6 overproduction and loss of function. Overexpression of TRIP6 in HEK293A cells reduced endogenous LATS1/2 activity as judged by probing the two sites of activating phosphorylation in LATS1, T1079 and S909 (which correspond to T1041 and S872 in LATS2) (Figure 2.1E & G) (note that T1079 is phosphorylated by MST1/2 and S909 is an autophosphorylation site). In contrast, TRIP6 overexpression did not affect MST2 activating phosphorylation (Figure 2.1I), suggesting that TRIP6 may regulate the ability of LATS1/2 to be phosphorylated by MST1/2. CRISPR mediated deletion of TRIP6 (TRIP6-KO) in HEK293A cells (Figure 2.1F & H) or shRNA mediated knockdown of TRIP6 in MCF10A cells (Figure 2.2A) increased LATS1/2 activating phosphorylation levels. Together these results show that TRIP6 acts to inhibit LATS1/2 activity.

Because LATS1/2 phosphorylate and inhibit YAP nuclear localization, stability, and activity, we tested the effect of modulating TRIP6 levels on YAP. Overexpression of

TRIP6 in HEK293A cells inhibited LATS1/2 phosphorylation of YAP on S127 (Figure 2.1E & G) and increased expression of YAP target genes (Figure 2.1J). In contrast, reduced levels of TRIP6 inhibited YAP function. Specifically, shRNA mediated knockdown of TRIP6 in MCF10A cells reduced expression of YAP target genes (Figure 2.2B) and diminished YAP nuclear localization (Figure 2.2C). These cells also had reduced levels of YAP protein (Figure 2.2D), presumably caused by LATS1/2 phosphorylation-dependent degradation (Liu et al., 2010b; Zhao et al., 2010b). TRIP6-KO HEK293A cells showed increased YAP S127 phosphorylation (Figure 2.1F & H), reduced expression of YAP target genes (Figure 2.1K), and reduced YAP nuclear localization (Figure 2.1L). Consistent with TRIP6 acting through LATS1/2 to affect YAP localization, depletion of LATS1/2 by siRNA in TRIP6-KO cells restored YAP nuclear localization (Figure 2.2E-F). Our observation that MCF10A, but not HEK293A, cells had reduced levels of YAP when TRIP6 was depleted (or eliminated) may reflect cell type differences in YAP degradation in response to LATS1/2 dependent phosphorylation. The TRIP6-KO HEK293A cells also displayed a defect in cell-cell adhesion as judged by the presence of frequent gaps between cells even at high density that were not observed in parental HEK293A cells (Figure 2.1L, Figure 2.2G). The cell-cell adhesion and YAP localization defect in TRIP6-KO HEK293A cells were rescued by re-expression of TRIP6 (Figure 2.2G-H). MCF10A cells knocked down for TRIP6 with shRNA did not show obvious cell-cell adhesion defects or changes in E-cadherin staining (Figure 2.8C), perhaps due to cell type differences or the presence of residual TRIP6. Overall, these results show that TRIP6 inhibition of LATS1/2 promotes YAP activity.

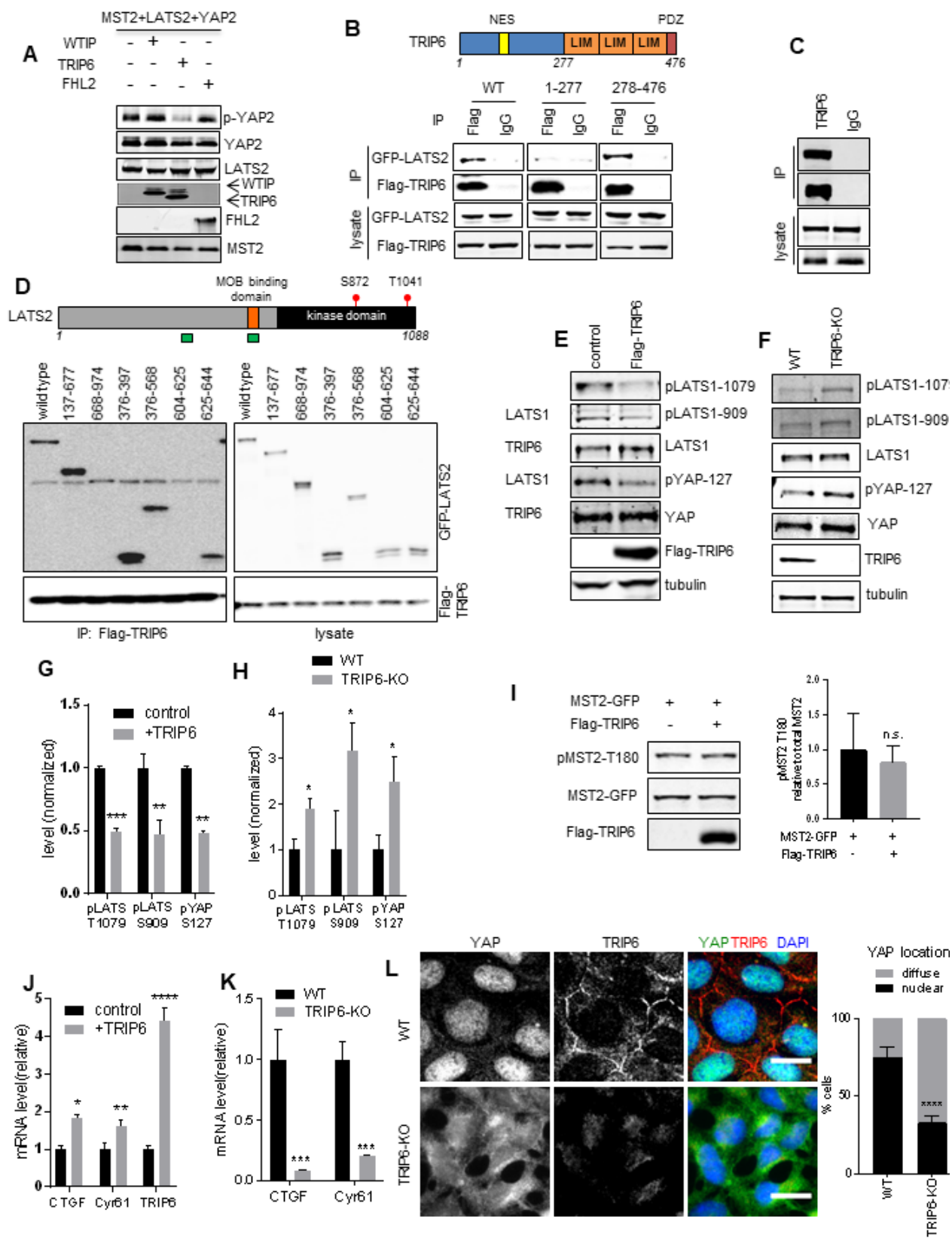


Figure 2.1 TRIP6 promotes YAP activity by inhibiting LATS1/2.

(A) MST2, LATS2 and YAP2 were co-expressed with or without TRIP6, FHL2 and WTIP in HEK293 cells and lysates were analyzed using western blotting for MST2, LATS2, YAP2 and YAP S127 inhibitory phosphorylation. YAP2 S127 phosphorylation was measured relative to YAP2 levels. (B) Full length (WT), the amino-terminal half (1-277), or the carboxy-terminal half (278-476) of TRIP6 were tested for binding to LATS2 by immunoprecipitation. FLAG-TRIP6 variants were co-expressed with LATS2-GFP in HEK293 cells, anti-FLAG or control (IgG) antibodies were used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for LATS2-GFP and FLAG-TRIP6. Schematic diagram depicts TRIP6 domains (NES: Nuclear Export Signal; LIM: LIM domain; PDZ: PDZ domain-binding motif). (C) Lysates from MCF10A cells were subjected to immunoprecipitation using anti-TRIP6 or control (IgG) antibodies, and immune complexes and lysates were probed for TRIP6 and LATS1. (D) FLAG-TRIP6 was tested for binding to various LATS2-GFP deletion mutants as described in part (B). Schematic diagram of LATS2 shows MOB1 binding domain and the autophosphorylation (S872) and MST1/2 phosphorylation sites (T1041) in the kinase domain. The regions marked in green depict TRIP6 binding sites on LATS2. (E) Lysates from HEK293A cells transfected with control or FLAG-TRIP6 plasmid was analyzed by western blotting using the indicated antibodies (quantification is shown in (G)). (F) Lysates from control (WT) or CRISPR generated TRIP6 null (TRIP6-KO) HEK293A cells were analyzed by western blotting using the indicated antibodies (quantification shown in (H)). (G) The relative levels of LATS1 activating phosphorylation (pLATS1-1079, 909) and YAP S127 inhibitory phosphorylation from (E)

were measured relative to LATS1 and YAP levels respectively. (Mean \pm SD; n=3; **P \leq 0.01, ***P \leq 0.001, T-test). (H) The levels of LATS1 activating phosphorylation and YAP inactivating phosphorylation in part (F) were quantified (Mean \pm SD; n=3; *P \leq 0.05, T-test). (I) GFP-MST2 was expressed with or without FLAG-TRIP6 in HEK293 cells and the levels of MST2, MST2 activating phosphorylation (pMST2-T180), and FLAG-TRIP6 were measured by western blotting with the indicated antibodies. (Mean \pm SD; n=3; n.s. \geq 0.05, T-test). (J) TRIP6 was overexpressed in HEK293A cells and the levels of TRIP6, and YAP target gene expression was analyzed using RT-qPCR. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ****P \leq 0.0001, T-test). (K) The levels of YAP target gene expression were analyzed using RT-qPCR in control (WT) and TRIP6-KO HEK293A cells. (Mean \pm SD; n=3; ***P \leq 0.001, T-test). (L) Control (WT) and TRIP6-KO HEK293A cells were stained for YAP and TRIP6. Merged image shows YAP (green), TRIP6 (red), and DNA (blue). Quantification of at least 100 cells is shown (Mean \pm SD; n=3; ****P \leq 0.0001, Fisher's test). Scale bar=20 μ m.

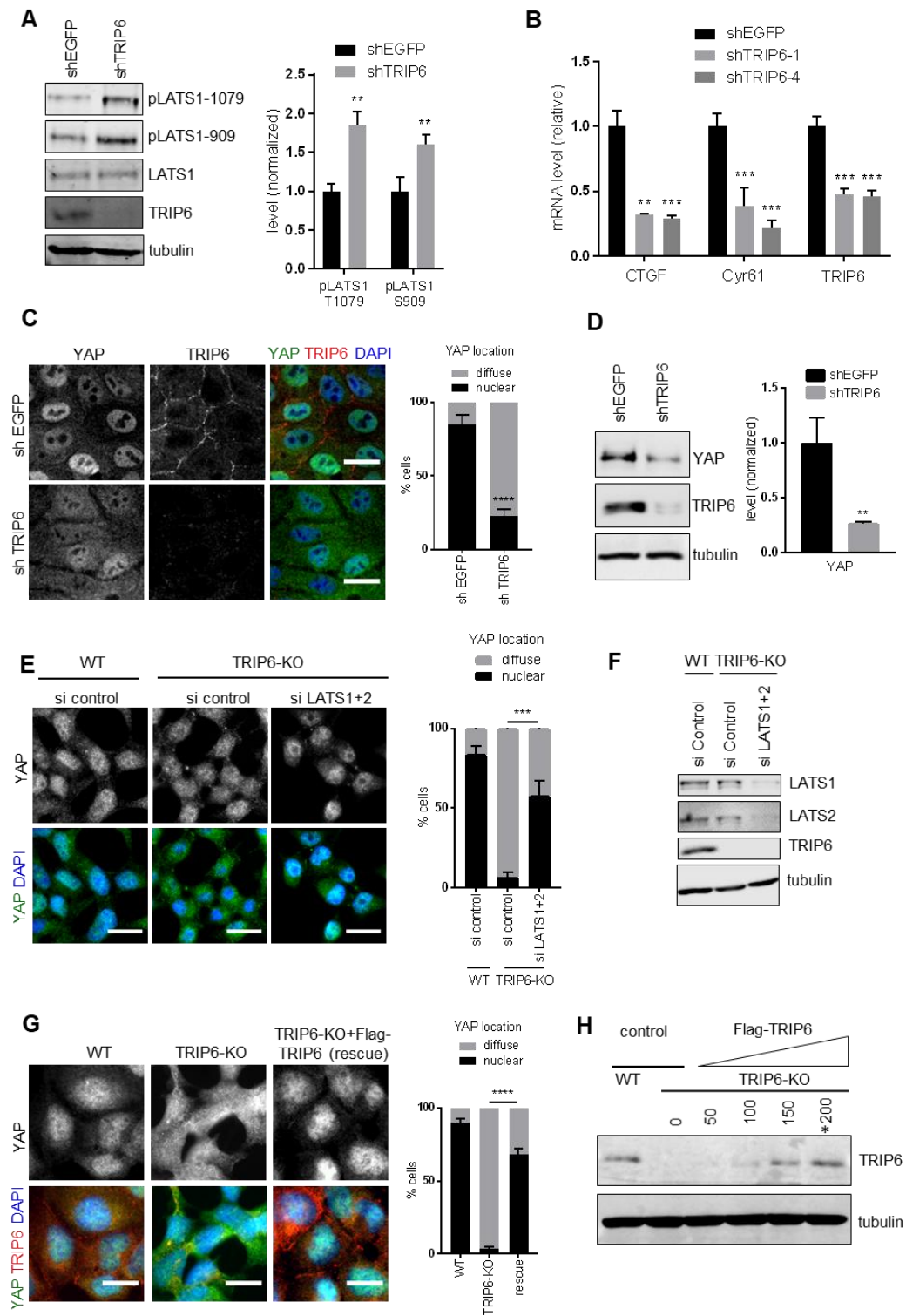


Figure 2.2 TRIP6 knockdown in MCF10A cells activates hippo signaling and TRIP6-KO knockout cells are rescued by FLAG-TRIP6 expression.

(A) Lysates from MCF10A cells infected with lentivirus with a control shRNA (shEGFP) or a mix of two different shRNA against TRIP6 (shTRIP6-1 and shTRIP6-4) were analyzed by western blotting using the indicated antibodies, and the levels of LATS1 activating phosphorylation was quantified (Mean \pm SD; n=3; **P \leq 0.01, T-test).

(B) MCF10A cells were infected with lentivirus carrying control shRNA (shEGFP), or two different shRNA against TRIP6 (shTRIP6-1, shTRIP6-4) and the levels of TRIP6 and YAP target gene expression was analyzed using RT-qPCR (Mean \pm SD; n=3; **P \leq 0.01, ***P \leq 0.001, T-test).

(C) MCF10A cells were infected with lentivirus carrying control shRNA (shEGFP), or a mix of two different shRNA against TRIP6 (shTRIP6-1 and shTRIP6-4) and were stained for YAP and TRIP6. Merged image shows YAP (green), TRIP6 (red), and DNA (blue). Quantification of YAP nuclear localization at least 100 cells is shown. (Mean \pm SD; n=3; ****P \leq 0.0001, Fisher's test). Scale bar=20 μ m.

(D) YAP, TRIP6, and tubulin levels were measured by western blotting in MCF10A cells infected with lentivirus carrying control shRNA (shEGFP), or shRNA against TRIP6 (shTRIP6-1) (Mean \pm SD; n=3; **P \leq 0.01, T-test).

(E) LATS1 and LATS2 were depleted from WT and TRIP6-KO 293A cells using siRNA as described (Figure 2.5B) and were stained for YAP. Merged image shows YAP (green) and DNA (blue). Quantification of YAP nuclear localization at least 100 cells is shown (Mean \pm SD; n=3; ****P \leq 0.0001, Fisher's test).

(F) The lysates from Figure 2.2E were probed by western blotting with LATS1, LATS2, TRIP6, and tubulin antibodies to test knockdown efficacy.

(G) Control (WT) and TRIP6-KO HEK293A cells were

transfected with 200ng of control and FLAG-TRIP6 plasmids. (Note that 200ng of FLAG-TRIP6 plasmid restores approximate wild-type levels (see Figure 2.2H) of TRIP6 expression). After 48 hours of transfection, cells were stained using anti-YAP and TRIP6 antibodies by immunofluorescence. Quantification of YAP nuclear localization is shown (Mean \pm SD; n=3; ****P \leq 0.0001, Fisher's test). Scale bar=20 μ m. We compare TRIP6-KO cells to TRIP6-KO cells rescued by FLAG-TRIP6 plasmid (rescue). (H) Different amounts (50, 100, 150, 200ng) of FLAG-TRIP6 plasmid were transfected into HEK293A TRIP6-KO cells and TRIP6 levels in lysates were analyzed by western blotting using anti-TRIP6 antibodies and compared to those in control HEK293A (WT) cells. 200ng of FLAG-TRIP6 plasmid (marked with an asterisk) was selected to perform the rescue experiment described in Figure 2.2G.

TRIP6 inhibits LATS1/2 by blocking binding to MOB1

We next investigated the mechanism for how TRIP6 inhibits LATS1/2. TRIP6-related LIM domain proteins have been shown to bind and inhibit LATS (Reddy and Irvine, 2013; Rauskolb et al., 2014; Hirota et al., 2000; Abe et al., 2006; Das Thakur et al., 2010; Rauskolb et al., 2011), however, it is not clear how they regulate LATS1/2 activity. Although zyxin was shown to promote degradation of LATS1/2 in response to hypoxia (Jagannathan et al., 2016), we did not observe any changes in LATS1 levels when TRIP6 levels were altered suggesting that TRIP6 uses a different mechanism. Because one of the TRIP6 binding sites in LATS2 (amino acids 625-644) overlaps with the binding site for its activator MOB1 (amino acids 595-662) (Ni et al., 2015), we wondered if TRIP6 and MOB1 compete for binding to LATS1/2. This mechanism would be consistent with our observations that TRIP6 inhibits the ability of MST1/2 to phosphorylate LATS1/2 because MOB1 activates LATS1/2 by promoting its association with and phosphorylation by MST1/2 (Ni et al., 2015). We first examined if TRIP6 could inhibit LATS1/2-MOB1 binding in vivo. We found that overexpression of full-length TRIP6 (but not a version of TRIP6 (1-277) lacking the LATS1/2 binding LIM domains) reduced LATS2-MOB1A association in HEK293 cells (Figure 2.3A). To determine whether TRIP6 directly competes with MOB1A for binding to LATS2, competition experiments were carried out using purified recombinant proteins. Initial results demonstrated that GST-TRIP6 bound directly to MBP-LATS2 but not MBP alone (Figure 2.3B, compare lanes 1 and 3). Competition experiments showed that MOB1A could compete with TRIP6 for binding to LATS2. 6HIS-MOB1A bound to MBP-LATS2 and inhibited GST-TRIP6 binding, with the highest levels of MOB1A reducing TRIP6-LATS2

binding to background levels (Figure 2.3B, lanes 3-6). Addition of non-specific competitor (BSA), at the same level as the highest amount of MOB1A used (Figure 2.4), did not cause any reduction in TRIP6-LATS2 binding (Figure 2.3B, lane 7). Together, these results show that that TRIP6 and MOB1 compete for binding to LATS2 and that TRIP6 likely inhibits LATS1/2 activity at least in part by blocking MOB1 binding.

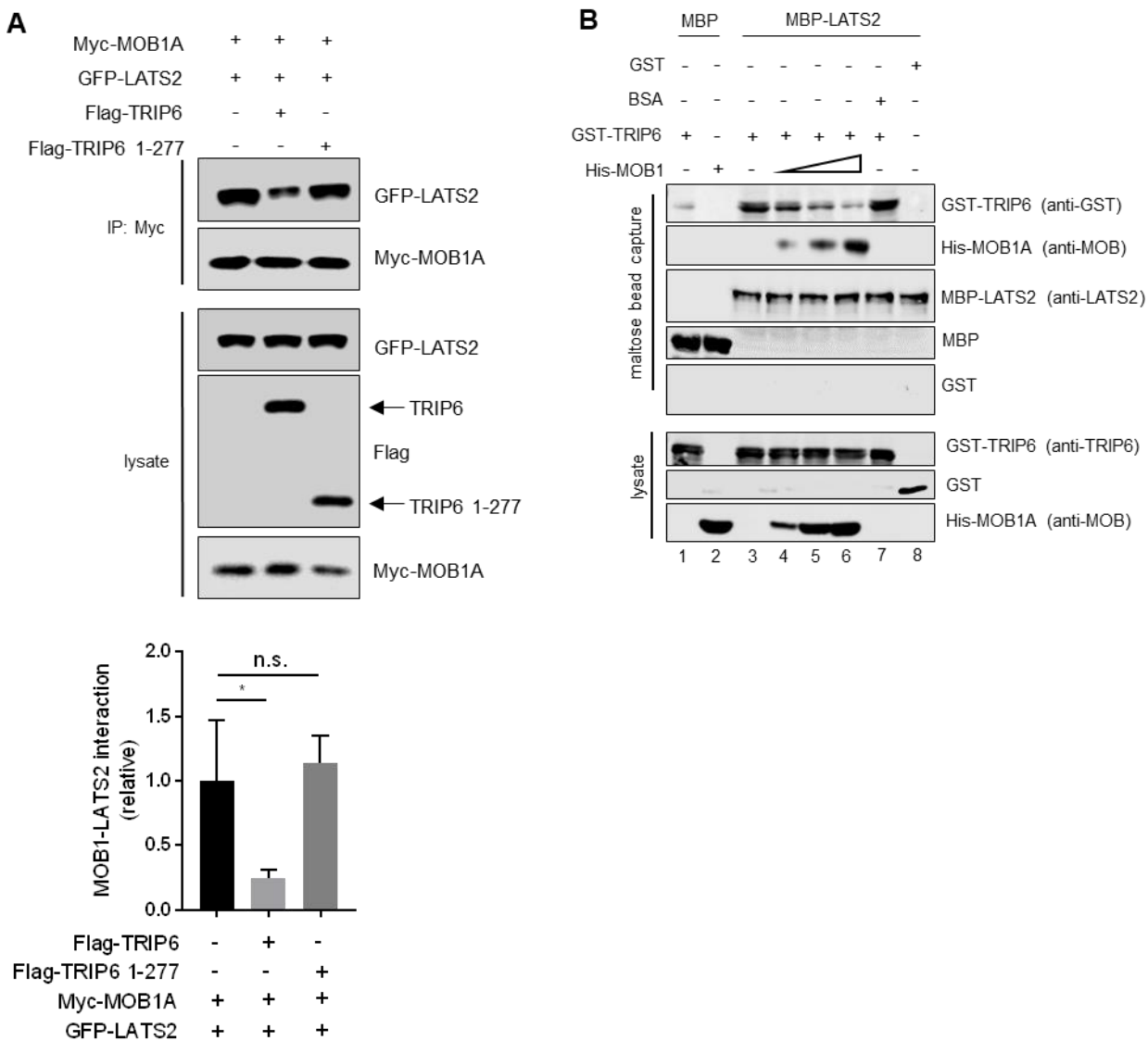


Figure 2.3 TRIP6 competes with MOB1 for binding to LATS2.

(A) LATS2-GFP and Myc-MOB1A were overexpressed in HEK293 cells with or without co-overexpression of full-length FLAG-TRIP6 and FLAG-TRIP6 1-277. Myc-MOB1A was immunoprecipitated using anti-Myc antibodies and immune complexes were assayed for Myc-MOB1A and LATS2-GFP levels. Levels of FLAG-TRIP6, FLAG-TRIP6 1-277, Myc-MOB1A, and LATS2-GFP in the lysate are also shown. The levels of

LATS2-GFP in immune complexes relative to the level of Myc-MOB1A are shown in the graph (Mean \pm SD; n=3; **P \leq 0.01, T-test). (B) Competitive binding experiments were done using purified recombinant MBP-LATS2, GST-TRIP6, and 6His-MOB1A. MBP-LATS2 bound to maltose beads was incubated with GST-TRIP6 with or without increasing amounts of 6His-MOB1A, and the levels of each protein bound to MBP-LATS2 on the beads at the end of the experiment was determined by western blotting. The levels of input proteins are shown (lysate). The binding of 6His-MOB1A and GST-TRIP6 to MBP alone and the use BSA as a competitor instead of 6His-MOB1A are shown as controls. The numbers at the bottom are referred to in the text.

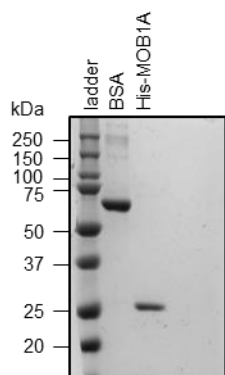


Figure 2.4 Coomassie-stained gel showing the amounts of BSA and highest amount of HIS-MOB1A used in Figure 2.3B.

TRIP6 modulates LATS1/2 activity and localization in response to tension at cell-cell junctions

To investigate what regulatory inputs might control TRIP6 inhibition of LATS1/2, we examined the localization of each protein. Endogenous TRIP6 and LATS1 co-localize to cell-cell junctions in MCF10A (Figure 2.5A, Figure 2.6A) and to a lesser extent in HEK293A (Figure 2.6B) cells. Although we have been unable to find LATS2 antibodies capable of detecting the endogenous protein, GFP-LATS2 fusions also localize to cell-cell junctions (Paramasivam et al., 2011). TRIP6 has been previously reported to localize to both cell-cell junctions (adherens junctions) (Guo et al., 2014) and to focal adhesions (Zhao et al., 1999; Wang et al., 1999). Although we could faintly observe TRIP6 at focal adhesions in MCF10A cells at low density or at the edge of monolayers, at densities typically used in this study (confluent but still proliferating), TRIP6 was primarily at adherens junctions, and we saw little focal adhesion staining for TRIP6 or the focal adhesion marker FAK (Figure 2.6C). LATS1 was not observed at focal adhesions in MCF10A cells at any low cell density (Figure 2.6D). We next assessed the mutual dependence of LATS1 and TRIP6 localization. Knockdown of TRIP6 in MCF10A cells (Figure 2.5A; Figure 2.6E) reduced localization of LATS1 to cell junctions. Deletion of TRIP6 in HEK293A cells (Figure 2.6B) also caused reduced localization of LATS1 to cell-cell junctions, although because of the reduced cell-cell adhesion in these cells, it is possible that effects on LATS1 localization could be due to defects in cell-cell junctions. When LATS1/2 were knocked down in MCF10A cells, TRIP6 remained at cell-cell junctions (Figure 2.5B; Figure 2.6F), but its localization was more punctate and less smooth, possibly reflecting a transition to a more mesenchymal state (Zhang et al.,

2008a). E-cadherin staining in TRIP6 and LATS1/2 knockdown cells looked similar to that in control cells (Figure 2.8C) suggesting that cell-cell adhesion remains intact, but we cannot rule out more subtle effects on junction architecture. Together these results show that TRIP6 is important for LATS1/2 localization to cell junctions.

We next examined whether recruitment of TRIP6 and LATS1 to cell junctions is regulated by stimuli that control LATS1/2 activity. Both TRIP6 and LATS1 localized to cell-cell junctions in cells that were confluent but still proliferating. However, in highly dense non-proliferating cells TRIP6 and LATS1 no longer localized to cell-cell junctions (Figure 2.5C, Figure 2.6B), despite unchanged levels of both proteins (Figure 2.5D), and cell-cell junctions remaining intact as judged by E-cadherin staining (Figure 2.8B). Interestingly we also observed a reduction in TRIP6-LATS1 binding in MCF10A cells at high cell density (Figure 2.5D), consistent with the increased LATS1/2 activity observed under these conditions (Meng et al., 2015). How cell density controls TRIP6-LATS1/2 binding and localization is not clear. However, a study in *Drosophila* tissue showed that tension at cell-cell junctions is reduced as cell density increases (Rauskolb et al., 2014). Therefore, we tested whether increasing tension at cell-cell junctions in dense cultures would restore localization of TRIP6 and LATS1 to cell-cell junctions. To do this, we examined TRIP6 and LATS1 localization in dense cultures grown on flexible PDMS substrates before and after a static stretch for 2 hours. We observed that stretch increased TRIP6-LATS1 binding (Figure 2.5E), localization of both proteins to cell-cell junctions (Figure 2.5F), and YAP activity (Figure 2.6G). Both tension dependent recruitment of LATS1 to cell-cell junctions and YAP activation in dense monolayers following stretch depended on TRIP6 (Figure 2.5G-H). Together these results show that

tension can trigger YAP activation through TRIP6 by increasing TRIP6 recruitment to cell-cell junctions, and TRIP6 binding to LATS1.

We also tested whether loss of tension across confluent (but not dense) monolayers of cells could trigger loss of LATS1-TRIP6 binding and co-localization at cell-cell junctions. Treatments that inhibit stress fibers such as type II myosin inhibition (Blebbistatin), Rho kinase inhibition (Y27632), or serum starvation are known to reduce tension at cell junctions (Yonemura et al., 2010). All of these treatments inhibited both LATS1-TRIP6 binding and cell-cell junction localization, as did the complete elimination of F-actin using Latrunculin B (Figure 2.7A-C, Figure 2.8A). These treatments (with the exception of Latrunculin B) did not obviously affect cell-cell adhesion and E-cadherin localization (Figure 2.8B). To reduce tension at cell junctions by blocking force transmission between cells, we disrupted cadherin complexes by treating cells with EGTA and found that this treatment also inhibited LATS1-TRIP6 binding and localization to cell-cell junctions (Figure 2.7A-C, Figure 2.8A). Together these observations suggest that TRIP6 responds to tension at cell-cell junctions to regulate LATS1 and YAP activity.

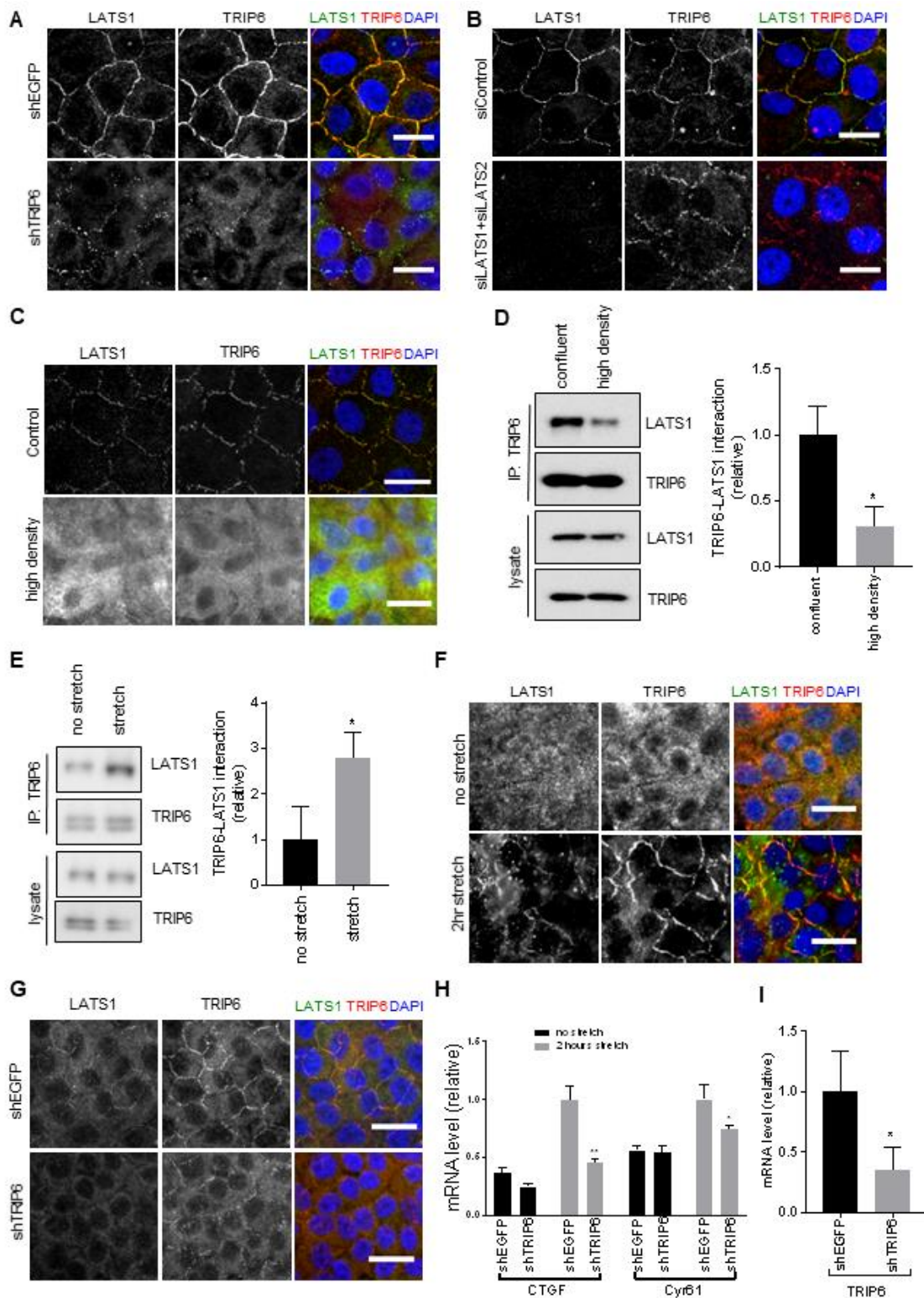


Figure 2.5 TRIP6-LATS binding and localization to cell-cell junctions is regulated by tension.

(A) MCF10A cells were infected with lentivirus carrying control shRNA (shEGFP), or a mix of two different shRNA against TRIP6 (shTRIP6-1 and shTRIP6-4) and were stained for TRIP6 and LATS1. Merged images show LATS1 (green), TRIP6 (red) and DNA (blue) (quantification of LATS1 localization at cell-cell junctions is shown in Figure 2.6E). Scale bar=20 μ m. (B) LATS1 and LATS2 were knocked down using siLATS1 and siLATS2 SMARTPools in MCF10A cells. MCF10A control cells were treated with control siRNA (siControl) against fire fly luciferase. Cells were stained with TRIP6 and LATS1 as in (A) (quantification of TRIP6 localization at cell-cell junctions is shown in Figure 2.6F). Scale bar=20 μ m. (C) MCF10A cells were grown to high density and were stained for TRIP6 and LATS1 as in (A). Scale bar=20 μ m. (D) Cells were grown as in (C), then lysed and anti-TRIP6 antibodies were used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for LATS1 and TRIP6. Quantification is shown. (Mean \pm SD; n=3; *P \leq 0.05, T-test). (E) MCF10A cells grown at high density on PDMS membranes and were stretched (or not) at 17% elongation for 2 hours and lysed while under tension. Anti-TRIP6 antibodies were used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for LATS1 and TRIP6. Quantification is shown. (Mean \pm SD; n=3; *P \leq 0.05, T-test). (F) Cells were treated as in (E), fixed while under tension, and stained for TRIP6 and LATS1 as in (A). Scale bar=20 μ m. (G) MCF10A cells infected with lentivirus carrying control shRNA (shEGFP), or a mix of two different shRNA against TRIP6 (shTRIP6-1 and shTRIP6-4), were grown at high density on PDMS membranes and were stretched

or not (only stretched cells shown) at 17% elongation for 2 hours, fixed while under tension, and were stained for TRIP6 and LATS1 as in (A). Scale bar=20 μ m. (H) Cells were treated as in (G) and YAP target gene (CTGF and Cyr61) and TRIP6 expression were analyzed using RT-qPCR. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, T-test). (I) TRIP6 expression levels in (H) in control (shEGFP) and TRIP6 (shTRIP6) knockdown cells just prior to stretch were analyzed using RT-qPCR. (Mean \pm SD; n=3; *P \leq 0.05, T-test).

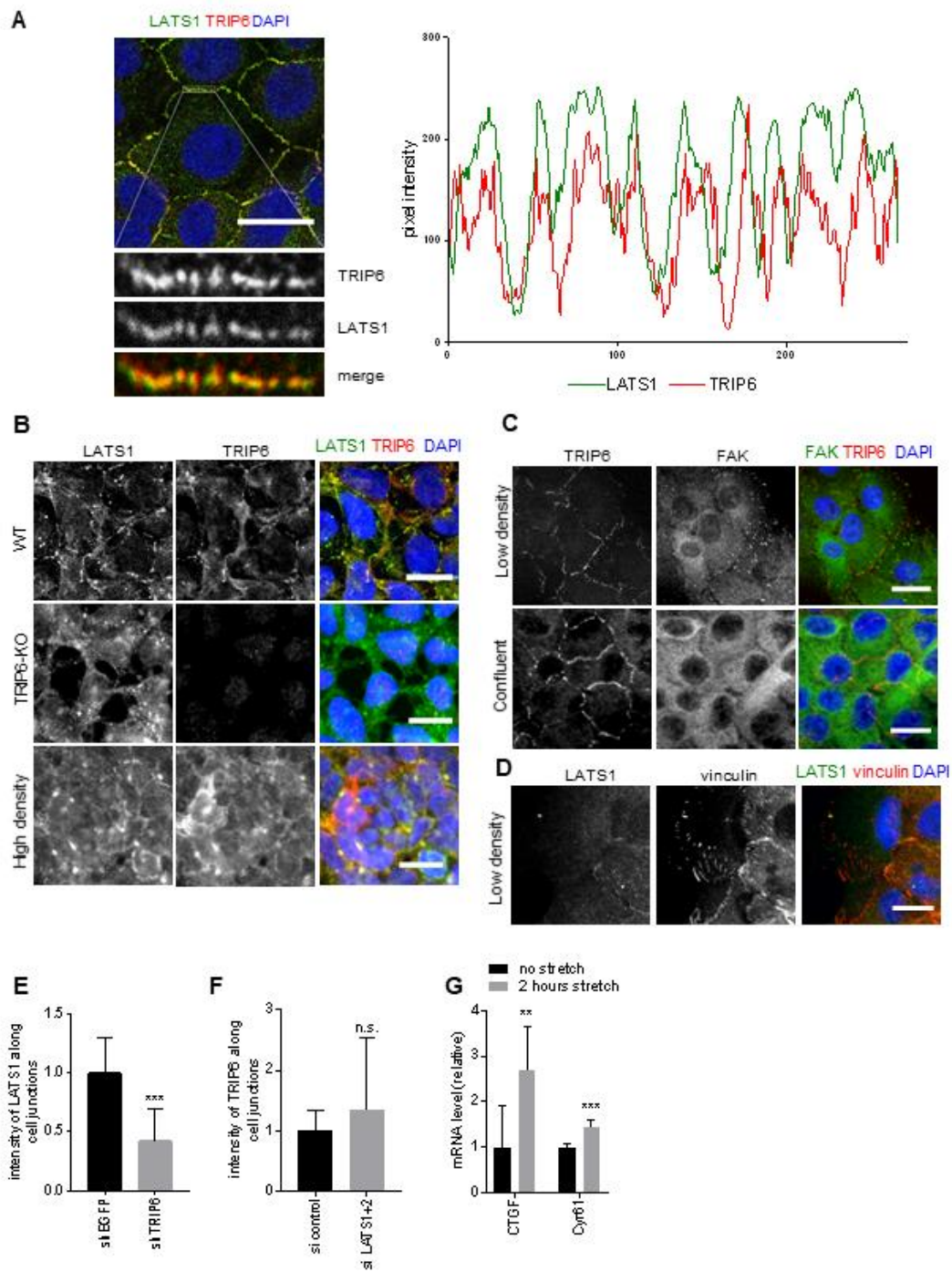


Figure 2.6 Regulation of TRIP6 and LATS1 localization and binding.

(A) MCF10A cells were stained with TRIP6 and LATS1 and the image was acquired with a confocal microscope. Merged images show LATS1 (green), TRIP6 (red) and DNA (blue). Scale bar=20 μ m. The LATS1-TRIP6 colocalization was confirmed by line scan analysis of the pixel intensity in different fluorescent channels. (B) HEK293A cells (WT and TRIP6-KO) were grown and TRIP6 and LATS1 intracellular localization were determined by immunofluorescence using anti-TRIP6 and anti-LATS1 antibodies. HEK293A (WT) cells were also grown at high density (High density) and treated similarly. Scale bar=20 μ m. (C) MCF10A cells were grown at low density and until confluency and stained for TRIP6 and FAK. Merged images show FAK (green), TRIP6 (red) and DNA (blue). (D) MCF10A cells were grown at low density and stained for LATS1 and vinculin. Merged images show LATS1 (green), vinculin (red) and DNA (blue). (E) Quantification of LATS1 at cell-cell junctions in MCF10A cells depleted of TRIP6 (see Figure 2.5A). The intensity of LATS1 was measured at individual cell-cell junctions ($n \geq 48$) (Mean \pm SD; $n=3$; $**P \leq 0.01$, T-test). (F) Quantification of TRIP6 at cell-cell junctions in MCF10A cells depleted of LATS1 and LATS2 (see Figure 2.5B). The average intensity of TRIP6 was measured at individual cell-cell junctions ($n \geq 48$) (Mean \pm SD; $n=3$; $n.s. \geq 0.05$, T-test). (G) MCF10A cells grown at high density on PDMS membranes and were stretched (or not) at 17% elongation for 2 hours, RNA was isolated and YAP target gene expression was analyzed using RT-qPCR. (Mean \pm SD; $n=3$; $**P \leq 0.01$, $****P \leq 0.0001$, T-test).

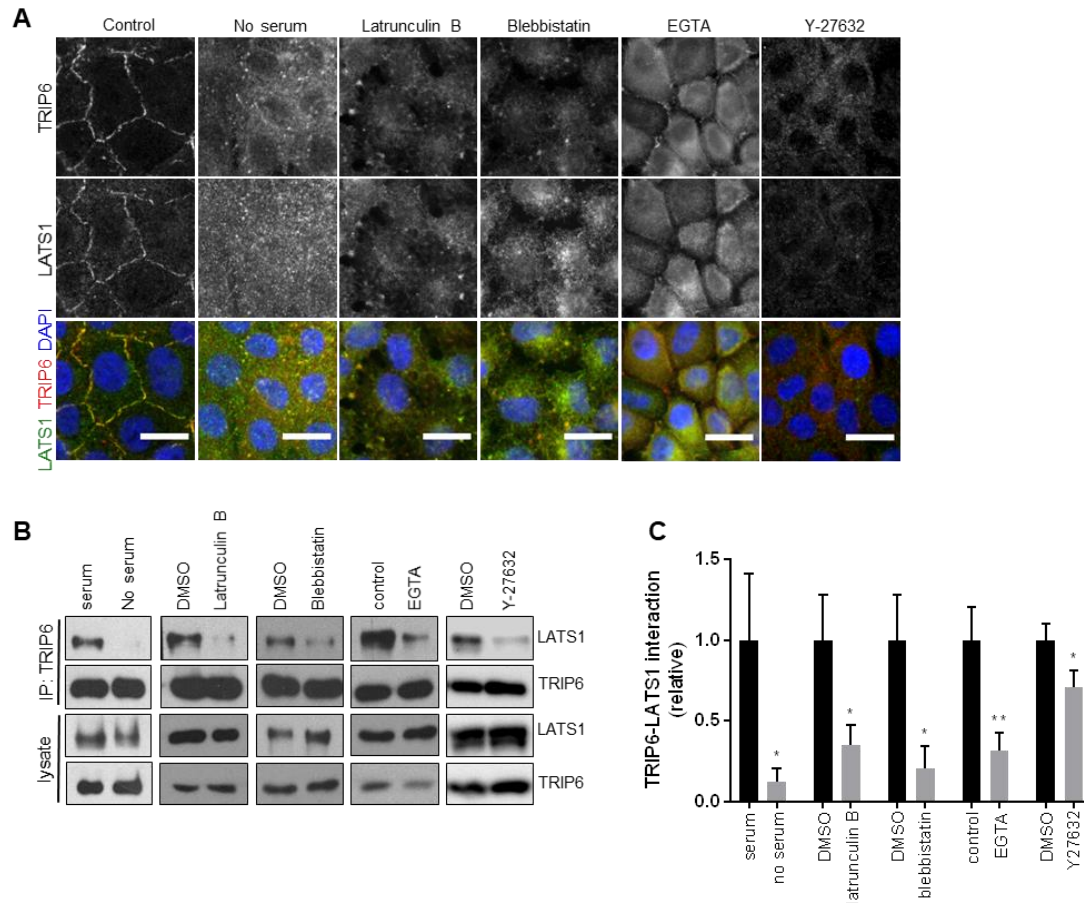


Figure 2.7 Perturbations of junctions and f-actin reduce TRIP6-LATS1 binding and localization to cell-cell junctions.

(A) MCF10A cells were either not treated or treated separately with Latrunculin B, Blebbistatin, EGTA, serum starvation, and Y27632, and were stained for TRIP6 and LATS1. Merged images show LATS1 (green), TRIP6 (red) and DNA (blue). Scale bar=20 μ m. (B) Cells were treated as in (A), then lysed and anti-TRIP6 antibodies were used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for LATS1 and TRIP6. (C) Quantification of (B) is shown. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, T-test).

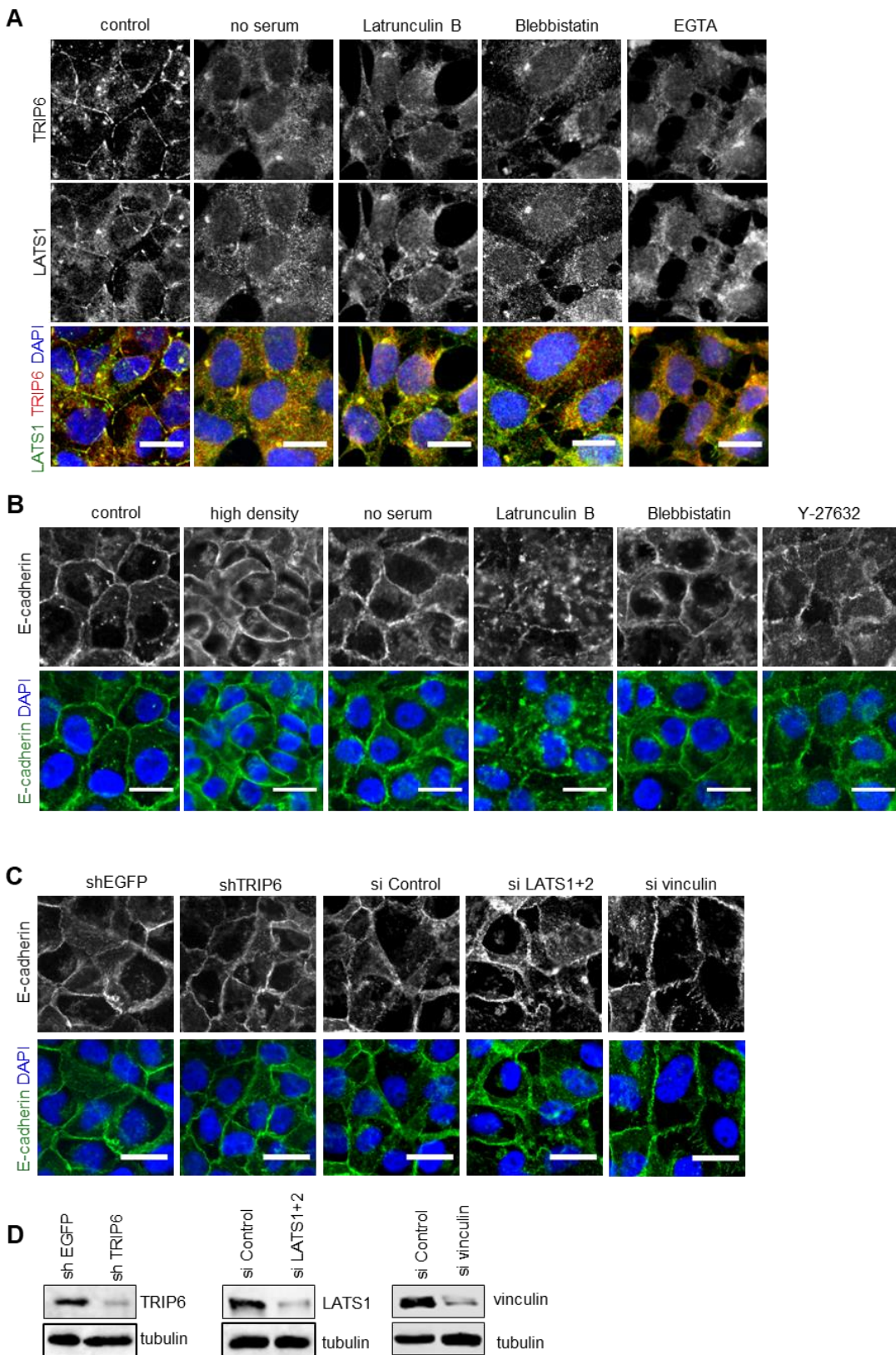


Figure 2.8 Regulation of TRIP6 and LATS1 localization in HEK293A cells and E-cadherin staining in MCF10A cells after various treatments, TRIP6, LATS1 and 2, and vinculin knockdown.

(A) HEK293A cells were either not treated (control) or treated separately by serum starvation (no serum), Latrunculin B, Blebbistatin, and EGTA, then TRIP6 and LATS1 intracellular localization were determined by immunofluorescence using anti-TRIP6 and anti-LATS1 antibodies. Scale bar=20 μ m. (B) MCF10A cells were either not treated (control) or treated separately by growth to high density, serum starvation, Latrunculin B, Blebbistatin, or Y27632 treatment, and stained using anti-E-cadherin antibodies by immunofluorescence. Scale bar=20 μ m. (C) TRIP6, vinculin and LATS1 and LATS2 were depleted from MCF10A cells as described (Figure 2.2A, Figure 2.9D, and Figure 2.5B) and stained using anti-E-cadherin antibodies by immunofluorescence. Scale bar=20 μ m. (D) The lysates from Figure 2.8C were probed by western blotting for LATS1, TRIP6 and vinculin antibodies to test knockdown efficacy.

TRIP6 is a part of the mechanoresponsive complex at adherens junctions

We next investigated whether TRIP6 could be part of a mechano-responsive complex at cadherin-catenin based adherens junctions (Huveneers and de Rooij, 2013). Previous studies showed that TRIP6 localizes to adherens junctions, and its association with the cadherin complex is dependent on engagement between the extracellular domains of cadherins on neighboring cells (Guo et al., 2014). How TRIP6 interacts with the cadherin complex is not known. Interestingly, two high throughput two-hybrid studies detected a binding interaction between TRIP6 and the adherens junction protein vinculin (Yu et al., 2011; Rual et al., 2005). Consistent with the high throughput studies, we detected vinculin in TRIP6 immune-complexes (Figure 2.9A). Vinculin and TRIP6 are known to respond to mechanical cues at focal adhesions (Schiller et al., 2011; Kuo et al., 2011; Bays and DeMali, 2017). In addition, vinculin localizes to adherens junctions in response to mechanical tension (Thomas et al., 2013; Leerberg et al., 2014; Huveneers et al., 2012; Yonemura et al., 2010). To test whether vinculin was involved with TRIP6 in tension dependent regulation of Hippo signaling, we examined whether vinculin localization to cell-cell junctions in MCF10A cells was tension dependent and whether vinculin regulated Hippo signaling. As with TRIP6, we observed vinculin at focal adhesions in MCF10A cells at low density or at the edge of monolayers, but at densities used in this study (confluent but still proliferating), vinculin was concentrated at adherens junctions (Figure 2.10A). Thus, we infer that TRIP6 is primarily interacting with vinculin at adherens junctions under these conditions. At high cell density, vinculin localization to adherens junctions was lost but could be restored by stretch (Figure 2.9B-C), as observed for TRIP6 and LATS1. Also, like TRIP6 and LATS1 treatments

that disrupt tension in confluent but proliferating MCF10A cells reduce vinculin localization to cell-cell junctions (Figure 2.9B). To test whether vinculin regulated Hippo signaling, we knocked down vinculin using siRNA in MCF10A cells. Depletion of vinculin resulted in increased LATS1 and YAP phosphorylation (Figure 2.9D) and reduced YAP nuclear localization (Figure 2.9E) and activity (Figure 2.9F) (as judged by a reduction in YAP target genes CTGF and Cyr61). This same effect on YAP was observed using two different siRNAs (Figure 2.10E-F). Like TRIP6, vinculin was required for stretch-induced YAP-dependent gene expression (Figure 2.9G). Knockdown of vinculin in HEK293A cells also reduced YAP activity (Figure 2.10G) and this reduction could be rescued by expression of chicken vinculin (Figure 2.10G-H). Together these results show that vinculin interacts with TRIP6 and, like TRIP6, participates in tension dependent regulation of Hippo signaling.

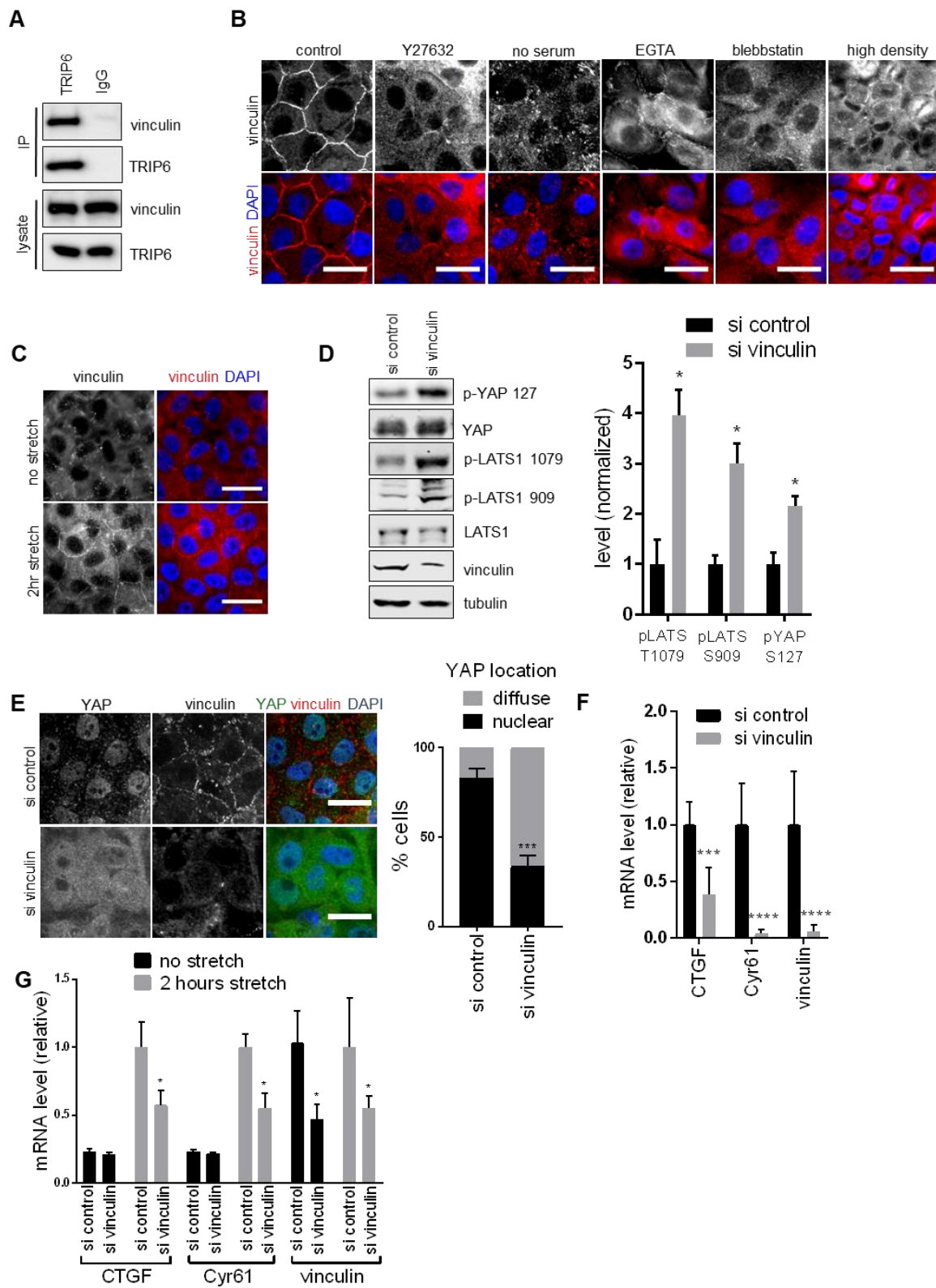


Figure 2.9 Vinculin interacts with TRIP6 and regulates LATS and YAP activity.

(A) MCF10A cells were lysed and anti-TRIP6 or control (IgG) antibodies were used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for vinculin and TRIP6. (B) MCF10A cells were either not treated (control) or treated separately by growth to high density, serum starvation, Latrunculin B, Blebbistatin, EGTA, or Y27632 treatment, and stained using anti-vinculin antibodies by immunofluorescence. Scale bar=20 μ m. (C) MCF10A cells grown at high density on PDMS membranes and were stretched (or not) at 17% elongation for 2 hours and fixed while under tension and stained for vinculin. Scale bar=20 μ m. (D) Vinculin was knocked down using two different siRNAs or control siRNA in MCF10A cells and the cell lysates were probed by western blotting for phospho-LATS1 (T1079 and S909), phospho-YAP (S127), LATS1, YAP, vinculin, and tubulin antibodies and the relative levels quantified (Mean \pm SD; n=3; **P \leq 0.01, T-test). (E) Vinculin was knocked down as described in (D) and cells were stained for YAP and vinculin. Merged image shows YAP (green), vinculin (red), and DNA (blue). Quantification of at least 100 cells is shown (Mean \pm SD; n=3; ***P \leq 0.001, Fisher's test). Scale bar=20 μ m. (F) Vinculin was knocked down as described in (D) and the levels of vinculin and YAP target gene expression was analyzed using RT-qPCR (Mean \pm SD; n=3; ***P \leq 0.001, ****P \leq 0.0001, T-test). (G) Vinculin was knocked down in MCF10A cells as described in (D), grown at high density on PDMS membranes and cells were stretched (or not) at 17% elongation for 2 hours and the levels of vinculin and YAP target gene expression was analyzed using RT-qPCR (Mean \pm SD; n=3; ***P \leq 0.001, ****P \leq 0.0001, T-test).

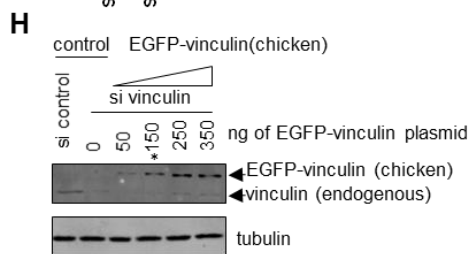
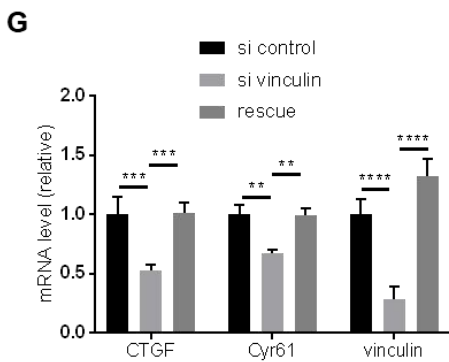
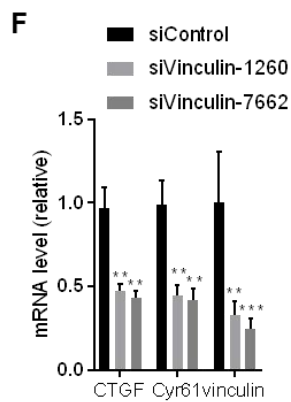
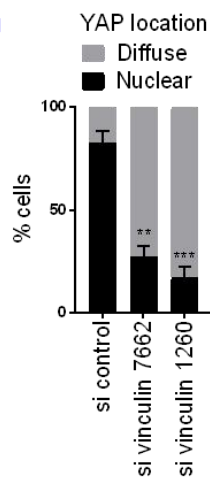
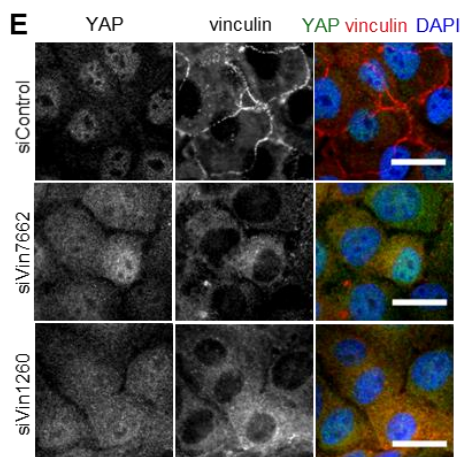
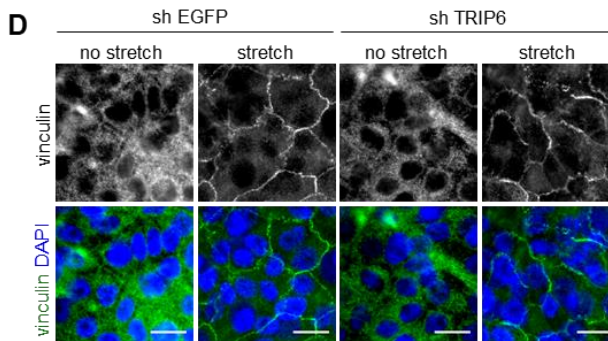
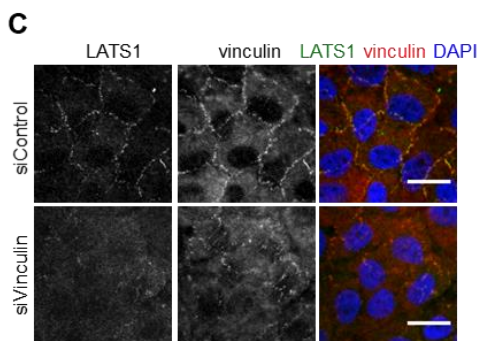
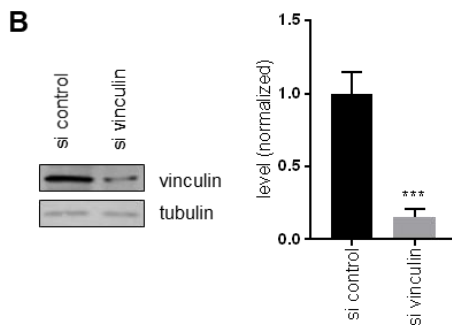
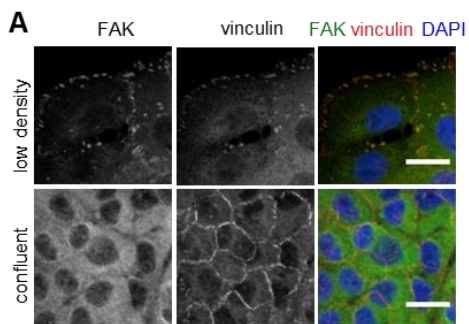


Figure 2.10 FAK and vinculin co-staining in MCF10A cells, vinculin knockdown efficacy, LATS1 and vinculin co-staining in MCF10A cells, the effect of TRIP6 knockdown on vinculin, the effect of vinculin knockdown by single siRNAs on YAP activity and localization and rescue of siRNA knockdown.

(A) MCF10A cells were grown at low density and until confluency and stained using FAK and vinculin antibodies by immunofluorescence. Scale bar=20 μ m. (B) Vinculin was knocked down using two different stealth siRNAs in MCF10A cells. MCF10A control cells were treated with control siRNA. Lysates were probed by western blotting for vinculin and tubulin. The levels of vinculin were determined. (Mean \pm SD; n=3; ***P \leq 0.001, T-test) (C) Vinculin was knocked down as described in (B) and cells were stained using vinculin and LATS1 antibody. Merged images show LATS1 (green), vinculin (red) and DNA (blue). Scale bar=20 μ m. (D) TRIP6 was depleted from MCF10A cells as described in Figure 2.2A and grown at high density on PDMS membranes and that was stretched (or not) at 17% elongation for 2 hours, fixed while under tension, and stained for vinculin. Merged images show vinculin (green) and DNA (blue). Scale bar=20 μ m. (E) Vinculin was knocked down by single stealth siRNAs (7662 and 1260) in MCF10A cells and stained using vinculin and YAP antibody. Merged images show YAP (green), vinculin (red) and DNA (blue). Quantification of YAP nuclear localization at least 100 cells is shown (Mean \pm SD; n=3; **P \leq 0.01, ***P \leq 0.001, Fisher's test). Scale bar=20 μ m. (F) Vinculin was knocked down as described in (E) and the levels of vinculin, and YAP target gene expression was analyzed using RT-qPCR. (Mean \pm SD; n=3; **P \leq 0.01, ***P \leq 0.001, T-test). (G) Vinculin was knocked down as described (Figure 2.10E) in HEK293A cells. Control

and vinculin depleted HEK293A cells were transfected with 150ng of control and EGFP-vinculin (chicken) plasmids. (Note that 150ng of EGFP-vinculin plasmid restores approximate wild-type levels (see Figure 2.10H) of vinculin expression). After 48 hours of transfection, the levels of vinculin, and YAP target gene expression was analyzed using RT-qPCR. We compare vinculin depleted cells to vinculin depleted cells rescued by EGFP-vinculin (rescue). (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ****P \leq 0.0001, T-test). (H) Vinculin was knocked down as described in Figure 2.9D in HEK293A cells. Different amounts (50, 150, 250, 350ng) of EGFP-vinculin plasmid were transfected into the vinculin depleted cells and vinculin levels in lysates were analyzed by western blotting using vinculin antibody and compared to those in control HEK293A cells. 150ng of EGFP-vinculin plasmid (marked with an asterisk) was selected to perform the rescue experiment described (Figure 2.10G).

We then investigated whether vinculin and TRIP6 function together to regulate Hippo signaling in response to tension. Several lines of evidence suggested that vinculin acts upstream of TRIP6 and LATS1/2 in response to tension. First, when vinculin was depleted by siRNA, TRIP6 and LATS1 localization to cell-cell junctions was reduced (Figure 2.11A; Figure 2.10B-C), without an obvious effect on E-cadherin staining (Figure 2.8C). In contrast, stretch-induced recruitment of vinculin to cell-cell junctions did not depend on TRIP6 (Figure 2.10D). Thus, vinculin is required to recruit TRIP6 and LATS1 to cell-cell junctions. Vinculin also promotes TRIP6-LATS1 binding since reduced TRIP6-LATS1 binding was observed after vinculin depletion (Figure 2.11B). Vinculin binding to TRIP6 appears to be tension dependent because it is reduced by treatments that disrupt tension (Figure 2.11C-F). Tension-dependent binding of upstream molecules can trigger vinculin to become activated by inducing a more open conformation (Choi et al., 2012). The vinculin-T12 mutation (Cohen et al., 2005) is thought to mimic the active (open) conformation. Therefore, we tested whether vinculin-T12 is better than wild-type vinculin at binding TRIP6 and promoting its association with LATS2. We co-expressed TRIP6, LATS2, and either wild-type or vinculin-T12 and immunoprecipitated TRIP6. This experiment showed that vinculin-T12 bound better to TRIP6 but did not increase the amount of LATS2 binding to TRIP6 as compared to wild-type vinculin (Figure 2.11G). These results indicate that TRIP6 binds better to the activated form of vinculin, but this binding alone is not sufficient to stimulate TRIP6-LATS2 binding. The inability of vinculin-T12 to stimulate TRIP6-LATS2 binding is surprising because we found that depletion of vinculin resulted in reduced TRIP6-LATS1 binding. These results could be explained if either vinculin-T12 does not fully

mimic the tension activated state of vinculin at adherens junctions, or other proteins at adherens junctions besides vinculin are required to promote TRIP6-LATS1/2 binding. A third possibility is that vinculin is necessary to generate tension, which could be sensed/transmitted to TRIP6-LATS1/2 by other proteins. To test this possibility, we stretched high-density cells that had been depleted of vinculin and stained them for TRIP6. These results showed that stretch was unable to trigger recruitment TRIP6 to cell-cell junctions when vinculin was depleted (Figure 2.11H), suggesting that vinculin does not solely affect TRIP6-LATS1/2 binding indirectly by promoting tension at junctions. Overall these results support a model where tension stimulates vinculin recruitment of TRIP6-LATS1/2 to adherens junctions to control YAP activity in response to changes in tension across tissues.

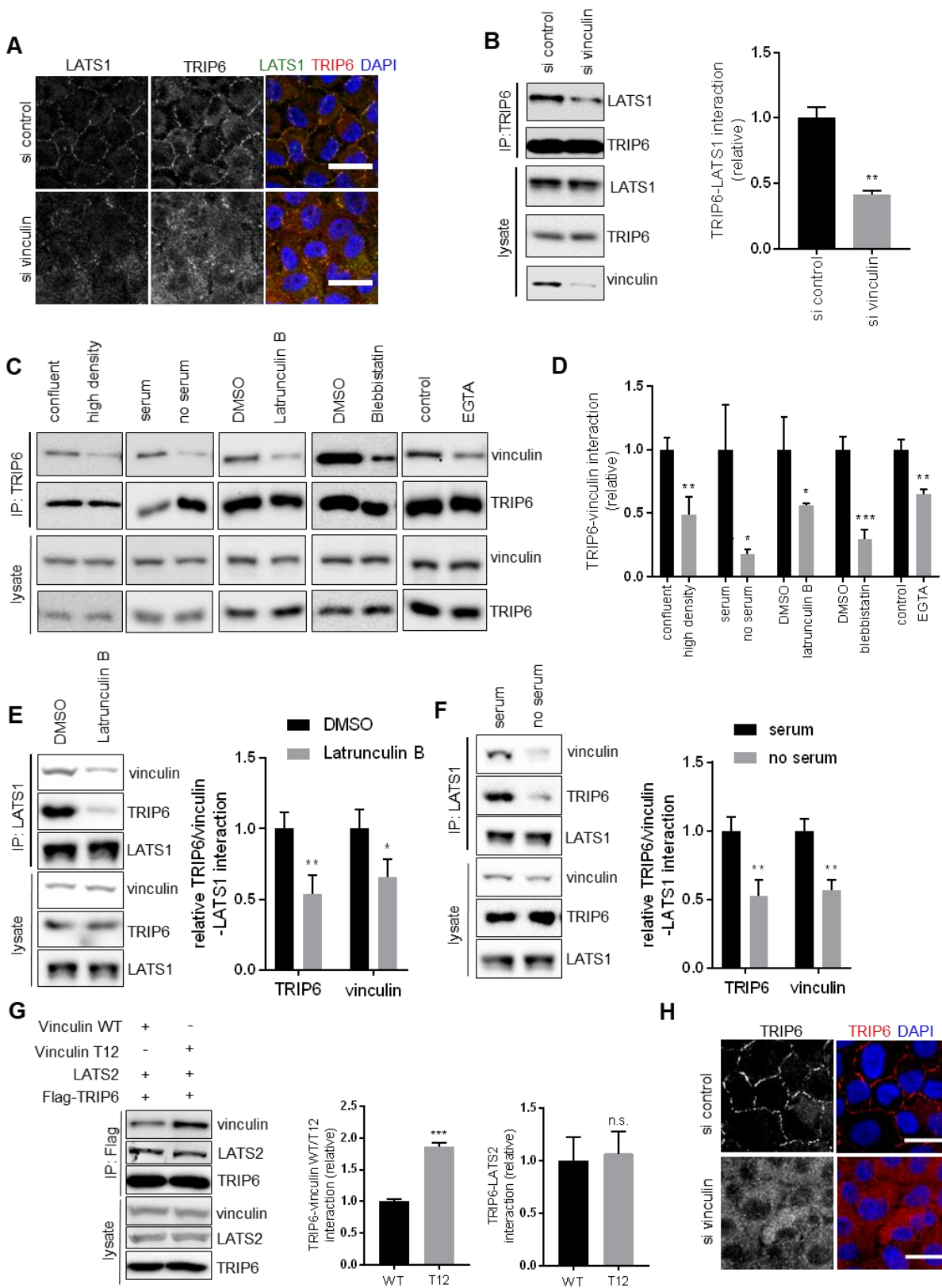


Figure 2.11 Vinculin regulates TRIP6-LATS1 interaction and localization.

(A) Vinculin was knocked down as described (Figure 2.9D) in MCF10A cells and cells were stained for LATS1 and TRIP6. Merged images show LATS1 (green), TRIP6 (red) and DNA (blue). Scale bar=20 μ m. (B) Vinculin was knocked down as described (Figure 2.9D) and TRIP6 immune complexes and lysates were probed by western blotting for LATS1, TRIP6, and vinculin and the relative levels quantified (Mean \pm SD; n=3; **P \leq 0.01, T-test). (C) MCF10A cells were subjected to different treatments as described (Figure 2.7A) and TRIP6 immune complexes and lysates were probed by western blotting for vinculin and TRIP6. (D) Quantification of TRIP6-vinculin binding from part (C) is shown. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, T-test). (E, F) MCF10A cells were treated separately (or not) with Latrunculin B (E) and by serum starvation (F) and LATS1 immune complexes and lysates were probed by western blotting for vinculin, LATS1, and TRIP6. Quantification of LATS1-TRIP6-vinculin binding is shown. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, T-test). (G) Wild-type and T12 mutant of vinculin were co-expressed with LATS2 and FLAG-TRIP6 and anti-FLAG antibody was used to isolate immune complexes. Immune complexes and lysates were probed by western blotting for vinculin, LATS2, and FLAG-TRIP6. Quantification of LATS2-TRIP6-vinculin (WT or T12) binding is shown. (Mean \pm SD; n=3; *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, T-test). (H) Vinculin was knocked down as described (Figure 2.9D) in MCF10A cells grown at high density on PDMS membranes. The membranes were stretched at 17% elongation for 2 hours and fixed while under tension and stained for TRIP6. Merged images show TRIP6 (red) and DNA (blue). Scale bar=20 μ m.

2.4 Discussion

This study provides new insight into the mechanism by which mechanical forces regulate cell growth and proliferation decisions via the Hippo signaling pathway. In particular, we report that the LIM domain protein TRIP6 functions as an intermediate between the LATS1/2 protein kinases, which transmit signals to YAP, and the mechano-responsive protein vinculin at the adherens junctions. Previous studies have shown that YAP activity can be stimulated by tension (Aragona et al., 2013; Rauskolb et al., 2014; Codelia et al., 2014; Benham-Pyle et al., 2015). However, the upstream signaling pathways remained uncertain. We found that tension stimulates TRIP6 binding to LATS1/2, and, once bound, TRIP6 inhibits LATS1/2 activity, reminiscent of work in *Drosophila* showing that the Ajuba LIM domain protein activates Yki (the YAP homolog) by inhibiting Warts (the LATS1/2 homolog) in response to tension (Rauskolb et al., 2014). Furthermore, we identified a specific molecular mechanism for how TRIP6 inhibits LATS1/2. We discovered that TRIP6 competes with MOB1 for binding to LATS1/2. MOB1 promotes LATS1/2 activation by scaffolding interactions between the LATS1/2 activating kinase MST1/2 and LATS1/2. The competition we observe between TRIP6 and MOB1 for binding to LATS1/2 is consistent with our other results showing TRIP6 interferes with MST1/2 phosphorylation of LATS1/2. This mechanism (explained in Figure 2.12) may be relevant for other LIM domain proteins that bind to a similar region of LATS1/2 (Abe et al., 2006; Hirota et al., 2000), and could function in conjunction with other proposed mechanisms of LATS1/2 inhibition by LIM domain proteins (Sun and Irvine, 2013; Jagannathan et al., 2016; Ma et al., 2016).

Our studies identified vinculin as acting upstream of TRIP6. Both TRIP6 and vinculin loss of function promotes LATS1/2 activity and inhibits YAP activity. Furthermore, vinculin can be co-immunoprecipitated with TRIP6 and is required to efficiently recruit TRIP6 and LATS1/2 to adherens junctions and to promote their binding to each other. It is not clear how vinculin promotes TRIP6-LATS binding, but one possibility is that vinculin directly or indirectly causes a conformational change in TRIP6 to allow it to bind LATS1/2. Vinculin itself responds to mechanical tension at adherens junctions since both vinculin localization to adherens junctions and the vinculin-TRIP6 association is dependent on mechanical tension. Previous studies have shown that vinculin is recruited to adherens junctions by α -catenin, which responds directly to mechanical tension. Alpha-catenin binding to vinculin stabilizes its open conformation, allowing it to bind actin and possibly other effectors like TRIP6 (Huveneers and de Rooij, 2013; Choi et al., 2012; Twiss et al., 2012; Yonemura et al., 2010). We found that the vinculin-T12 mutant (Cohen et al., 2005), which is thought to mimic the open conformation of vinculin, associates more strongly with TRIP6 than wild-type vinculin suggesting that tension induced activation of vinculin may stimulate its binding to TRIP6. Surprisingly, vinculin-T12 did not increase the binding of co-expressed TRIP6 to LATS2 even though vinculin loss of function reduces TRIP6-LATS1 binding. Although we do not observe obvious differences in E-cadherin staining when vinculin (or TRIP6 or LATS1/2) is knocked down in MCF10A cells we cannot rule out the possibility that subtle effects on cell-cell junction architecture when vinculin is depleted affect TRIP6-LATS1/2 binding. Alternatively, it is possible that either vinculin-T12 does not fully mimic activation of

vinculin at adherens junctions or the ability of vinculin to activate TRIP6-LATS1/2 binding requires additional proteins.

Given that α -catenin is thought to act upstream of vinculin, one would expect α -catenin and vinculin loss of function to have similar effects on YAP activity. However, previous studies showed that α -catenin loss of function stimulates YAP activity (Schlegelmilch et al., 2011; Silvis et al., 2011), in contrast to the decreased YAP activity we observe when vinculin is knocked down. This apparent discrepancy could be resolved if adherens junctions and α -catenin had different functions in cells at lower density (higher tension) compared to cells at high density (low tension). It should be noted that the earlier studies showing α -catenin acting as an inhibitor of YAP were done at high cell density where tension would be low and vinculin and TRIP6 would not be at adherens junctions. At lower cell density, when cells are confluent but still proliferating (and presumably under more tension), α -catenin may recruit vinculin to adherens junctions to enhance YAP activity (via TRIP6 inhibition of LATS1/2) and drive cell proliferation. Thus, as cell density increases and tension decreases the vinculin-TRIP6 system turns off, and the YAP inhibitory function of adherens junctions could become dominant. It will be interesting in the future to determine how these two systems interact with each other to tune YAP regulation in response to changes in cell density and/or tension. In summary, we showed that TRIP6 acts as an intermediary connecting tension monitoring at adherens junctions to Hippo signaling, which has implications for how tension contributes to the growth of organs and tissues during development, tissue repair during injury and to pathological conditions such as cancer.

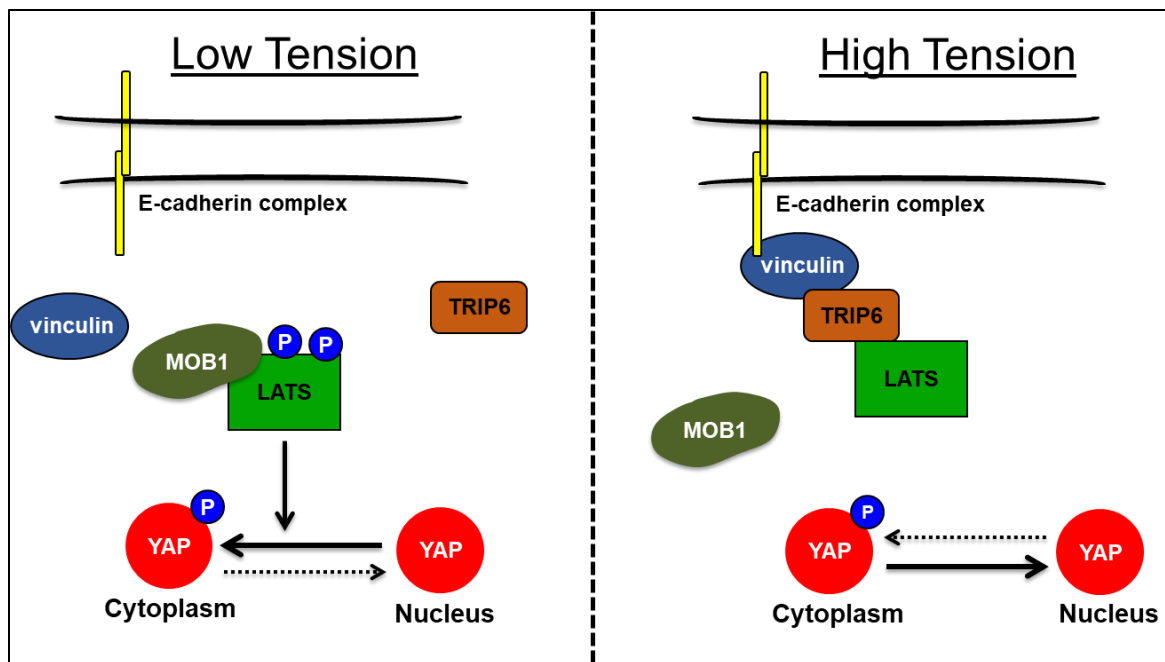


Figure 2.12

TRIP6 inhibits the Hippo signaling pathway in response to tension at adherens junctions.

Under low tension, vinculin releases TRIP6 from adherens junctions. This inhibits TRIP6-LATS binding. LATS can then be activated by MOB1 which then phosphorylates and inhibits YAP. Under high mechanical tension, vinculin binds and sequesters TRIP6 at adherens junctions which promotes TRIP6-LATS binding. LATS no longer binds MOB and remains inactive which promotes YAP activity.

CHAPTER 3

DISCUSSION

Mechanical forces regulate cell proliferation and cell differentiation *in vitro* (Curtis and Seehar, 1978; Huang and Ingber, 1999) and have been implicated in the regulation of organ growth *in vivo* (Hufnagel et al., 2007; Aegerter-Wilmsen et al., 2007, 2012). They have also been shown to correlate with tumor progression (Butcher et al., 2009). The Hippo pathway and other growth regulatory pathways contribute to responses to mechanical stress (Halder et al., 2012; Samuel et al., 2011). Computational and experimental approaches show that transmission of mechanical forces across tissues involves cell-cell connections mediated by cadherins at adherens junctions, which then connect to actomyosin stress fibers via an α -catenin-vinculin complex (Ng et al., 2014). How these complexes at adherens junctions mediate the crosstalk between mechanical tension, cell junctions, actin and Hippo signaling is not well understood.

We unravel a novel mechanism by which vinculin regulates the Hippo signaling pathway through TRIP6 in response to mechanical tension at adherens junctions. Two previous studies showed that vinculin regulates YAP localization and activity in response to mechanical tension at focal adhesions and one of them showed that the effect of vinculin on YAP is independent of LATS1/2 (Elosegui-Artola et al., 2016; Kuroda et al., 2017). We show that at the adherens junctions vinculin is able to inhibit LATS1/2 by promoting TRIP6 binding to LATS1/2 under high mechanical tension, thereby activating YAP. Disruption of tension relieves TRIP6 inhibition of LATS1/2 preventing YAP function. It was previously shown that LATS1/2 was activated at the cell membranes by NF2 (Yin et al., 2013). However, we and others show that LATS1/2 is also inhibited at

the membranes (Rauskolb et al., 2014; Dutta et al., 2017). Therefore, it would be interesting to understand under what conditions LATS1/2 is activated or inhibited at cell membranes. One explanation could be that these two opposing processes are compartmentalized where LATS1/2 activation by NF2, SAV and MOB could happen in one region on the membrane and LATS1/2 inhibition by vinculin and TRIP6 could happen in a different region on the membrane. Another explanation could be that different conditions could trigger these two opposite phenomena. We show tension promotes LATS1/2 inhibition by vinculin and TRIP6. It could happen that once tension is relieved and LATS1/2 no longer binds TRIP6, it could be free for NF2 to rapidly activate LATS1/2 at the cell membranes. Therefore, it would be interesting to study the localization and interaction of NF2 and LATS1/2 by modulating mechanical tension and also compare the membrane localization of vinculin-TRIP6 and NF2 to understand whether compartmentalization is important.

Angiomotins (AMOTL1, AMOTL2 and AMOT p130) have also been shown to activate LATS1/2 under low mechanical tension (Zhao et al., 2011; Paramasivam et al., 2011; Chan et al., 2011a; Mana-Capelli et al., 2014). It is shown that under high mechanical tension angiomotins bind to F-actin stress fibres and when tension is reduced angiomotins dissociate from actin and activate LATS1/2 (Mana-Capelli et al., 2014). The mechanism by which angiomotins activate LATS1/2 is not clearly known. Since angiomotins bind both MST1/2 and LATS1/2, it could be hypothesized that angiomotins could act as a scaffold which brings MST1/2 and LATS1/2 closer to each other for MST1/2 mediated LATS1/2 activation. Previous reports have suggested that MOB1 acts as a scaffold similar to angiomotins and is crucial for MST2 mediated LATS1/2

activation where MOB1 brings MST1/2 and LATS1/2 closer for MST1/2 mediated LATS1/2 activation (Ni et al., 2015). Further studies are needed to determine whether MOB1 is crucial angiomin mediated LATS1/2 activation and whether mechanical tension could regulate their activity.

NF2 is important for LATS1/2 activation at the cell membranes and actin cytoskeleton disruption promotes the interaction between NF2-LATS1/2 (Yin et al., 2013). One report shows that NF2 interacts with angiomin at tight junctions and regulate NF2's function as a tumor suppressor (Yi et al., 2011). A recent report shows that angiomin relieve NF2 of its autoinhibition and promote NF2-LATS1/2 interaction (Li et al., 2015). Since it is not clearly known how angiomin activate LATS1/2 we could speculate that angiomin might activate LATS1/2 in part by promoting NF2 activity at cell membranes and requires further investigations. It would be also interesting to examine under what conditions inhibitors (TRIP6, Ajuba, Zyxin) and activators (NF2, Angiomin) of LATS1/2 regulate LATS1/2 activity at the adherens junctions to control YAP function.

Previous studies have identified α -catenin and vinculin as force transducers at adherens junction. It was shown that under high mechanical tension conformational changes in α -catenin expose vinculin binding sites thus recruiting vinculin. Subsequently, vinculin also undergoes conformational changes and this stabilizes their interaction and helps in actin stress fiber recruitment to the adherens junctions (Huvneers and de Rooij, 2013; Yonemura et al., 2010; Leerberg et al., 2014; Yao et al., 2014; Bays et al., 2014; Thomas et al., 2013; Bays and DeMali, 2017). We show that under high mechanical tension TRIP6 binds vinculin and disruption of tension releases TRIP6 from vinculin.

Interestingly, the constitutively active “open” form of vinculin called T12 (Marg et al., 2010; Cohen et al., 2005) is able to bind more TRIP6 than the wild-type form. This suggests that tension induced conformational change of vinculin could open up cryptic TRIP6 binding sites on vinculin. Further studies to find out the TRIP6 binding domains on vinculin (and vice versa) can shed light on the mechanism of TRIP6-vinculin interaction under mechanical tension. Understanding the role of upstream factors such as α -catenin and β -catenin is also important to clearly define the role of vinculin and TRIP6 on how mechanical tension regulates the Hippo signaling pathway.

The Ajuba and Zyxin family of LIM domain proteins are known to inhibit LATS1/2 in flies and mammalian cells (Rauskolb et al., 2011; Reddy and Irvine, 2013; Sun and Irvine, 2013; Rauskolb et al., 2014). The mechanism by which these proteins inhibit Warts/LATS is not clearly understood. Based on localization experiments in flies, it was proposed that Ajuba inhibit Warts by sequestering it away from its activators (Rauskolb et al., 2014). Our results demonstrate that TRIP6 likely functions at least in part by competing with MOB1 for binding to LATS1/2. MOB1 is a scaffold protein that connects MST1/2 with its substrate LATS1/2 and is key to LATS1/2 activation (Rauskolb et al., 2014; Ni et al., 2015; Lai et al., 2005). A competition between TRIP6 and MOB1 for binding to LATS1/2 is consistent with our results showing TRIP6 interferes with MST1/2 phosphorylation of LATS1/2. Other LIM domain proteins such as Zyxin and Ajuba bind to a similar region of LATS1/2 (Rauskolb et al., 2011; Reddy and Irvine, 2013; Sun and Irvine, 2013). These proteins could also inhibit LATS1/2 in a similar fashion and it would be interesting to understand the mechanism.

One of our key findings is that tension dictates the interaction between LATS1/2 and TRIP6. High mechanical tension increases TRIP6-LATS1/2 interaction and low tension reduces the interaction. In order to explain this phenomenon, we came up with a simple model where we speculate that tension could modulate the structural conformation of either TRIP6 and/ or LATS1/2 or both to regulate binding. Interestingly, it is seen that zyxin, a LIM domain protein closely related to TRIP6, undergoes autoinhibition in which the C-terminus LIM domains are masked by its N-terminus region (Moody et al., 2009; Hirota et al., 2000). So, it could be speculated that low tension keeps TRIP6 in its autoinhibited state and high tension triggers some unknown mechanism that relieves TRIP6 of its autoinhibition allowing LATS1/2 to bind its C-terminus LIM domain. One possibility is that its interaction with vinculin or some other unknown protein might trigger a conformational change in TRIP6 exposing its LIM domains for LATS1/2 interaction. Since TRIP6 has also been shown to be phosphorylated by Src and AMPK (Solaz-Fuster et al., 2006; Chastre et al., 2009), another possibility is that phosphorylation by these kinases could relieve autoinhibition of TRIP6 promoting LATS1/2 binding. Further studies are required to understand these phenomena.

In our studies we have used two epithelial cell lines namely Human Embryonic Kidney cell line or HEK 293A and Mammary epithelial cell line or MCF 10A. Previous reports have shown that fibroblasts cells also undergo contact inhibition and hyperactive YAP can cause overgrowth (Zhao et al., 2007). It is not known how these cells sense mechanical tension at adherens junction. The process by which other types of cell lines (such as endothelial and mesothelial cells lines) sense tension not clearly understood.

Further studies are required to address how tension is sensed by these cell lines and whether the role of TRIP6 is universal or restricted to only epithelial cells.

Various studies have shown the importance of mechanical tension in development and cancer. Mechanical tension promotes morphological changes in the tissues and cells which not only regulates gene expression but also protein function (Mammoto and Ingber, 2010; Wozniak and Chen, 2009; Brouzés and Farge, 2004). In vitro studies on cultured cells and in vivo studies on embryonic cells shows how mechanotransduction of external forces regulate cell fate and differentiation (Engler et al., 2006; McBeath et al., 2004; Farge, 2003). Recent studies have also linked mechanical tension with cancer progression and malignancy. Cancer progression has been closely linked with remodeling of the microenvironment, extracellular matrix, and abnormal adherens junctions (Barriere et al., 2015). Epithelial-Mesenchymal transition (EMT) is another hallmark of cancer which leads to disruption of adherens junctions thus promoting integrin-mediated cell migration (Nguyen-Ngoc et al., 2012; Provenzano et al., 2009). Substrate stiffness also regulates tumorigenic cell migration where stiffer substrates promote cell migration and vice versa (Nguyen-Ngoc et al., 2012; Provenzano et al., 2009). Interestingly, tumor growth alters the composition of the ECM to increase tissue stiffness which changes force balance to regulate metastasis (Levental et al., 2009; Lu et al., 2012). Over the years YAP and the Hippo pathway emerged as important intracellular factors that respond to changes in mechanical tension and regulate cell proliferation. Several studies have implicated their role in breast cancer, gastrointestinal tumors, thoracic tumors, melanomas, primary brain tumors and sarcomas (Maugeri-Saccà and De Maria, 2018). Although pharmacological inhibitors of YAP such as

verteporfin (Wang et al., 2016; Liu-Chittenden et al., 2012), statins (Wang et al., 2014; Sorrentino et al., 2014), and VGLL4-mimicking peptides (Koontz et al., 2013; Zhang et al., 2009) have shown some promise against YAP mediated tumorigenesis, further studies are required to clearly understand the role of mechanical forces on cancer progression through YAP. We show for the first time the involvement of LIM domain protein TRIP6 in connecting junctional mechanosensors (vinculin) to the effectors (Hippo signaling components and YAP) in mammalian system. TRIP6 has already been shown to be involved in cell proliferation and tumorigenesis (Miao et al., 2016; Zhao et al., 2017; Shiozawa et al., 2018). However, whether TRIP6 functions through regulating LATS1/2 in vivo needs further investigation. Additionally, the function of other LIM domain proteins closely related to TRIP6 could also be studied with respect to mechanosensing and cancer progression. These studies may help explain how tension contributes to growth of organs and tissues during development, tissue repair during injury and how tension can contribute to pathological conditions such as cancer.

APPENDIX

4.1 ROLE OF ANGIOMOTINS IN REGULATION OF THE HIPPO SIGNALING PATHWAY

4.1.1 Introduction

The Hippo signaling pathway plays an important role in organ growth regulation, contact inhibition and stem cell differentiation and maintenance (Halder and Johnson, 2011). The core kinases in the Hippo signaling pathway MST1/2 and LATS1/2 is important in regulating transcription co-activator YAP. YAP promotes cell proliferation, stem cell maintenance, and stem cell fate. A large number of mutagenic screens in *Drosophila* identified various upstream factors that regulate the Hippo signaling pathway. However, in the mammalian system the upstream components of the Hippo signaling pathway were not known.

In order to identify the upstream regulators, LAP-tagged (Cheeseman and Desai, 2005) LATS2 was stably expressed in U2OS cells and purified using immunoprecipitation. The isolated protein complexes were then identified using mass-spectroscopy. A large number of proteins were identified and subsequent experiments identified AMOTL2 (a member of the angiomin family of proteins) as a potent activator of LATS2 (Paramasivam et al., 2011). The angiomin family consists of three proteins namely AMOTL1, AMOTL2 and AMOT (AMOT has two isoforms: a short isoform called AMOT p80 and a long isoform called AMOT p130; see Figure 4.1). Angiominins associate with tight junctions and are involved in actin cytoskeleton remodeling, growth regulation and cell motility (Patrie, 2005; Gagné et al., 2009; Zheng et al., 2009; Ranahan et al., 2011). Angiominins have been shown to bind and activate the hippo signaling kinase LATS to phosphorylate and inactivate YAP (Paramasivam et al., 2011). It is widely known that

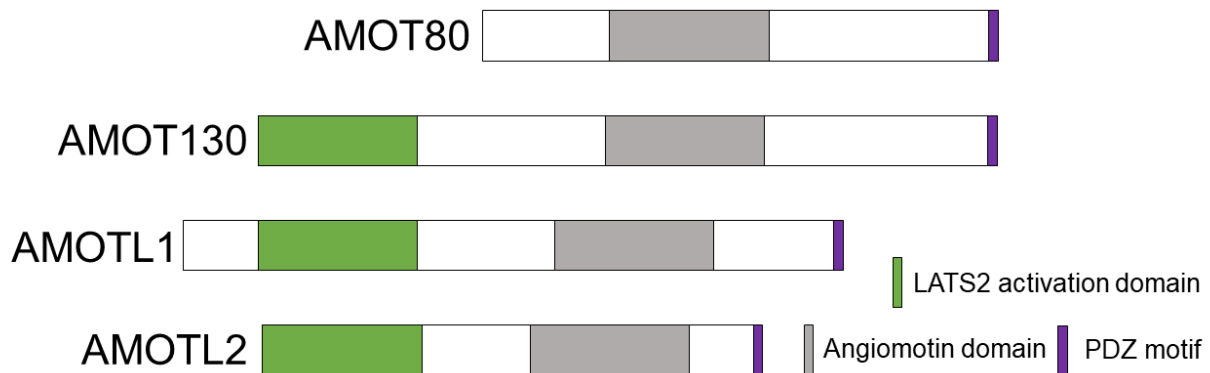


Figure 4.1 A diagram of AMOT80, AMOT130, AMOTL1, and AMOTL2.

The LATS2 activation domain (green), the angiomotin domain (grey) and C-terminus PDZ domain (purple) are shown. The LATS2 activation domain is not found in AMOT80.

cell density and actin cytoskeleton regulate YAP activity (Reddy et al., 2008; Dupont et al., 2011; Sansores-Garcia et al., 2011; Zhao et al., 2012). The Hippo signaling pathway is also controlled by GPCRs through the regulation of the actin cytoskeleton (Miller et al., 2012; Yu et al., 2012), but how the actin cytoskeleton regulates the Hippo signaling pathway was not clearly known. Interestingly, angiomotins also localize to actin stress fibers (Ernkvist et al., 2008; Gagné et al., 2009). As a result, we wanted to investigate whether angiomotins could be a missing link that connects the actin cytoskeleton and Hippo signaling. The results that will be discussed in this section are part of a paper published from our lab (Mana-Capelli et al., 2014).

4.1.2 Results

N-terminus of angiomotins activate LATS2

Angiomotins bind and activate LATS2 which promotes YAP phosphorylation and inhibition (Chan et al., 2011b; Zhao et al., 2011; Paramasivam et al., 2011). However, it was not clearly known which domains on angiomotin are responsible for LATS2 activation. To determine the LATS2 activation domain (LAD) we deleted various regions in the N-terminal half based on conserved domains that were determined by aligning all the three angiomotin proteins (AMOT p130, AMOTL1, AMOTL2) (Figure 4.2A). Then we co-expressed wild-type (WT) and the deletion constructs of angiomotin p130 with LATS2 and YAP and looked at YAP phosphorylation. We saw that YAP phosphorylation significantly decreased when AMOT p130 Δ 13-27 (called as AMOT130- Δ LAD) was used compared to WT AMOT p130. LATS2 phosphorylation was also reduced when AMOT p130- Δ LAD was expressed compared to the WT (Figure 4.2B; this figure also shows AMOT130- Δ AB which is referred in Figure 4.3). These results are consistent with previous results from the lab showing that the first 100 amino acids of AMOTL2 were sufficient to activate LATS2 (Paramasivam et al., 2011).

Determination of the actin-binding domain (AB) on angiomotins

Overexpression experiments with AMOT130 in U2OS cells show that it localizes to F-actin and promotes their bundling. Specifically, the N-terminus of AMOT p130, AMOTL2 and AMOTL1 localize to F-actin stress fibers (Figure 4.3). In order to determine the actin-binding domain (AB) of angiomotin p130, we used the deletion constructs described in Figure 4.2B. We saw that AMOT p130-AB (deletion of residues 169-178)

did not localize to actin (Figure 4.3B). Deletion of the same region in the shorter form of AMOTL2 also had the same effect (Figure 4.3A).

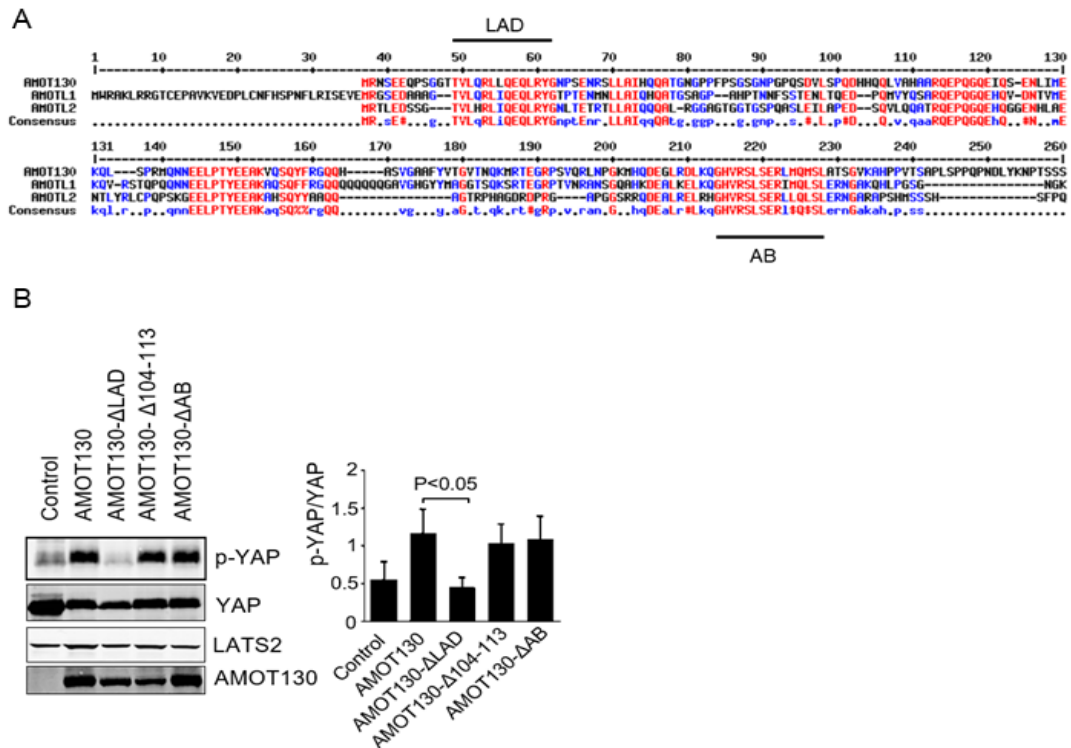


Figure 4.2 Determining conserved domains in angiomotins and testing their role in LATS2 activity

(A) AMOT130, AMOTL1 and AMOTL2 were aligned and the conserved domains in their N-terminus are shown in red. LAD = LATS2 activation domain and AB = Actin binding domain are shown. (B) LATS2, YAP, and the indicated AMOT130 plasmids were transfected into HEK293 cells, and the levels of AMOT130, LATS2, YAP, and phospho-YAP were analyzed by Western blotting. The experiment was done in triplicate, and error bars indicate the SD of the averages.

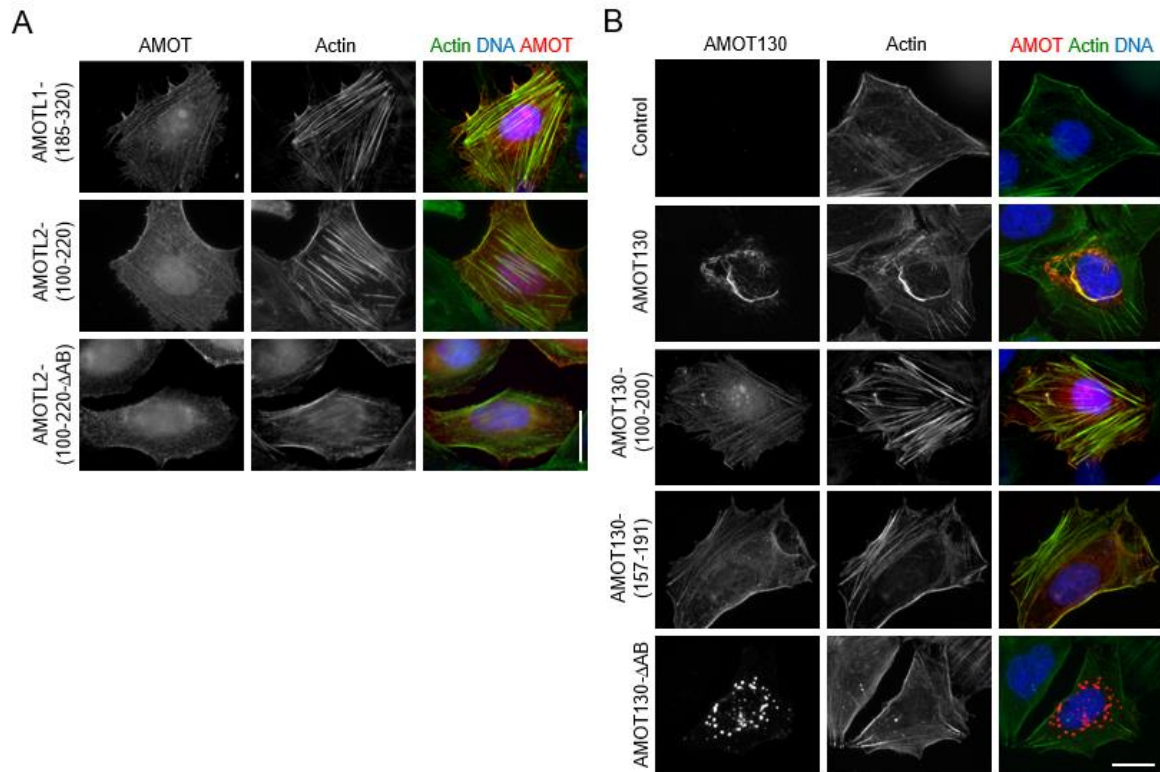


Figure 4.3 Mapping actin binding sites on angiomotins

(A) U2OS cells were transfected with plasmids for expression of amino acids 185-320 of AMOTL1 or 100-220 of AMOTL2 with or without the ABD sequence. Cells were stained for the Myc-tagged AMOTL1 and AMOTL2 using anti-Myc antibodies and for F-actin using phalloidin. Merged images with DNA in blue are shown. In all cases, nuclei were visualized with DAPI. Bar: 20 μ m. (B) U2OS cells were transfected with plasmids for expression of Myc-tagged full-length AMOT130, amino acids 100–200 of AMOT130 (AMOT130 (100-200)), AMOT130 with a deletion in the actin-binding region (AMOT130- Δ AB), or a fragment containing the actin-binding region fused to GFP (AMOT130-(157-191)) and imaged at low densities. Cells were stained for AMOT130 using anti-GFP antibodies and for F-actin using phalloidin. DNA was stained with DAPI. Bar, 20 μ m.

LATS2 regulate the actin binding of angiomotins

The consensus LATS2 phosphorylation site is the HXRXXS motif. Interestingly, angiomotins have a HXRXXS site in the middle of the actin binding (AB) site for AMOT p130 (Figure 4.4A). LATS2 could phosphorylate WT AMOT p130 and AMOTL2 but not the S175A mutant version in vitro (Figure 4.4B). LATS2 phosphorylation of AMOT130 prevents its actin localization whereas the kinase dead (KD) does not (Figure 4.4C). The alanine mutant localizes and bundles F-actin even in the presence of LATS2 whereas the phosphomimetic glutamic acid mutation does not (Figure 4.4C). This data was entirely done by Sebastian Mana-Capelli (Mana-Capelli et al., 2014).

Actin-binding deficient angiomotins inhibit YAP activity

Disruption of F-actin inhibits YAP activity and promotes its cytoplasmic localization. AMOT p130 has 3 YAP binding L/PPxY domains flanking the AB. Therefore, a possibility could be that AMOT p130 would localize to intact F-actin, which would mask its L/PPxY domains, keeping YAP active. F-actin disruption would release AMOT p130 unmasking L/PPxY domains which could now bind, sequester and inactivate YAP. To study the effect of AMOT p130 on YAP, we expressed WT, S175E, Δ AB, and Δ AB- Δ L/PPxY (double mutant) versions of AMOT p130 and looked at YAP location. We saw that WT AMOT p130 that binds f-actin only weakly affects YAP location and YAP is mostly nuclear (Figure 4.5). The actin-binding deficient versions of AMOT p130 (S175E and Δ AB) reduce YAP nuclear location since it is now free from actin to bind and sequester YAP in the cytoplasm (Figure 4.5). However, the Δ AB- Δ L/PPxY mutant that cannot bind both actin and YAP has no effect on YAP location (Figure 4.5). These

results suggest that the binding of AMOT p130 to actin fibers interferes with its binding and inhibition of YAP. (Please note: I made the deletion constructs).

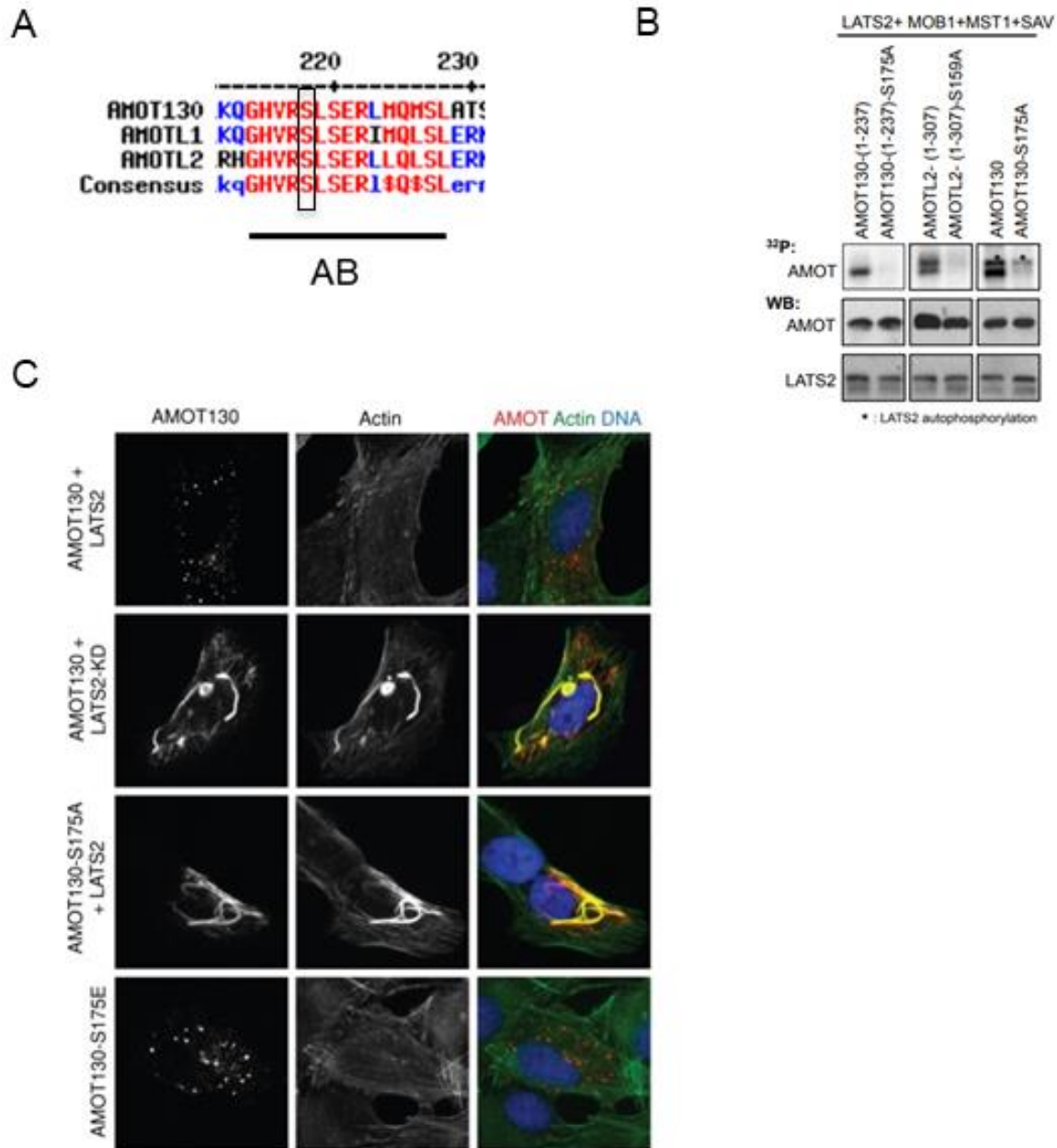


Figure 4.4 LATS2 regulates AMOT130 function

(A) An alignment of the amino-terminal region of human AMOT130, AMOTL1, and AMOTL2 is shown. The region containing the actin-binding region (underlined) and

LATS phosphorylation site are indicated (box). (B) HEK293 cells were co-transfected with LATS2, its activators (MST1, MOB1, SAV) and Myc-tagged angiomin constructs (AMOT130, AMOT130-(1-237), or AMOTL2-(1-307)) with or without the conserved LATS2 phosphorylation site mutated. LATS2 and each AMOT protein were immunoprecipitated on the same beads and immune-complex kinase assays were carried out (^{32}P). The levels of angiomin proteins (Myc) and LATS2 (GFP) are shown. (C) U2OS cells were transfected with the indicated AMOT130 and LATS2 plasmids and imaged at low densities. Cells were stained for AMOT130 (Myc), F-actin using phalloidin, and LATS2 or LATS2-KD (FLAG). DNA was stained with DAPI. Bar, 20 μm .

4.1.3 Discussion

Studies have implicated the actin cytoskeleton as a major regulator of the Hippo signaling pathway. The actin cytoskeleton is regulated in turn by GPCRs, cell density and mechanical tension in the cellular microenvironment (Meng et al., 2016; Panciera et al., 2017). However, the factors that connect F-actin regulation of the Hippo pathway was not known. We showed that angiominins mediate effects of the actin cytoskeleton on the Hippo signaling pathway and regulate LATS1/2 activity. High mechanical tension promotes F-actin stress fibers formation (Deshpande et al., 2006; Sugimoto et al., 1991), which sequesters angiominins keeping LATS1/2 inactive and YAP active. Disruption of mechanical tension (Latrunculin B, cytochalasin D, Blebbistatin and high cell density) activates LATS which phosphorylates angiominins reducing their binding to F-actin and allowing them to bind and sequester YAP in the cytoplasm. Free phosphorylated angiominins can alternatively activate LATS1/2 which in turn

phosphorylate and inhibit YAP. Together these results show how F-actin is connected to the Hippo signaling pathway through angiomotins and regulates YAP in response to changes in mechanical tension in the cellular microenvironment.

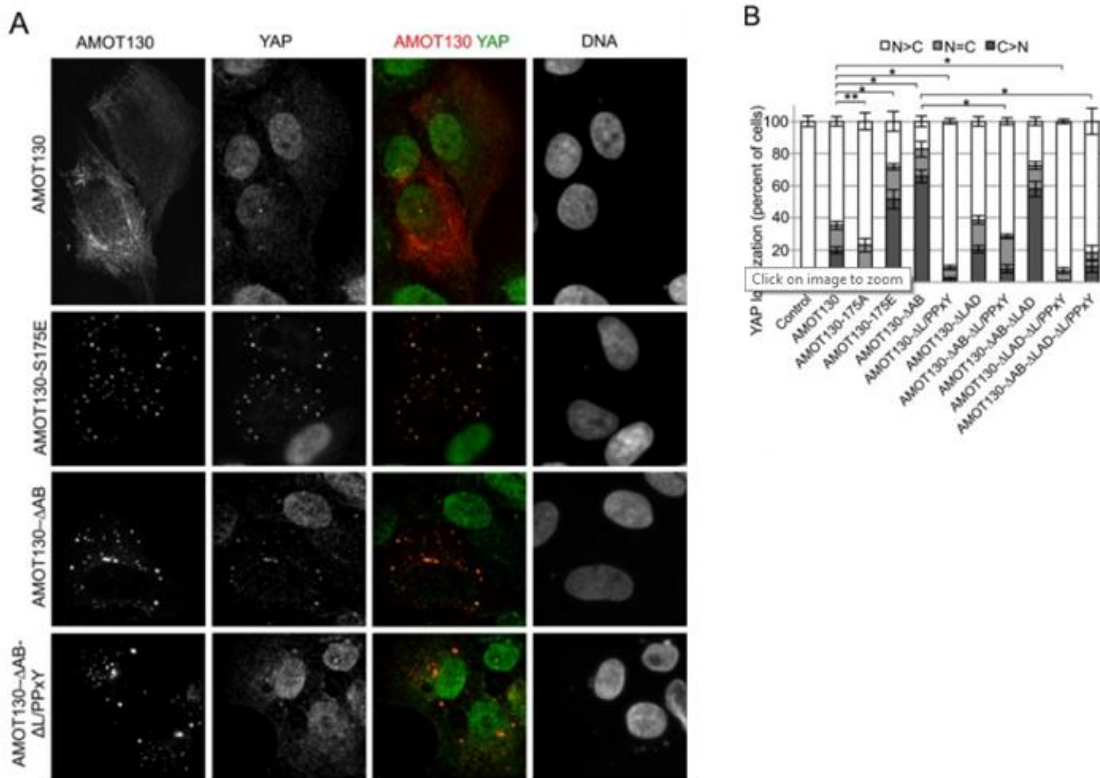


Figure 4.5 AMOT130 regulates YAP localization.

(A, B) U2OS cells were transfected with either control plasmid or one of the indicated AMOT130 plasmids. The next day, cells were stained for endogenous YAP and scored for the percent of cells with more YAP in the nucleus than the cytoplasm ($N > C$), more in the cytoplasm than the nucleus ($C > N$), or equal signal in the cytoplasm and nucleus ($C = N$). (A) Example images. (B) Average from three experiments ($n \geq 100$ each), and the error bars indicate SD of the averages. Brackets on top of bars represent statistical significance (Fisher test, $*p < 0.00001$, $**p < 0.02$). Bar, 20 μm .

4.2 MATERIALS AND METHODS

Cell Lines

Human Embryonic Kidney (HEK293, HEK293A, U2OS) cell lines were grown in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO) and 1% (v/v) penicillin/streptomycin (Invitrogen). Human mammary epithelial cell line MCF10A was cultured in DMEM/F12 (1:1) media supplemented with 5% (v/v) fetal horse serum (GIBCO), 20ng/ml Epidermal Growth Factor (Peprotech), 0.5 mg/ml Hydrocortisone (Sigma), 100 ng/ml Cholera toxin (Sigma), 10µg/ml Insulin (Sigma) and 1% (v/v) penicillin/streptomycin (Invitrogen). Cell lines were cultured in a humidified incubator at 37°C with 5% CO₂.

Expression Plasmids and shRNAs

The Flag-trip6 plasmid is obtained from Addgene (Plasmid #27255). shRNAs for TRIP6 (shRNA1: TRCN0000061438, shRNA4: TRCN0000061441) and control shRNA (shEGFP) were obtained from the UMass RNAi core facility.

CRISPR mediated deletion of TRIP6 in 293A cell line

The target sequence to knock-out the TRIP6 gene was selected with the web tool developed by the Zhang lab (<http://crispr.mit.edu/>). Oligos complementary to the target sequence with appropriate overhangs (See Table 2) were annealed and cloned into a variant of the px330 plasmid with puromycin resistance (Hainer et al., 2015). HEK293A and HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer directions and using 500 ng of plasmid per well of a 12-well plate. The next day, cells were placed under selection with 2 µg/ml of Puromycin (GIBCO) for 48

hours. Puromycin-resistant cells were then heavily diluted and plated on 10 cm plates for colony isolation. Colonies were picked 7-10 days later by depleting the media from the plate and using a P20 pipette loaded with 1 μ l of media to dislodge the colony from the plate by rapid back and forth movements. Clonal lines were then expanded and the expression of TRIP6 was determined by Western blot. Clonal lines that lacked expression of TRIP6 were again expanded from single cells by dilution followed by colony isolation and tested by Western blot to ensure that they were true clonal lines. At least two independent clonal lines were kept for further analysis. HEK293A cells were maintained at low densities as much as possible to prevent morphological changes associated with cell crowding.

Cell starvation and drug treatments

MCF10A and HEK293A cells were starved overnight in DMEM/F12 (1:1) and DMEM respectively supplemented with 1% of penicillin and streptomycin (Invitrogen) before adding complete cell culture medium described above for 1 hour. Latrunculin B was used at 1 μ M for 1 hour. Blebbistatin was used at 25 μ M for 2 hours on MCF10A and 1 hour on 293A. Both MCF10A and 293A cell lines were treated with 0.5mM EGTA for 30 minutes. MCF10A cells were treated with 50 μ M of Y27632 for 1 hour.

Stretching experiments

MCF10A cells were cultured on collagen I coated bioflex plates (Flexcell International Corporation (# BF-3001C)) at high density before stretching them with a Flexcell FX-4000 machine (Flexcell International, Burlington, NC) using 22mm diameter posts under maximum vacuum pressure, resulting in a 17% equibiaxial stretch for 2 hours in a

humidified incubator at 37°C with 5% CO₂. Cells were either lysed for RNA preparation (see RT-qPCR), protein preparation (Cell lysis, Immunoprecipitation, and Western Blotting) or fixed while under stretch with 3.7% PFA for 10 minutes before performing immunofluorescence. Control plates were not stretched. For stretched plates, the stretch level was validated by measuring deformation before and after stretch using multiple fiduciary markers.

Immunofluorescence

HEK 293A and MCF10A cells were cultured on coverslips and fixed with 3.7% paraformaldehyde in PBS for 10 minutes, permeabilized in 0.5% Triton-X in UB (UB; 150 mM NaCl, 50 mM Tris pH 7.6, 0.01% NaN₃) for 3 minutes at 37°C, then blocked with 10% BSA in UB for 30 minutes at 37°C. Cells were then incubated for 1 hour at 37°C with appropriate primary antibodies, washed three times in UB and incubated with Alexa Fluor-conjugated secondary antibodies (Molecular Probes) for 1 hour at 37°C.

U2OS cells cultured on coverslips were fixed in phosphate-buffered saline (PBS)/4% paraformaldehyde for 10 min and permeabilized/blocked with 0.1% Triton X-100 and 5% normal goat serum (Invitrogen) for 30 min. Cells were subsequently incubated with appropriate primary antibodies for 1–2 h at room temperature. They were washed three times in PBS with 0.1% Triton X-100 and incubated with Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Grand Island, NY) for 1 h at room temperature. 4',6-Diamidino-2-phenylindole (DAPI) staining and Alexa-conjugated phalloidin (488 or 568; Invitrogen) were also added to the secondary antibody solution when appropriate.

After three washes in UB, coverslips were mounted on glass slides using Prolong Gold Antifade reagent with DAPI (Invitrogen) and left at 4°C overnight. The next day slides were viewed using fluorescent microscopy (Nikon Eclipse E600) and images were acquired using a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ). The confocal image was acquired using a Leica SP5 AOBS second generation laser scanning confocal microscope. Image processing and analysis were carried out with IPLab Spectrum software (Signal Analytics, Vienna, VA) and ImageJ software (Schneider et al., 2012).

Cell lysis, Immunoprecipitation, and Western Blotting

HEK293 and HEK293A cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the rescue assay, HEK293A (WT) and HEK293A TRIP6-KO knockout cells were transfected with empty plasmid and increasing amounts of FLAG-TRIP6 plasmid (50ng-200ng) respectively and vinculin depleted HEK293A cells were transfected with empty plasmid and increasing amounts of GFP-vinculin (chicken) plasmid (50ng-350ng) respectively, using FuGENE® HD Transfection Reagent (#E2311, Promega) according to manufacturer's protocol. 200ng of the FLAG-TRIP6 and 150ng of GFP-vinculin (chicken) plasmid was used for the final rescue experiment. Cells were collected after 48 hours and lysed with lysis buffer (10% Glycerol (Invitrogen), 20mM Tris-HCl-pH 7.0, 137mM NaCl, 2mM EDTA, 1% NP-40 (Invitrogen), 1mM PMSF(Sigma), 1mM Na₃VO₄ (Sigma) and 1x mammalian protease inhibitor cocktail (Sigma)). MCF10A cells were additionally passed through a 26G $\frac{1}{2}$ needle. Cells were then incubated for 10 minutes at 4°C and lysates were cleared by

centrifugation at 10,000 rpm for 10 min at 4°C. For immunoprecipitation, Dynabeads (Invitrogen) were used according to the manufacturer's protocol.

siRNA/shRNA transfection

Knockdowns in MCF10A cells were performed using 30 nM of control siRNA (fire fly luciferase) or SMARTpool siRNA from Dharmacon (for LATS1 and 2) or stealth siRNA from Thermo-Fisher (for vinculin). RNAiMAX Lipofectamine (Invitrogen) was used according to the manufacturer's protocol. After 48 hours cells were either used for western blotting or fixed for immunofluorescence. Stable knockdowns in MCF10A cells were done using lentiviral infection of shRNA and cells were selected with puromycin for 3 days. Experiments were performed immediately after puromycin selection. Viral supernatants were generated by the shRNA Core Facility (UMASS) to target TRIP6.

RT-qPCR

RNA was prepared using Quick-RNA MiniPrep kit (Zymo Research) according to the manufacturer's protocol. cDNA was prepared using qScript cDNA super mix (Quanta Biosciences, Inc) according to the manufacturer's protocol. RT-qPCR was performed using KAPA SYBR Fast- Master Mix Universal kit (Kapa Biosystems). Target mRNA levels were measured relative to GAPDH mRNA levels. Oligo sequences used are listed in Table 1.

Recombinant protein expression and in vitro competition assays

TRIP6 and LATS2 were cloned in pDEST-GST and pDEST-MBP respectively (provided by Dr. Marian Walhout's lab) using Gateway (ThermoFisher Scientific) directions. MOB1A was cloned in pET28a through standard cloning. GST-TRIP6, MOB1A-6xHis,

and MBP-LATS2 plasmids were transformed into BL21 DE3 cells and recombinant protein expression was induced with 1mM IPTG for 4 hours and 30 minutes at 25° C. Bacterial pellets were resuspended in lysis buffer (1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 150 mM NaCl, 10 mM β-mercapto-ethanol, 0.05% Triton X-100, 1 mg/ml of lysozyme, 5 µg/ml of nuclease, and 1mM PMSF) and incubated for 30 minutes at 4° C. Cells were lysed on ice with 6 rounds of 10 sonications each using a VWR Sonifier 450 fitted with a microtip set to an output of 2 and a duty cycle of 80. Lysates were cleared by centrifugation at 21,000g for 10 minutes at 4° C. GST-TRIP6 was purified using Glutathione beads (GE) and eluted with 20 mM glutathione for 30 minutes at 4° C in elution buffer (1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 150 mM NaCl, 0.05% Triton X-100, and 1mM PMSF). MOB1A-6His was purified using Ni-IDA beads (Biotool) and eluted with 300 mM Imidazole for 30 minutes at 4° C in the elution buffer. MBP-LATS2 was pulled down with magnetic maltose beads (NEB). For the in vitro competition assay, GST-TRIP6, MOB1A-6xHis, and control proteins were mixed as indicated in Figure 3B and then adjusted to a volume of 60µl using elution buffer. A constant amount of GST-TRIP6 (approximately 0.7 µg) was used in each sample, and either 1, 4, or 10-fold molar ratios of MOB1A-6xHis were added as a competitor. The different protein solutions were then added with 20 µl of 10 mM Tris-HCl, pH 7.4 (to ensure an equal pH) to bead-bound MBP-LATS2, and incubated for 20 minutes at room temperature with mixing. MBP-LATS2 bound beads were separated using a magnetic stand, washed 3 times in elution buffer, and boiled in SDS-PAGE sample buffer. Protein samples were then subjected to SDS-PAGE and Western blotting with the specified antibodies.

Quantification and Statistical Analysis

Data are presented as Mean \pm SD. Each experiment was done in triplicate except where indicated. Student's t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) were performed using Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). For YAP localization studies, we counted 100 cells each from three different experiments and used Fisher's test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) using GraphPad Quickcalcs (<http://graphpad.com/quickcalcs/contingency1/>) to calculate the significance. For TRIP6 and LATS1 fluorescent intensity measurements at cell-cell junctions, the average intensity for each protein at individual cell-cell junctions (n=48) was measured using Image J. Intensity measurements for each junction were normalized to the total average fluorescence of the field of cells. For western blots, we performed background subtraction and densitometric analysis of respective bands using Image J (Schneider et al., 2012) and normalized to loading control (either actin or tubulin).

4.3 TABLES

Table 1: RT-qPCR primers

GAPDH-F	CTCCTGCACCACCAACTGCT
GAPDH-R	GGCCATCCACAGTCTTCTG
CTGF-F	AGGAGTGGGTGTGTGACGA
CTGF-R	CCAGGCAGTTGGCTCTAATC
Cyr61-F	GAGTGGGTCTGTGACGAGGAT
Cyr61-R	GGTTGTATAGGATGCGAGGCT
TRIP6-F	CCTTCACAGTGGATGCTACG
TRIP6-R	CTTGGGGCAAACCTTCCTGT
vinculin-F	TCAGATGAGGTGACTCGGTTGG
vinculin-R	GGGTGCTTATGGTTGGGATTGG
AMOT-F	ACTACCACCACCTCCAGTCA
AMOT-R	ACAAGGTGACGACTCTCTGC
AMOTL1-F	GCAGACAGGAAAACCTGAGGA
AMOTL1-R	AAATGTGGTGGGAACAGAGA
AMOTL2-F	GCTACTGGGGTAGCAACTGA
AMOTL2-R	GAAGGCAGTGAGGAACTGAA

Table 2: TRIP6 CRISPR primers (designed using <http://crispr.mit.edu/>)

Oligo 1	CACCGGCGATCCCCGCGGCACCC
Oligo 2	AAACGGGTGCCGCGGGGGATCGCC

Table 3: Reagent and resource table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse TRIP6	Santa Cruz	Cat# sc-365122
Rabbit YAP	Santa Cruz	Cat# sc-15407
Mouse YAP	Santa Cruz	Cat# sc-101199
Mouse GFP	Santa Cruz	Cat# sc-9996
Mouse Myc	Santa Cruz	Cat# sc-40
Rabbit Myc	Santa Cruz	Cat# sc-789
Rabbit LATS1	Cell Signaling	Cat# 3477S
Rabbit LATS2	Cell Signaling	Cat# 5888S
Rabbit phospho LATS Ser 909	Cell Signaling	Cat# 9157S
Rabbit phospho LATS Thr 1079	Cell Signaling	Cat# 8654S
Rabbit phospho MST	Cell Signaling	Cat# 3681S
Rabbit MOB1	Cell Signaling	Cat# 13730S
Rabbit phospho YAP Ser 127	Cell Signaling	Cat# 4911S
Rabbit LATS2	Proteintech	Cat# 20276-1-AP
Mouse MBP	Proteintech	Cat# 66003-1-Ig

Rabbit GST	Proteintech	Cat# 10000-0-AP
Mouse Tubulin	Proteintech	Cat# 66031-1-Ig
Mouse E-cadherin	BD Biosciences	Cat# 610181
Mouse FLAG	Sigma	Cat# F1804
Mouse vinculin	Sigma	Cat# V9131, kindly provided by Beth Luna
Bacterial and Virus Strains		
DH5 α Competent Cells	Thermo Fisher	Cat# 18265017
Rosetta™(DE3) Competent Cells - Novagen	EMD Millipore	Cat# 70954
Chemicals, Peptides, and Recombinant Proteins		
Penicillin-Streptomycin	Thermo Fisher	Cat# 15140-122
Epidermal Growth Factor	Peptotech	Cat# AF-100-15
Hydrocortisone	Sigma	Cat# H-0888
Cholera toxin	Sigma	Cat# C-8052
Insulin	Sigma	Cat# I-1882
Lipofectamine 2000	Thermo Fisher	Cat# 11668019
RNAiMAX Lipofectamine	Thermo Fisher	Cat# 13778150
FuGENE HD Transfection Reagent	Promega	Cat# E2311
Protease Inhibitor Cocktail	Sigma	Cat# P8340
Dynabeads Protein G	Thermo Fisher	Cat# 10004D
Collagen I	Corning	Cat# 354231
Latrunculin B	Sigma	Cat# L5288
Blebbistatin	Sigma	Cat# B0560
Y27632	Tocris	Cat# 1254
Puromycin	Thermo Fisher	Cat# A1113803
Critical Commercial Assays		
Quick RNA Mini Prep Kit	Zymo Research	Cat# R1054
qScript cDNA super mix	Quanta Biosciences	Cat# 101414-102
KAPA SYBR Fast- Master Mix Universal kit	Kapa Biosystems	Cat# KK4651
Qiagen Plasmid Mini Prep Kit	Qiagen	Cat# 27104
Experimental Models: Cell Lines		
MCF10A	ATCC	CRL-10317
HEK 293	ATCC	CRL-1573
HEK 293A	Thermo Fisher	#R70507
HEK 293A TRIP6 CRISPR mediated knockout line	This study	N/A
Oligonucleotides		
control siRNA CGUACGCGGAUACUUCGA	GE Lifesciences	D-001100-01-20
SMARTpool: ON-TARGETplus LATS1 siRNA	GE Lifesciences	L-004632-00-0005
SMARTpool: ON-TARGETplus LATS2 siRNA	GE Lifesciences	L-003865-00-0005

Vinculin siRNAs, oligo IDs: HSS111260 and HSS187662	Invitrogen	Cat# 1299001
RT-qPCR primers, see table 1	N/A	N/A
TRIP6 CRISPR primers, see table 2	N/A	N/A
Recombinant DNA		
pDONR221 LATS2	This study	N/A
pDONR221 TRIP6	This study	N/A
pDEST-MBP LATS2	This study	N/A
pDEST-GST TRIP6	This study	N/A
pET28a MOB1A	This study	N/A
pcDNA-FLAG-Hu-Trip6	Addgene	Addgene #27255
pcDNA-FLAG-Hu-Trip6 1-277	This study	N/A
pcDNA-FLAG-Hu-Trip6 278-476	This study	N/A
pCI113-LAP-GFP-LATS2	Paramsivam et al., 2011	N/A
pCI113-LAP-GFP-LATS2 137-677	This study	N/A
pCI113-LAP-GFP-LATS2 668-947	This study	N/A
pCI113-LAP-GFP-LATS2 376-397	This study	N/A
pCI113-LAP-GFP-LATS2 376-568	This study	N/A
pCI113-LAP-GFP-LATS2 604-625	This study	N/A
pCI113-LAP-GFP-LATS2 625-644	This study	N/A
pcDNA3.1-Myc-MOB1	MOB1 was cloned in pcDNA3.1 from pSIRN-HA-MOB1 plasmid.	pSIRN-HA-MOB1 plasmid DNA was a gift of Thanos Halazonetis (University of Geneva, Geneva, Switzerland)
pCDNA4-Myc-His AMOT130	Mana-Capelli et al. 2014	N/A
pEGFP Vinculin	Addgene	Addgene# 46265
pEGFP Vinculin T12	Addgene	Addgene# 46266
pGEX-4T-1	GE Lifesciences	Cat# 28-9545-49
TRIP6 GCTGCTTTGTATGTTCTACAT, GAAGCTGGTTCACGACATGAA	shRNA1: shRNA4: shRNA Core Facility (UMASS)	shRNA1:T TRCN000006143 8, shRNA4: TRCN000006144 1
EGFP shRNA-Control	shRNA Core Facility (UMASS)	N/A
Software and Algorithms		
Image J	https://imagej.nih.gov/ij/	N/A

IPLab Spectrum software	Signal Analytics, Vienna, VA	N/A
Leica Application Suite X	Leica Microsystems	N/A

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