UNIVERSITY OF CALIFORNIA SAN DIEGO

Regulation of the Hippo Pathway Transcription Factor TEAD

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by

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2019

DEDICATION

To my parents, whose unwavering support made completion of this dissertation possible.

EPIGRAPH

Endurance is one of the most difficult disciplines,
but it is to the one who endures
that the final victory comes.

-Buddha

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LIST OF ABBREVIATIONS

α-MHC	Alpha-myosin heavy chain
2-DG	Deoxy-D-glucose
AMOT	Angiomotin
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
CAC	Colitis-associated cancer
CHX	Cyclohexamide
CRISPR	Clustered regularly interspaced short palindromic repeats
CRM1	Chromosomal maintenance 1
CTGF	Connective tissue growth factor
CTNNA1	Alpha-catenin
CTNNB1	Beta-catenin
CYR61	Cysteine-rich angiogenic inducer 61
DSP	Desmoplakin
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
GPCR	G-Coupled protein receptor
Hpo	Hippo
HPV-16	Human papillomavirus-16
ICM	Inner cell mass
JUP	Plakoglobin
KO	Knockout
LatB	Latrunculin B
LATS1/2	Large tumor suppressor kinases 1/2
LPA	Lysophosphatidic acid
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MAX	Myc-associated factor X
mESC	Mouse embryonic stem cells
MOB1	Mob1 Homolog
MST/12	Mammalian STE20-like kinases 1/2
mTSC	Mouse trophoblast stem cells
NF-κB	Nuclear factor light chain enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
PARP	Poly-ADP ribose polymerase
PKA	Protein kinase A
PKC	Protein kinase C
ROCK	Rho-associated protein kinase
SAV1	Salvador Homolog

Sd	Scalloped
SRC1	Steroid receptor coactivator 1
SRF	serum response factor
STAT	Signal transducer and activator of transcription
TAZ	Transcriptional activator with PDZ binding domain
TCF4	Transcription factor 4
TE	Trophectoderm
TEAD	TEA/ATTS domain
TGFβ	Transforming growth factor beta
Tgi	Tondu-domain-containing growth inhibitor
UM	Uveal melanoma
Vg	Vestigial
VGLL	Vestigial-like protein
YAP	Yes-associated protein
Yki	Yorkie

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FIELDS OF STUDY

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ABSTRACT OF THE DISSERTATION

Regulation of the Hippo Pathway Transcription Factor TEAD

by

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Doctor of Philosophy in Biomedical Sciences

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The Hippo pathway is a master regulator of tissue growth and homeostasis. As such, the Hippo pathway plays important roles in processes such as cell proliferation, growth, differentiation, and survival. In the past few decades, a slew of studies has characterized a network of proteins that crosstalk with the Hippo pathway, the signals regulating the Hippo pathway, and the biological outputs of the pathway in development, tissue regeneration, and cancer. The transcriptional components of the Hippo pathway

consist of transcription co-activators YAP and TAZ as well as transcription factor family TEAD. Past studies of the Hippo pathway largely focus on regulation of the Hippo transcription co-activators YAP and TAZ and studies characterizing mechanisms of TEAD regulation are largely lacking. Thus, studies uncovering new mechanisms of TEAD regulation will provide insight into Hippo signaling and Hippo related pathogeneses.

We demonstrate that upon environmental stresses, such as osmotic stress and high cell density, TEAD localization can be dynamically regulated. Upon osmotic stress, TEAD is translocated to the cytoplasm through direct binding with p38 through its D domain. Importantly, this cytoplasmic translocation of TEAD can override YAP/TAZ activating signals and inhibit Hippo signaling output. Furthermore, in YAP/TAZ driven cancer cell lines, TEAD cytoplasmic translocation can inhibit cell growth *in vitro* and *in vivo*.

TEAD cytoplasmic translocation is also seen upon tissue repair and in response to extracellular matrix (ECM) composition. High cell density promoted TEAD cytoplasmic localization can be reversed by inducing a wound in the cell monolayer. This cytoplasmic-nuclear translocation is also seen in an intestinal injury model. Furthermore, TEAD nuclear accumulation occurs in response to composition of ECM proteins, namely laminin and fibronectin. TEAD nuclear localization in response to fibronectin is mediated through FAK/Src signaling. Here we show several Hippo independent mechanisms of TEAD regulation and provide evidence that regulation of TEAD is an alternative mechanism of regulating Hippo signaling output.

Chapter 1: Introduction to the TEAD family of transcription factors and its implications in cancer

Studies of the TEAD (TEA/ATTS domain) transcription factor family began with the identification of TEAD1 which was first discovered in an attempt to identify nuclear proteins that could bind to the SV40 enhancer and activate transcription(Xiao et al 1987). Further studies showed that TEAD could not only bind to GT-IIC and Sph motifs on the SV40 enhancer but also human papillomavirus-16 (HPV-16) oncogenes(Davidson et al 1988, Ishiji et al 1992, Xiao et al 1991) and M-CAT motifs(Azakie et al 1996). Since their initial discovery, TEADs have been found to be evolutionarily conserved, and have been shown to play important roles in various biological processes and human disease (Jin et al 2011, Pobbati & Hong 2013).

Mammals express four *TEAD* genes, *TEAD1-4*. TEADs are broadly expressed but each TEAD has tissue specific expression, which indicates tissue specific roles for each TEAD(Anbanandam et al 2006, Azakie et al 1996, Jacquemin et al 1996, Jacquemin et al 1997). In particular, TEADs have been shown to play important roles in development, with activity detected at the two-cell embryo stage and maintained for cardiogenesis(Chen et al 1994), neural crest and notochord development(Kaneko et al 2007, Sawada et al 2008), and trophectoderm lineage determination(Yagi et al 2007). *Tead1* null mice are embryonic lethal due to defective maturation during cardiac development(Chen et al 1994). Knockout of *Tead2* in mice leads to defects in neural development with an increased risk for defects in neural tube closure(Kaneko et al 2007). In contrast, another study showed redundant functions for TEAD1 and TEAD2;

Tead2 null mice showed no phenotype but knockout of both *Tead1* and *Tead2* was embryonic lethal with embryos lacking a closed neural tube, notochord, and somites(Sawada et al 2008). *Tead4* null mice are also embryonic lethal due to failure in embryo implantation, however, disruption of *Tead4* after embryo implantation results in normal development(Nishioka et al 2008, Yagi et al 2007). In humans, an inactivating missense mutation of *TEAD1* (Y421H) is associated with Sveinsson's chorioretinal atrophy, a genetic disorder that results in degeneration of the choroid and retina (Fossdal et al 2004, Kitagawa 2007).

TEADs seem to have important biological functions, but studies thoroughly characterizing TEAD function and regulation are lacking. Our knowledge of TEADs has developed largely from work that focuses on TEADs in the context of the Hippo pathway signaling (Meng et al 2016). TEAD transcriptional activity is broadly believed to be regulated by the presence or the absence of nuclear YAP/TAZ. However, accumulating evidence shows that TEAD itself is regulated through other mechanisms.

1.1 Regulation of TEAD by coactivators

When TEADs were identified, they were found to have little transcriptional activity by themselves and were predicted to require the presence of coactivators to induce target gene transcription (Xiao et al 1991). TEAD proteins have an N terminal TEA/ATTS domain which binds to DNA as a homeodomain fold and a C terminal transactivation domain with which coactivators bind in order to transcribe target genes (Anbanandam et al 2006, Burglin 1991, Jiao et al 2014, Vassilev et al 2001). The

TEA/ATTS domain of TEAD is highly conserved in all TEAD family members and recognizes the sequence motif 5′-GGAATG-3′ (Anbanandam et al 2006, Farrance et al 1992, Xiao et al 1987). The C terminal transactivation domain of TEAD is also highly conserved, especially residues that are necessary for coactivator binding (Chen et al 2010, Li et al 2010). The structure of the TEA domain of TEAD4 bound to DNA has recently been resolved and shows that the α3 helix formed by the TEA domain is the most important interface for DNA binding. While mutations at residues Ser100 and Gln103 completely abolished TEAD4's DNA binding ability, mutations at other interface residues did not significantly inhibit DNA binding ability. The flexibility of these mutated residues indicates that TEAD-DNA binding sites may be diverse and specificity may be regulated by binding of different coactivators (Shi et al 2017). Several TEAD binding proteins and cofactors have been identified and are discussed in the sections below.

Hippo-dependent coactivators

The Hippo pathway is a regulator of cell growth, proliferation and homeostasis and has been shown to be essential in development, stem cell function, and tissue regeneration(Johnson & Halder 2014, Mo et al 2014). In recent years, studies have revealed a vast array of regulators upstream of the Hippo pathway(Hansen et al 2015), however, the main components are comprised of serine/threonine kinases Mammalian STE20-like kinases (MST1/2), mitogen activated protein kinase kinase kinase kinases (MAP4Ks), Large Tumor Suppressor kinases (LATS1/2), and transcription co-activators Yes-associated protein (YAP) and its paralog Transcriptional Activator with PDZ binding domain (TAZ). When complexed with adaptor protein Salvador Homolog (SAV1),

MST1/2 phosphorylate and activate LATS1/2 and their adaptor protein Mob1 Homolog (MOB1) (Callus et al 2006, Praskova et al 2008, Tapon et al 2002, Wu et al 2003). LATS1/2 have also been shown to be phosphorylated by the MAP4K4 family(Meng et al 2015). Phosphorylation of YAP/TAZ by activated LATS1/2 results in cytoplasmic sequestration due to binding to 14-3-3 or ubiquitinylation and degradation of YAP/TAZ (Hao et al 2008, Lei et al 2008, Liu et al 2010, Zhao et al 2010, Zhao et al 2007). When the Hippo pathway is turned off, LATS1/2 are inactive, YAP/TAZ are dephosphorylated and accumulate in the nucleus where they bind to TEAD to drive expression of target genes such as CTGF and Cyr61 (Lai et al 2011, Zhao et al 2008).

The Hippo pathway transcriptional coactivators, YAP and its paralog TAZ, were among the cofactors identified (Mahoney et al 2005, Vassilev et al 2001) and are now the most well-established activators of TEAD. When phosphorylated by LATS 1/2, YAP/TAZ are localized in the cytoplasm and incapable of binding TEAD, thus rendering TEAD transcriptionally inactive. Upon dephosphorylation, YAP/TAZ are translocated to the nucleus to bind TEAD and drive transcription of target genes that are critical for cell growth, proliferation, and survival (Lai et al 2011, Lei et al 2008, Zhao et al 2008) (Figure 1.1). Structural studies show that the TEAD binding domain of YAP is located in the protein's N terminus, while the YAP binding region is located in the C terminal, transactivation domain of TEAD. One molecule of YAP and one molecule of TEAD bind to form a heterodimer complex. The N-terminus of YAP wraps around the globular C-terminal structure of TEAD and binds through three major interfaces (Chen et al 2010, Li et al 2010). Importantly, mutations at Y421 of TEAD1, found in Sveinsson's

chorioretinal atrophy, were discovered to disrupt a hydrogen bond that is essential in mediating TEAD-YAP interaction (Chen et al 2010, Fossdal et al 2004, Kitagawa 2007, Li et al 2010). Residues critical for the interactions are evolutionarily conserved on both YAP and TEAD (Chen et al 2010, Li et al 2010). TAZ and TEAD binding has been shown to have two conformations. In one conformation, one molecule of TAZ binds to one molecule of TEAD forming a heterodimer similar to that of YAP-TEAD. In a second conformation, two molecules of TAZ bind two molecules of TEAD. In addition, the two TAZ molecules interact with each other to bridge the two TEAD molecules, forming a heterotetramer complex (Kaan et al 2017). Further studies are needed to validate the physiological and functional significance of the difference in YAP-TEAD and TAZ-TEAD complexes. However, human interactome studies show interaction between TEADs, suggesting that homo- and hetero- complexes may differentially regulate TEAD transcriptional activity (Huttlin et al 2017, Huttlin et al 2015). As TEAD is the major transcriptional partner of YAP/TAZ (Chan et al 2009, Zhang et al 2009, Zhao et al 2008), Hippo-regulated YAP/TAZ nuclear-cytoplasmic shuttling has served as a proxy for regulation of TEAD activity.

Hippo-independent coactivators

Though YAP/TAZ are currently the most well-studied coactivators and regulators of TEAD transcriptional activity, several other cofactors have been identified as TEAD binding partners. The Vestigial-like (VGLL) protein family consists of four members, VGLL1-4. VGLL has been shown to interact with TEAD to regulate gene expression (Chen et al 2004a, Chen et al 2004b, Gunther et al 2004, Koontz et al 2013). Studies

show that VGLL family proteins have binding sites on TEAD that overlap with YAP/TAZ binding sites and thus compete with YAP/TAZ for TEAD binding (Jiao et al 2014, Pobbati et al 2012). Binding of VGLL4 to TEAD inhibits YAP/TAZ-TEAD target gene expression and suppresses tumor growth (Jiao et al 2014, Zhang et al 2014). In contrast to VGLL4, overexpression of VGLL1 promoted anchorage-independent cell growth and upregulated target genes different from that of YAP/TAZ-TEAD4 (Pobbati et al 2012). Suppression of canonical YAP/TAZ target genes was not analyzed with overexpression of VGLL1, however as VGLL1 was shown compete with YAP/TAZ for TEAD binding, upregulation of VGLL1 target genes by VGLL1 overexpression likely suppresses YAP/TAZ target genes. Though there are a few studies implicating the functional role of VGLL and TEAD (Chen et al 2004a, Chen et al 2004b, Gunther et al 2004, Jiao et al 2014, Koontz et al 2013), further studies are needed to understand the opposing effects on cell growth by VGLL1 and VGLL4. It is not clear if all VGLL proteins broadly compete with YAP/TAZ for TEAD binding or if under different physiological contexts TEAD preferentially binds to different VGLL proteins to carry out YAP/TAZ independent cellular functions. However, binding of VGLL to TEAD occupies the YAP/TAZ-TEAD binding site and prevents TEAD from binding to YAP or TAZ, thereby inhibiting YAP/TAZ-TEAD specific transcriptional activity (Figure 1). As in the case with VGLL1, TEADs may not only regulate YAP/TAZ-driven target gene expression, but under different contexts may mediate transcriptional output of YAP/TAZ independent signaling pathways. In line with this notion, TEAD4 was recently implicated in the regulation of Wnt target genes. TEAD4 directly interacts with transcription factor 4 (TCF4) through its TEA domain to facilitate transactivation of TCF4 and mediate

expression of Wnt target genes. VGLL4 binding to TEAD4 inhibits TEAD4-TCF4 driven target gene expression as it does for TEAD-YAP/TAZ target gene expression, but does not compete with TCF4 for TEAD binding. Instead, VGLL4 inhibition of TEAD4-TCF4 transcriptional activity is due to formation of a TEAD4/TCF4/VGLL4 ternary complex (Jiao et al 2017). IFN-γ signaling has also been shown to inhibit cell proliferation by inhibiting TEAD activity. IFN-γ promotes the binding of guanylate-binding protein 1 (GBP-1) and TEAD via TEAD's DNA binding domain (Unterer et al 2018). The p160 family of steroid receptor coactivators was also identified to interact with TEAD. In a yeast two-hybrid screen using the bHLH-PAS domain of steroid receptor coactivator 1 (SRC1), TEAD was identified as an interacting partner (Belandia & Parker 2000). Moreover, all members of the p160 family could potentiate TEAD transcriptional activity (Belandia & Parker 2000). In recent studies activator protein-1 (AP-1) was demonstrated to directly interact with TEAD (Liu et al 2016a, Zanconato et al 2015). AP-1 was also shown to co-occupy the same chromatin sites as TEAD and presence of AP-1 was necessary to activate target genes important for tumor growth and progression(Liu et al 2016a, Zanconato et al 2015). Other cofactors identified include poly-ADP ribose polymerase (PARP) (Butler & Ordahl 1999), serum response factor (SRF) (MacLellan et al 1994), myocyte enhancer factor 2 (MEF2) (Maeda et al 2002), and myc-associated factor X (MAX) (Gupta et al 1997). These cofactors have been shown to aid TEAD transcriptional activity and regulate the transcriptional program necessary for muscle homeostasis and differentiation(Butler & Ordahl 1999, Gupta et al 1997, MacLellan et al 1994, Maeda et al 2002). Although many TEAD interacting proteins have been implicated, it is clear that YAP/TAZ are the most important in

stimulating TEAD transcriptional activity as binding of YAP/TAZ potently enhances TEAD reporter activity by several hundred folds. Moreover, inhibition of YAP/TAZ by either knockdown or knockout strongly abolishes endogenous expression of TEAD target genes (Zhao et al 2008).

1.2 Regulation of TEAD by post-translational modifications

The regulation of TEAD through binding of coactivators has, until now, been the primary mechanism of modulating TEAD transcriptional activity. However, recent studies suggest that TEAD transcriptional activity is also regulated by post-translational modifications as well as changes in subcellular localization.

Phosphorylation

In cardiac myocytes, an overlapping Max binding, E-box motif and a TEAD binding, M-CAT motif were identified on the α-myosin heavy chain (α-MHC) promoter, a promoter that is responsible for cAMP-induced gene expression (Gupta et al 1994). This hybrid motif, found in several muscle specific genes, is regulated by a TEAD1-MAX complex (Gupta et al 1997). Though interaction with MAX regulates TEAD1 by potentiating target gene expression (Gupta et al 1997), TEAD1-MAX target gene expression is also regulated by TEAD1 phosphorylation (Gupta et al 2000). Protein kinase A (PKA) phosphorylation of TEAD1 at serine 102 inhibited TEAD1 DNA binding ability but did not disrupt TEAD1-MAX interaction (Gupta et al 2000). In addition to phosphorylation by PKA, TEAD has also been shown to be a phosphorylation substrate

of Protein Kinase C (PKC) (Jiang et al 2001). Phosphorylation of TEAD by PKC also resulted in a decrease in its DNA binding ability (Jiang et al 2001). Thus, phosphorylation is an alternative mechanism of modulating TEAD activity independent of interaction with coactivators.

Palmitoylation

Recent studies have identified S-palmitoylation as a post-translational modification of the TEAD family and that the palmitoylation of TEADs regulates protein stability (Noland et al 2016) and transcriptional activity (Chan et al 2016). Interestingly, TEAD palmitoylation is autocatalytic as the abundance of palmitoylation increased significantly with the addition of palmitoyl-CoA to purified TEAD in vitro, despite the absence of palmitoyltransferases (Chan et al 2016). However, it is possible that palmitoylation of TEAD may require palmitoyltransferase in vivo because bacterially expressed TEAD is not efficiently palmitoylated. Three cysteine residues, conserved among TEADs, were identified as sites of palmitoylation. Mutation of any one cysteine residue on TEAD1 decreased palmitoylation while mutations at all three residues completely ablated TEAD1 palmitoylation (Chan et al 2016). Functionally, S-palmitoylation of TEAD1 is important for YAP/TAZ binding and transcriptional activity. Palmitoylation deficient mutant TEAD1 showed a substantial decrease in YAP binding, diminished transcriptional activity as assessed by a TEAD reporter assay, and inhibited C2C12 myoblast cell differentiation by blocking expression of muscle differentiation genes (Chan et al 2016). Interestingly, despite loss of YAP binding, the TEAD1 palmitoylation mutants retained VGLL4 binding ability (Chan et al 2016). Disruption of

TEAD2 palmitoylation decreased protein stability and resulted in a significant loss of TEAD2 protein abundance (Noland et al 2016). Palmitoylation is important for protein trafficking and membrane localization (Resh 2006), however, palmitoylation of TEAD does not affect TEAD localization or membrane binding (Chan et al 2016, Noland et al 2016). Consistently, the palmitoyl group is buried inside a deep hydrophobic pocket of TEAD as revealed by structure studies. It is still unknown whether TEAD palmitoylation is a dynamic process and whether mechanisms of TEAD depalmitoylation may be manipulated to regulate TEAD coactivator binding and transcriptional activity.

1.3 Regulation of TEAD during embryonic differentiation

Mouse knockout studies showed that *Tead4* is specifically required for embryo implantation and trophectoderm lineage determination (Nishioka et al 2008, Yagi et al 2007). To elucidate how TEAD4 regulates trophectoderm (TE) and inner cell mass (ICM) lineage in the preimplantation mouse embryo, Home et al. performed ChIP-seq to determine TEAD4 target genes in mouse trophoblast stem cells (mTSCs) and preimplantation mouse embryos. TEAD4 was shown to directly regulate a trophectoderm specific transcriptional program that included genes such as *Gata3* and *Cdx2*. Although TEAD4 was found to be expressed in both the TE and the ICM, *Gata3* and *Cdx2* were not expressed in the mouse ICM or ICM-derived mouse embryonic stem cells (mESCs). Interestingly, TEAD4 was found to be localized exclusively in the cytoplasm of mESCs compared to mTSCs in which TEAD4 was enriched in the nucleus. Importantly, YAP remained nuclear in both mESCs and mTSCs, indicating that regulation of TEAD by subcellular localization is the primary mechanism of TE and ICM

cell lineage determination. In human ESCs that were induced to a trophoblast fate, TEAD4 was found to localize to the nucleus along with an increase of GATA3 expression. Forced expression of nuclear TEAD4 in the inner blastomeres of a developing embryo activated CDX2 and inhibited proper blastocyst formation. At different developmental stages of the embryo, TEAD4 nuclear localization correlated with TE lineage cells expressing CDX2 while TEAD4 cytoplasmic localization correlated with ICM lineage cells. This TEAD4 expression pattern was conserved across various mammalian species, including human. The data suggests that TEAD subcellular localization regulates its transcriptional activity, turning TE-specific transcriptional programs on or off to determine specification of TE vs. ICM lineage differentiation for embryo maturation (Home et al 2012). However, neither the signal nor the molecular mechanism that regulate the subcellular localization of TEADs in embryos is known.

1.4 Regulation of *Drosophila* homolog of TEAD, Scalloped

Studies of the Hippo pathway in drosophila have characterized Scalloped (Sd), the homolog of TEAD, to bind to Yorkie (Yki), the homolog of YAP, and mediate the growth regulatory effects of the Hippo pathway (Goulev et al 2008, Huang et al 2005, Wu et al 2008, Zhang et al 2008). Interestingly, when analyzing *sd;yki* double mutant clones, loss of *sd* rescued *yki* mutant undergrowth phenotypes in the eye and in ovarian follicle cells (Koontz et al 2013). This observation indicates that Sd has a repressor function when not bound with Yki. Thus, Yki may promote normal tissue growth by relieving the default repressor activity of Sd. Furthermore, Tondu-domain-containing

growth inhibitor (Tgi), the homolog of VGLL4, was identified as a cofactor mediating Sd default repressor function (Koontz et al 2013). Vestigial (Vg) the homolog of VGLL1, did not induce Sd repressor function (Koontz et al 2013). Currently, it is unclear whether this mechanism of default repression by Sd is conserved in mammalian TEADs. In addition, Hippo (Hpo), the homolog of MST1/2, promotes cytoplasmic translocation of Sd to suppress Sd-Vg mediated proliferation in the wing, independently of Yki (Cagliero et al 2013).

1.5 TEAD in Cancers

Numerous studies have suggested the importance of Hippo signaling in the development of cancer. These studies have emphasized the role of YAP/TAZ amplification and hyperactivity in various cancers (Moroishi et al 2015), however increased TEAD expression and activity, both dependent and independent of YAP/TAZ, have also been implicated in the progression of several solid tumors (Pobbati & Hong 2013) (Table 1). High TEAD expression levels are seen in prostate, colorectal, and breast cancers and, concordantly, are an indicator of poor clinical outcome (Diepenbruck et al 2014, Han et al 2008, Knight et al 2008, Liu et al 2016b, Richardson et al 2006). In breast cancer cells, induction of epithelial to mesenchymal transition (EMT) resulted in upregulation of TEAD2 and a marked increase in YAP/TAZ nuclear accumulation despite decreases in overall YAP/TAZ protein levels (Diepenbruck et al 2014). The increase in TEAD2 expression resulted in increased YAP/TAZ binding which retained YAP/TAZ in the nucleus and drove TEAD transcriptional activity (Diepenbruck

et al 2014). TEAD2 and TEAD4 were also found to be overexpressed in colorectal cancer, particularly in metastatic tissues, and knockdown of TEAD4 in vitro and in vivo reduced cell migration and metastasis (Liu et al 2016b). Furthermore, the increase in metastatic potential in colorectal cancer was YAP independent as both wild-type TEAD4 and the YAP-binding deficient Y429 TEAD4 mutant rescued the effects of TEAD4 knockdown(Liu et al 2016b). TEAD1 has been reported to play a role in conferring resistance to apoptosis in a YAP independent manner (Landin Malt et al 2012). Additionally, TEAD1 was shown to regulate mesothelin, a gene that serves as a cancer biomarker due to its overexpression in many tumors (Hucl et al 2007). The importance of TEAD-driven transcriptional programs has further been highlighted in several recent studies. ChIP-seq studies have shown that TEAD binds not only promoters but also distal enhancer elements (Hu et al 2016, Liu et al 2016a, Zanconato et al 2015). Binding of YAP/TAZ-TEAD and AP-1 to enhancers synergistically activates target genes important for oncogenic growth, invasion, and migration in vitro and in vivo (Liu et al 2016a, Zanconato et al 2015). In pre-B cells, YAP-TEAD binds superenhancer networks and contributes to aberrant pre-B cell phenotypes (Hu et al 2016). TEADs have also been reported to drive the transcriptional program responsible for increased invasiveness and resistance to MAPK inhibition in melanomas (Verfaillie et al 2015) (Table 1.5).

Due to the important roles TEAD plays in cancer development and progression, inhibition of TEAD activity in cancers via small molecules and peptides has shown some efficacy in treating cancer in vivo and in vitro. Structural studies of TEAD reveal a

central hydrophobic pocket in the transactivation domain (Pobbati et al 2015). Flufenmate drugs were found to bind in this hydrophobic pocket and inhibit TEAD transcriptional activity, without disrupting YAP-TEAD interaction, leading to decreases in cell migration and proliferation (Pobbati et al 2015). Palmitoylation of TEAD occurs in this hydrophobic pocket suggesting that flufenmate drugs, despite a low binding affinity, may inhibit TEAD activity by displacing TEAD palmitoylation (Chan et al 2016, Noland et al 2016). In YAP/TAZ driven cancers, studies have explored the effects of disrupting YAP/TAZ-TEAD interaction. Treatment with a VGLL4 mimicking peptide was found to inhibit gastric cancer growth in vitro and in vivo by outcompeting YAP for TEAD binding (Jiao et al 2014). Verteporfin, a small molecule found to inhibit YAP-TEAD interaction, also suppressed cancer cell growth (Liu-Chittenden et al 2012). Thus, these attempts at inhibiting TEAD activity show that development of TEAD inhibitors is feasible and is a promising therapeutic strategy for cancer treatment.

1.6 Concluding remarks

TEADs play an important role in development, differentiation, cell growth and proliferation, and tumorigenesis. Though the activity of TEAD is traditionally thought to be regulated through coactivator binding, with the majority of studies placing an emphasis on YAP/TAZ, several studies demonstrate Hippo independent mechanisms of TEAD regulation. Post-translational modifications such as phosphorylation and palmitoylation have been shown to effect TEAD DNA binding ability, protein stability, and coactivator interaction (Chan et al 2016, Noland et al 2016, Yu et al 2013). In addition, changes in TEAD subcellular localization represent an important mechanism to

modulate TEAD transcriptional activity in a Hippo-independent manner (Home et al 2012) (Figure 1.6). Though exciting progress has been made towards understanding TEAD regulation, many key questions remain to be answered (see Outstanding Questions). Future work that elucidates mechanisms of TEAD regulation may be important in developing therapeutic options for cancers, particularly those that rely heavily on TEAD transcriptional activity.

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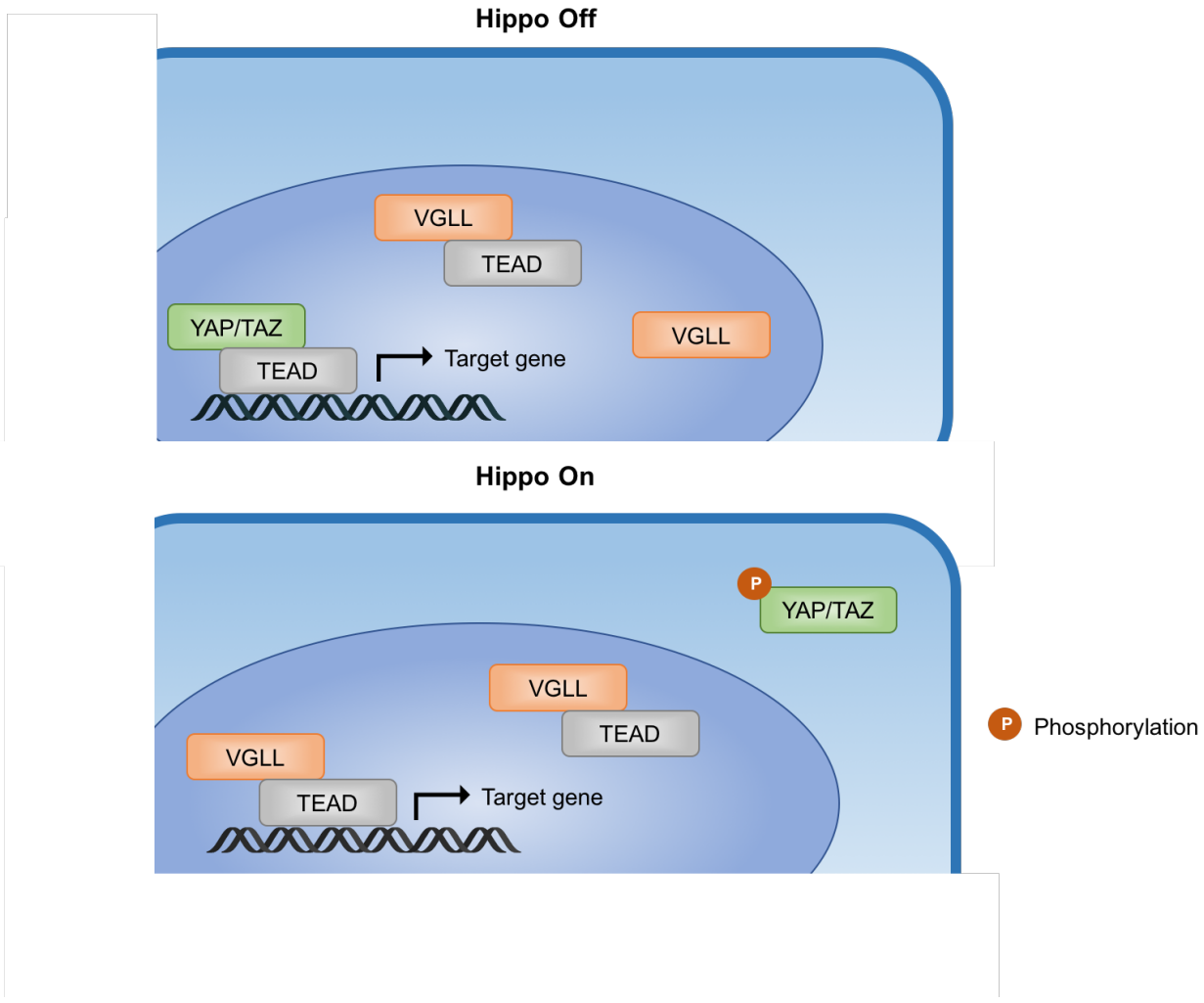

Figure 1.1: Coactivator binding stimulates TEAD transcriptional activity.

VGLL competitively bind TEAD to regulate its transcriptional activity. When the Hippo pathway is “off” YAP/TAZ are dephosphorylated and translocated to the nucleus to bind TEAD and activate transcription of downstream target genes. Abundance of nuclear YAP/TAZ outcompetes VGLL-TEAD binding. When the Hippo pathway is “on”, YAP/TAZ are phosphorylated and sequestered in the cytoplasm. Absence of YAP/TAZ in the nucleus allows VGLL-TEAD binding.

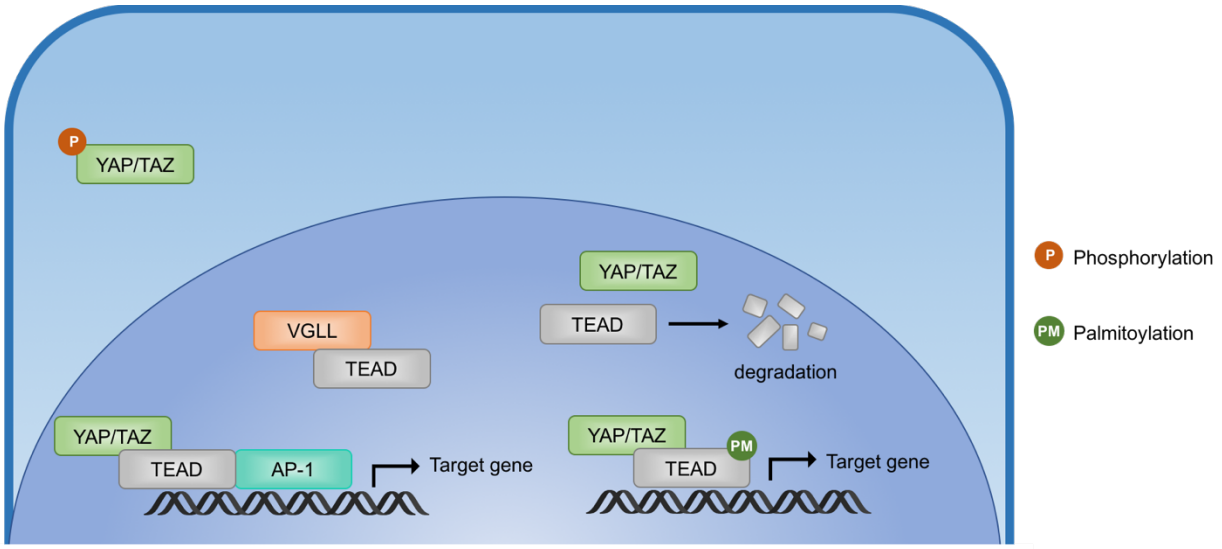

Figure 1.2: Mechanisms of TEAD Regulation

Several mechanisms have been shown to regulate TEAD transcriptional activity. Coactivator binding is the most important mechanism of altering TEAD transcriptional activity. YAP/TAZ bind TEAD along with AP-1 to activate transcription of downstream target genes. The transcriptional program driven by the YAP/TAZ, TEAD, AP-1 complex has been shown to be important for cancer progression. VGLL has been shown to compete with YAP/TAZ for TEAD binding. Availability of and competition between coactivators drive different TEAD transcriptional programs. Palmitoylation of TEAD in the central hydrophobic pocket is necessary for protein stability and is also suggested to be important for YAP binding

Table 1.1: TEADs in disease

Disease	Gene	Alteration	Effects	Target Gene	References
Sveinsson's chorioretinal atrophy	TEAD1	Y421H inactivating mutation	choroidal and retinal degeneration		(Fossdal et al 2004)
Breast cancer, squamous cell carcinoma	TEAD4	increased expression	increased cell proliferation and tumorigenesis, decreased survival		(Liu et al 2016a)
Prostate cancer	TEAD1	increased expression	decreased survival		(Knight et al 2008)
Breast cancer	TEAD4	increased expression			(Han et al 2008, Richardson et al 2006)
Breast cancer	TEAD2	increased expression	EMT	zyxin	(Diepenbruck et al 2014)
Colorectal cancer	TEAD2, TEAD4	increased expression	EMT, correlated with decreased survival	vimentin	(Liu et al 2016b)
Colorectal cancer	TEAD1		resistance to apoptosis	livin	(Landin Malt et al 2012)
Pancreatic cancer	TEAD1	increased expression		mesothelin	(Hucl et al 2007)
Melanoma	TEAD4	increased invasiveness, resistance to MAPK inhibition			(Verfaillie et al 2015)

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Chapter 2: Regulation of Hippo pathway transcription factor TEAD by p38 MAPK-induced cytoplasmic translocation

2.1 Introduction

The Hippo pathway controls organ size, and tissue homeostasis with deregulation leading to cancer. The core hippo components in mammals are composed of upstream serine/threonine kinases Mst1/2, MAPK4Ks, and Lats1/2. Inactivation of these upstream kinases leads to dephosphorylation, stabilization, nuclear translocation, and thus activation of the major functional transducers of the Hippo pathway, YAP and its paralog TAZ(Johnson & Halder 2014, Yu et al 2015). YAP/TAZ are transcription co-activators that regulate gene expression primarily through interaction with TEA domain DNA-binding family of transcription factors (TEAD)(Zhao et al 2008). The current paradigm for regulation of this pathway centers on phosphorylation-dependent nucleocytoplasmic shuttling of YAP/TAZ through a complex network of upstream components(Yu et al 2015). However, unlike other transcription factors, such as SMAD, NF-κB, NFAT, and STAT, the regulation of TEAD nucleocytoplasmic shuttling has been largely overlooked. In the present study, we show that environmental stress promotes TEAD cytoplasmic translocation via p38 MAPK in a Hippo-independent manner. Importantly, stress-induced TEAD inhibition predominates YAP activating signals and selectively suppresses YAP-driven cancer cell growth. Our data reveal a mechanism governing TEAD nucleocytoplasmic shuttling and show that TEAD localization is the final determinant of Hippo signaling output.

2.2 Results

p38-mediates stress-induced TEAD cytoplasmic translocation.

We set out to identify signals that may regulate TEAD subcellular localization by focusing on conditions known to inhibit YAP/TAZ such as serum starvation(Yu et al 2012), energy stress by glucose starvation(Mo et al 2015, Wang et al 2015), PKA activation by forskolin(Yu et al 2012), disruption of the actin cytoskeleton by latrunculin B(Dupont et al 2011, Zhao et al 2012), Src inhibition by dasatinib(Kim & Gumbiner 2015), and inhibition of mevalonate synthesis by cerivastatin(Sorrentino et al 2014). These well-known YAP/TAZ inhibitory stimuli indeed induced YAP/TAZ cytoplasmic localization, but failed to alter TEAD subcellular localization (Fig. 2.1a). In contrast, environmental stresses such as osmotic stress, high cell density and cell detachment induced cytoplasmic translocation of TEAD and YAP/TAZ (Fig. 2.1b and Supplementary Fig. 2.1a, b), demonstrating that only a subset of signals that induce YAP/TAZ cytoplasmic localization are capable of driving TEAD cytoplasmic localization.

The p38 MAP kinase is activated by stress, including hyperosmotic conditions; therefore, we examined whether p38 plays a role in regulation of TEAD during stress. Treatment with p38 inhibitors (SB203580 or PH797840) blocked osmotic stress-induced, but not high density-induced, TEAD cytoplasmic localization, indicating that p38 is specifically involved in TEAD cytoplasmic translocation upon osmotic stress (Fig. 2.1c and Supplementary Fig. 2.1c). Activation of p38 by ectopic expression of p38 and its upstream kinase MKK3 also induced cytoplasmic translocation of TEAD and this effect was blocked by p38 inhibitor treatment (Fig. 2.1d and Supplementary Fig. 2.1d).

We predicted that all four isoforms of p38 may play compensatory roles as ablating TEAD translocation required concentrations of p38 inhibitor which were sufficient for inhibiting all p38 isoforms (Supplementary Fig. 2.1e). Deletion of p38α/β (p38 2KO) resulted in p38 upregulation and did not impede TEAD cytoplasmic translocation (Supplementary Fig. 2.1f, g), further supporting the pharmacological evidence that all four isoforms of p38γ/δ play a role in TEAD regulation. When all four p38 genes were deleted in the p38$\alpha/\beta/\gamma/\delta$ knockout (KO) (p38 4KO) cells, TEAD localization was insensitive to osmotic stress and largely retained in the nucleus (Fig. 2.1e, f and Supplementary Fig. 2.1f, h). Under basal conditions, deletion of p38 had no effect on TEAD localization and marginally increased YAP-TEAD activity, indicating that p38 plays a role in regulation of TEAD mainly under conditions of cellular stress. (Supplementary Fig. 2.1i, j). The specific role of p38 in osmotic stress is further supported by the result that p38 inhibition or knockout had no effect on density-induced TEAD cytoplasmic localization (Supplementary Fig. 2.1c). Collectively, the above observations demonstrate a critical role of p38 in stress-induced TEAD nucleocytoplasmic translocation. Consequently, TEAD cytoplasmic translocation by osmotic stress suppressed YAP/TAZ target gene expression induced by YAP-activating signals, such as lysophosphatidic acid (LPA) and serum(Yu et al 2012), which was rescued by p38 inhibition (Fig. 2.1g, h). The NaCl-induced cytoplasmic localization of TEAD was slower than p38 phosphorylation and activation (Fig. 2.1i, j) but occurred concurrently with p38 dephosphorylation and cytoplasmic translocation (Fig. 2.1k). It is well established that post osmotic stress, p38 undergoes dephosphorylation and

cytoplasmic translocation, indicating that TEAD cytoplasmic translocation occurs during the adaptation phase of stress response(de Nadal et al 2011).

p38 mediates stress-induced TEAD cytoplasmic translocation via protein-protein interaction.

To further gain mechanistic insight into TEAD regulation by p38, we investigated the role of p38 protein-protein interaction and kinase activity, both of which are critically involved in p38 signal transduction(Cargnello & Roux 2011). We sought to determine whether p38 directly interacts with TEAD to promote cytoplasmic translocation. Interestingly, osmotic stress induced endogenous TEAD-p38 interaction, whereas serum-induced TEAD-YAP interaction was abolished by osmotic stress (Fig. 2.2a-c). In addition, exogenous p38 and MKK3 both showed interaction with TEAD (Fig. 2.2d). Using bacterially-purified proteins in an *in vitro* binding assay, we show TEAD can interact directly with p38 without scaffold proteins (Fig. 2.2e). Furthermore, p38 does not bind to YAP and thus regulates TEAD independently of YAP (Fig. 2.2f). The D domain, found in p38 binding partners, serves as a docking site for p38 protein-protein interactions(Cargnello & Roux 2011). We identified a putative D domain that is highly conserved within the TEAD family (Fig. 2.2g). Deletion of the D domain in TEAD abolished TEAD-p38 interaction (Fig. 2.2h). Consistently, p38 CD/ED, a p38 mutant that has lost its ability to interact with D domain-containing substrates(Tanoue et al 2000, Tanoue et al 2001), significantly dampened TEAD-p38 interaction and was unable to induce TEAD cytoplasmic translocation (Fig. 2.2i-l). Ectopic expression of kinase deficient mutant, p38 KM, was also insufficient in binding to TEAD and driving

cytoplasmic translocation (Fig. 2.2m, n). To determine whether TEAD cytoplasmic translocation is due to p38-mediated phosphorylation, we constructed TEAD4-4SP, in which the four putative p38 phosphorylation sites were mutated to alanine (Supplementary Fig. 2.2a). Using an *in vitro* kinase assay, we found TEAD4 to be a poor substrate for p38 phosphorylation with complete ablation of phosphorylation in the TEAD4-4SP mutant, indicating the absence of alternative phosphorylation sites (Supplementary Fig. 2.2b-d). Additionally, TEAD4-4SP displayed normal cytoplasmic translocation upon osmotic stress (Supplementary Fig. 2.2e), suggesting that p38 kinase activity is required for TEAD interaction but does not directly phosphorylate TEAD to regulate its subcellular localization. Disruption of a putative TEAD nuclear export signal, as well as inhibition of Chromosomal Maintenance 1 (CRM1) using Leptomycin B (LMB), largely ablated TEAD translocation, indicating that TEAD cytoplasmic translocation is an active, CRM1-mediated process (Supplementary Fig. 2.2f-h). In contrast to TEAD cytoplasmic localization, osmotic stress stimulates nuclear translocation of the transcription factor Nuclear Factor of Activated T-cells 5 (NFAT5)(Estrada-Gelonch et al 2009), thus TEAD cytoplasmic translocation is a specific cellular response upon osmotic stress (Supplementary Fig. 2.2i).

TEAD cytoplasmic translocation prevents YAP activation.

Next, we tested the effect of stress-induced TEAD cytoplasmic sequestration on YAP activation. Under osmotic stress, YAP activating signals, such as serum and LPA, failed to induce YAP dephosphorylation and nuclear accumulation (Fig. 2.3a, b). ERK phosphorylation, however, was not affected, suggesting specificity of osmotic stress on

YAP inhibition. Unexpectedly, stress evoked TEAD and YAP/TAZ cytoplasmic translocation in MAP4K 4/6/7, Mst1/2 and Lats1/2 KO cells, despite constitutively dephosphorylated YAP (Fig. 2.3c, d and Supplementary Fig. 2.3a-g). These results indicate that stress-induced TEAD and YAP/TAZ cytoplasmic retention is a Hippo-independent process and uncouples YAP dephosphorylation from its nuclear localization. Consistently, p38 inhibition restored TEAD in the nucleus in Lats1/2 KO cells (Fig. 2.3d). Compared to WT cells, p38 inhibition enhanced YAP/TAZ nuclear accumulation and target gene expression in the absence of Lats (Fig. 2.3d, e and Supplementary Fig. 2.3g). To test whether nuclear TEAD is required for YAP nuclear translocation upon activating signals, we generated TEAD1/2/4 KO cells. YAP-activating signals promoted normal YAP dephosphorylation in the TEAD KO cells, but failed to elicit nuclear YAP/TAZ accumulation (Fig. 2.3f, g), suggesting that nuclear localization of TEAD is a prerequisite for proper YAP nuclear localization. No interaction was detected between YAP and p38, indicating that YAP cytoplasmic translocation is a consequence of TEAD regulation by p38 (Fig. 2.2f). Our data suggests that YAP nuclear localization is contingent upon two conditions, dephosphorylation and nuclear localization of TEAD.

TEAD inhibition restricts YAP-driven cancer cell growth.

YAP is highly active in many cancers, particularly in uveal melanoma (UM) and mesothelioma, due to mutations in upstream components of the Hippo pathway(Moroishi et al 2015, Murakami et al 2011, Yu et al 2014). YAP was constitutively hypophosphorylated and nuclear in mesothelioma cells MSTO-211H

(Lats2 mutation) and H2373 (NF2 mutation), even under YAP-inhibitory conditions (Fig. 2.4a, b and Supplementary Fig. 2.4a, b). However, osmotic stress promoted cytoplasmic translocation of both TEAD and YAP/TAZ, and consequently suppressed anchorage-independent growth (Fig. 2.4a-c and Supplementary Fig. 2.4a, b). Importantly, ectopic expression of a fusion of the TEAD DNA binding domain to the VP16 transactivation domain, which is constitutively active and p38 binding deficient, restored colony forming ability of mesothelioma cells (Fig. 2.4c-e Supplementary Fig. 2.4c), suggesting that osmotic stress-induced growth arrest is due to TEAD inhibition. To further examine whether stress-induced TEAD inhibition selectively suppresses YAP-driven cancer cell growth, we compared a series of UM cell lines with mutations in either GNAQ or BRAF. The GNAQ-mutant UM cells are YAP-dependent, while the BRAF-mutant UM cells are YAP-independent (Fig. 2.4f and Supplementary Fig. 2.4d, e)(Yu et al 2014). We observed that osmotic stress evoked p38-dependent cytoplasmic translocation of TEAD and YAP/TAZ in both 92.1 ($GNAQ^{Q209L}$) and OCM1 ($BRAF^{V600E}$) cells (Fig. 2.4g and Supplementary Fig. 2.4d-g). However, TEAD inhibition by osmotic stress or stable expression of p38 preferentially suppressed anchorage-independent growth of GNAQ-mutant UM cell lines, 92.1, OMM2.2, OMM2.3, Mel202, Mel270, but not BRAF mutant UM cell lines, OCM1 and OCM8 (Fig. 2.4h and Supplementary Fig. 2.4h). Consistently, TEAD translocation induced apoptosis specifically in the YAP-driven 92.1 cells but not the YAP-independent OCM1 cells (Fig. 2.4i). Furthermore, promotion of anchorage independent growth by YAP-5SA transformation of MCF10A cells was also stunted by TEAD inhibition (Supplementary Fig. 2.4i). These results indicate that YAP-driven cancer cells are highly susceptible to stress-induced TEAD inhibition. To

further elucidate the role of TEAD inhibition on YAP-driven cancers, MSTO-211H cells were used as an isogenic model. Growth inhibitory effects resulting from stable expression of p38 were rescued by expression of constitutively active TEAD *in vitro* and *in vivo*. (Fig. 2.4j-m and Supplementary Fig. 2.4j). Under physiological osmotic stress, TEAD was cytoplasmic in tubule cells of normal kidney tissue, but was nuclear in malignant renal clear cell carcinoma as well as other normal tissues not exposed to osmotic stress (Supplementary Fig. 2.4k-m). Taken together, our results suggest that regulation of TEAD is important for modulating cancer growth and indicate TEAD as a potential therapeutic target.

2.3 Discussion

In the present study, we report that the Hippo pathway transcription factor TEAD is regulated through nucleocytoplasmic shuttling. TEAD is regulated by different upstream signals with distinct mechanisms as compared to YAP/TAZ. Many signals, such as serum and energy status, which modulate the localization of YAP, have no effect on TEAD localization. We identified certain environmental stresses that can induce cytoplasmic TEAD translocation. In the case of osmotic stress, TEAD cytoplasmic translocation is mediated by p38 MAPK and independent of the Hippo core kinases. It should be noted that osmotic stress initially induces acute nuclear accumulation of YAP (Hong et al 2017, Moon et al 2017), then at later stages, induces cytoplasmic translocation of TEAD, and consequently YAP, as an adaptive response to stress. Mechanistically, osmotic stress-induced cytoplasmic TEAD translocation occurs

via direct protein-protein interaction with p38, independent of Hippo. Disruption of TEAD-p38 interaction abolishes TEAD cytoplasmic translocation, resulting in nuclear retention of transcriptionally active TEAD. Cytoplasmic localization of TEAD is also observed in different cellular contexts, such as cell density that is p38-independent, as well as developmental contexts (Cagliero et al 2013, Home et al 2012). Importantly, stress-induced TEAD inhibition predominates YAP-activating signals by preventing YAP nuclear accumulation, regardless of the phosphorylation status of YAP. Thus, inhibition of TEAD presents a Hippo pathway independent avenue of regulating YAP activity, thereby providing a mechanism of controlling its functional output without targeting Hippo core components Mst and Lats. Moreover, stress-induced TEAD nucleocytoplasmic shuttling is intact in cancer cells that harbor mutations in Hippo pathway upstream components and renders YAP-driven cancer cells highly susceptible to stress-induced growth inhibition. Therefore, pharmacological agents that promote TEAD cytoplasmic localization may be a viable therapeutic strategy for treatment of cancers, especially those with high YAP activity.

2.4 Experimental procedures

Cell Culture

All cell lines were maintained at 37°C with 5% CO2. HEK293A cells were cultured in DMEM (Invitrogen, 11965118) and uveal melanoma and mesothelioma cell lines were cultured in RPMI (Invitrogen, 11875119) containing 10% FBS (Gibco, 10437028) and 50 μg/ml penicillin/streptomycin (Invitrogen, 15140122). MCF10A cells were cultured in DMEM-F12 supplemented with 5% horse serum (Invitrogen,

26050088), 20 ng/ml EGF (Peprotech, AF-100-15), 0.5 μg/ml hydrocortisone (Sigma, H4001-25G), 100 ng/ml cholera toxin (Sigma, C8052-2MG), and 10 μg/ml insulin (Sigma, I1882-100MG). YAP inhibitory signals and environmental stresses included the following: serum starvation (16hr), glucose starvation (2-DG, 25mM, 2hr), PKA activation (forskolin, 10μM, 1hr), disruption of F-actin (latrunculin B, 0.1μg/ml, 1hr), Src inhibition (dasatinib, 5μM, 6hr), inhibition of the mevalonate synthesis (cerivastatin, 2μM, 6hr), NaCl (200mM, 6hr), sorbitol (0.5M, 6hr), high cell density (2 day post-confluent), and cell detachment (1hr). No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. The cell lines were not authenticated. Cells lines were tested and confirmed to be free of mycoplasma.

Induction of osmotic stress and p38 inhibitor treatment

Cells were treated with either 200mM NaCl or 0.5M sorbitol for 6 hr. p38 inhibitors SB203580 (S1076) (40μM) and PH-797840 (S2726) (30μM) were purchased from Selleckchem and cells were treated 2 hr prior to osmotic stress exposure.

Transfection and viral infection

Cells were transfected with plasmid DNA using PolyJet Reagent (Signagen Laboratories) according to manufacturer's protocol. Cells were transfected with (pCDNA3) Flag-p38 or (pCDNA3) HA-p38, (pCDNA3) Flag-MKK3, and (pRK5) Myc-TEAD4. 92.1, OCM1, and MSTO-211H cells stably expressing empty vector; p38 and MKK3; and p38, MKK3, and TEAD1/4-VP16 were generated by retroviral and lentiviral

infection. HEK293T packaging cells were transfected with empty vector, (pHIV puro) p38, (pQCXIH) MKK3, and (pHIV GFP) TEAD1/4-VP16 constructs. 48 hr after transfection, retroviral and lentiviral supernatant was filtered through 0.45 μm filter, supplemented with 8 μg/ml polybrene, and used for infection. 48 hr after infection, cells were selected with puromycin (2μg/mL) and hygromycin (200μg/mL) and FACS sorted for GFP expression.

Animal Work

NU/J (nude mice) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). For tumor xenograft models, MSTO-211H cells ($5x10^6$) were injected subcutaneously into both flanks of 8-12 week old female nude mice. Four mice were assigned to each group. The investigators were not blinded to allocation during experiments and outcome assessment. Tumor height and width were measured with a caliper every 2–3 days to calculate tumor volume (= $width^2$ × height × 0.523). Mice were sacrificed 4 weeks post engraftment. All animal experiments were approved by the University of California San Diego, Institutional Animal Care and Use Committee.

Antibodies

The following antibodies were purchased from Cell Signaling and used at the indicated dilution for western blot analysis, immunohistochemistry, and immunofluorescence: pan-TEAD (13295, 1:1000), p38 MAPK (8690, 1:1000), phospho-p38 MAPK (4511, 1:1000), YAP (14074, 1:1000), TAZ (4883, 1:1000), Lats1 (3477, 1:1000), p-MK2 (3007, 1:1000), p-ERK (4370, 1:1000), DYKDDDDK tag (2368, 1:1000),

Myc tag (2276, 1:1000), p38α (2371, 1:1000), p38β (2339, 1:1000), p38γ (2307, 1:1000), and p38δ (2308, 1:1000). The following antibodies were purchased from Santa Cruz Biotechnology and used at the indicated dilution for western blot analysis and immunofluorescence: YAP (sc-101199, 1:1000), HA (sc-7392, 1:5000), Myc (sc-40, 1:5000), GAPDH (sc-25778, 1:1000). TEAD4 (ab58310, 1:1000) was purchased from Abcam, Flag (A8592, 1:10,000) and vinculin (V9131, 1:5000) was purchased from Sigma, TEAD1 (610923, 1:1000) was purchased from BD Biosciences, Lats2 (A300-479A, 1:1000) was purchased from Bethyl Laboratories, and NFAT5 (bs-9473R OWL 1:1000) was purchased from One World Lab.

Generation of knockout cells and mutagenesis

pSpCas9(BB)-2A-Puro (PX459) was a gift from Dr. Feng Zhang (Addgene plasmid #48139)(Ran et al 2013). The nucleotide guide sequences were designed using the CRISPR design tool at http://www.genome-engineering.org/crispr. Single-guide RNAs (sgRNAs) were cloned into PX459 expression vector. HEK293A cells were transfected using PolyJet DNA in vitro Transfection Reagent according to the manufacturer's instructions. 24 hr post transfection, cells were selected with puromycin for 2–3 days. Following removal of puromycin, cells were allowed to recover in regular growth media for 24 hr before being single-cell sorted by FACs (UCSD; Human Embryonic Stem Cell Core, BDInflux) into a 96-well plate format. Single clones were expanded and screened by protein immunoblotting, genomic sequencing, and functional assays. Lats KO, Mst KO, and MAP4K KO cells were generated as previously described(Meng et al 2015, Park et al 2015).

Guide sequences:

p38α: 5'-3' AGCTCCTGCCGGTAGAACGT

p38β: 5'-3' CCACGCGCGCAGAACGTACC

p38γ: 5'-3' GGACGGCCGCACCGGCGCTA

p38δ: 5'-3' TCCCCGACGCACGTCGGCAG

TEAD1: 5'-3' TGGCAGTGGCCGAGACGATC

TEAD2: 5'-3' AGATAGGTGGGACGCCGGCG

TEAD4: 5'-3' CTCAAGGATCTCTTCGAACG

p38 and TEAD site-directed mutagenesis was carried out using Q5 Hot Start High Fidelity DNA Polymerase from New England Biolabs (M0494) per manufacturer protocol.

RNA extraction, cDNA synthesis and quantitative real-time PCR analysis

Cells were harvested for RNA extraction using RNeasy Plus mini kit (QIAGEN, 74136). RNA samples were reverse-transcribed to complementary DNA (cDNA) using iScript reverse transcriptase (Bio-Rad, 1708891). qRT-PCR was performed using KAPA SYBR FAST qPCR kit (Kapa Biosystems, KK4605) and the 7300 real-time PCR system (Applied Biosystems). Primer sequences used were as previously described(Park et al 2015, Yu et al 2012).

Immunofluorescence microscopy

Cells were seeded in 12 well plates on coverslips 2 days prior to experimentation. Coverslips were pretreated with Poly-L-ornithine solution (Sigma,

P4957) diluted 1:20 at 37°C for 15 mins with a quick phosphate-buffered saline (PBS) wash prior to cell seeding. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, 2280) for 15 min followed by permeabilization with 0.1% Triton-X for 5 mins. Cells were blocked in 3% BSA for 1 hr and incubated overnight at 4°C in primary antibodies diluted in 3% BSA. Secondary antibodies were diluted in 3% BSA and incubated for 1 hr. Slides were mounted with prolong gold antifade reagent with DAPI (Invitrogen, P36931). Each image is a single *Z* section at the same cellular level. Images were captured with a Nikon Eclipse T*i* confocal microscope. Images depicted in figures were exported from NIS elements imaging software.

Immunohistochemistry

Kidney tissue arrays were purchased from U.S. Biomax Inc. Tissues were subject to heat induced antigen retrieval using 10mM sodium citrate buffer followed by 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Sections were incubated overnight at 4°C with pan-TEAD antibody and detected using Vectastain elite ABC kit and DAB Peroxidase Substrate kit (Vector Laboratories) as per manufacturer protocol.

Western blot and immunoprecipitation

Immunoblotting was performed using a standard protocol. Phos-tag reagents were purchased from Wako Chemicals, gels containing phos-tag were prepared according to manufacturer's instructions. For immunoprecipitations, cells were rinsed twice with ice-cold PBS and lysed in ice-cold lysis buffer (0.15M NaCl, 0.05M Tris-HCl,

0.5% Triton X-100, and one tablet each of EDTA-free protease inhibitors (Roche, 11873580001) and phosphatase inhibitor (Thermo Fisher, 88667) per 50 ml). For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation overnight at 4°C. 10 µl magnetic protein A/G beads (Thermo Fisher, 88802) were added and incubated for an additional 2 hr. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of sample buffer and boiling for 5 mins, resolved by 9% SDS-PAGE, and analyzed via Western blot analysis.

In vitro kinase assay

To analyze p38 kinase activity, HEK293A cells were transfected with WT or kinase mutant p38α. p38γ and p38δ were purchased from SignalChem. Cells were collected and p38 was immunoprecipitated as described above. Immunoprecipitates were washed with kinase assay buffer (25mM HEPES pH 7.4, 25mM $MgCl_2$, and 2mM DTT) and subjected to a kinase assay in kinase assay buffer along with 500µM ATP-γ-S. GST–ATF2, GST-TEAD4, or GST-TEAD4-4SP fusion proteins were used as substrates. Reactions were incubated for 30 min at 30°C. p-Nitrobenzyl mesylate (Sigma, A1388) was added to the kinase reactions and incubated for 1 hr to alkylate the thiophosphorylation sites on the substrates. Reactions were terminated with sample buffer and resolved on 9% SDS-PAGE. A thiophosphate ester antibody (Abcam, ab92570) was used to detect substrate phosphorylation.

Soft agar colony growth

Each 6-well plate was coated with 1.5 ml of bottom agar (DMEM containing 10% FBS and 0.5% Difco agar noble). Various cells (5×10^3) were suspended in 1.5 ml of top agar (DMEM containing 10% FBS and 0.35% Difco agar noble) into each well. Cells were incubated for approximately three weeks and replaced with fresh medium containing 50 mM NaCl every three days. Colonies were stained using 0.005% crystal violet.

Statistics and Reproducibility

The experiments shown in Fig. 2b, 2f, 4j, and Supplementary Fig. 1h, 2b-d, 3f, 4h-j, 4l-m are representative of 2 independent experiments performed with similar results. All other experiments are representative of at least 3 independent repeats. Data are presented as mean ± s.e.m. p values were determined using one-way ANOVA followed by Tukey's multiple comparison test or two-way ANOVA as noted in the figure legends.

2.5 Acknowledgments

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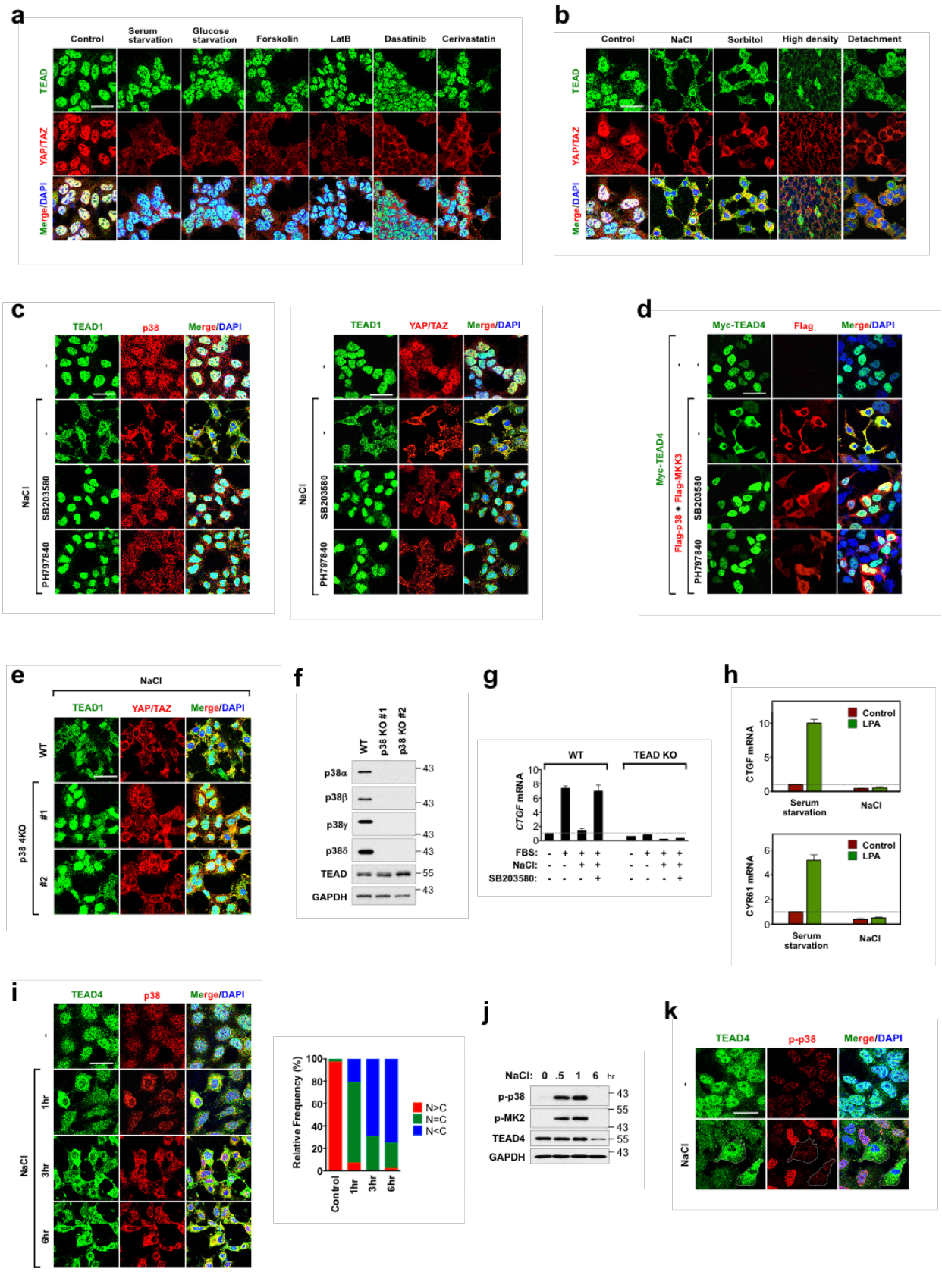
Figure 2.1: p38 mediates stress-induced TEAD cytoplasmic translocation.

a, Immunofluorescence staining of TEAD and YAP/TAZ in HEK293A cells treated with YAP-inhibiting signals. **b,** Immunofluorescence detects TEAD cytoplasmic translocation by environmental stress. **c,** Effect of p38 inhibitors on osmotic stress induced-TEAD cytoplasmic translocation. HEK293A cells were pretreated with p38 inhibitors, and stimulated with NaCl and stained for immunofluorescence. **d,** Ectopic expression of MKK3/p38 promotes TEAD4 cytoplasmic translocation. 24 hr after transfection, cells were treated with p38 inhibitors for 8hr and stained for immunofluorescence. **e,** Immunofluorescence staining shows deletion of p38 impairs TEAD nucleocytoplasmic shuttling by osmotic stress. Data for two independent p38 4KO clones are shown. **f,** Western blotting of p38 isoforms in p38 4KO cells. **g,** p38 mediates inhibition of YAP-TEAD target gene expression by stress. WT and TEAD KO cells were pretreated with NaCl and SB203580 as indicated, and then stimulated with 10% serum. CTGF mRNA expression was measured by qRT-PCR. Data are presented as mean ± s.e.m. from *n=3* independent experiments. **h,** Osmotic stress inhibits YAP-TEAD target gene expression. Cells were subject to serum starvation or NaCl, and then LPA-induced CTGF and CYR61 mRNA expression was measured by qRT-PCR. Data are presented as mean ± s.e.m. from *n=3* independent experiments. **i,** Correlation between stress-induced cytoplasmic translocation of TEAD and p38. Cells were stimulated with NaCl for the indicated times and then subjected to immunofluorescence to detect p38. Quantification of TEAD nuclear localization (N) and cytoplasmic localization (C) is provided. Random views (~100 cells) were selected for quantification. **j,** Time course of p38 activation by NaCl. Western blotting of phospho-p38 and its substrate phospho-MK2 upon NaCl treatment. **k,** Inverse correlation between stress-induced cytoplasmic translocation of TEAD and phospho-p38. Cells were stimulated with NaCl for 1 hr and then subjected to immunofluorescence using a phospho-p38 antibody. Scale bars in a-e, i, and k are 20μm.


a
Control
Serum starvation
Glucose starvation
Forskolin
LatB
Dasatinib
Cerivastatin
TEAD
YAP/TAZ
Merge/DAPI
b
Control
NaCl
Sorbitol
High density
Detachment
c
TEAD1
p38
YAP/TAZ
NaCl
SB203580
PH797840
d
Myc-TEAD4
Flag
Flag-p38 + Flag-MKK3
e
WT
p38 4KO
#1
#2
f
p38 KO #1
p38 KO #2
p38α
p38β
p38γ
p38δ
TEAD
GAPDH
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WT
TEAD KO
CTGF mRNA
FBS:
NaCl:
SB203580:
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Control
LPA
CTGF mRNA
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i
1hr
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N>C
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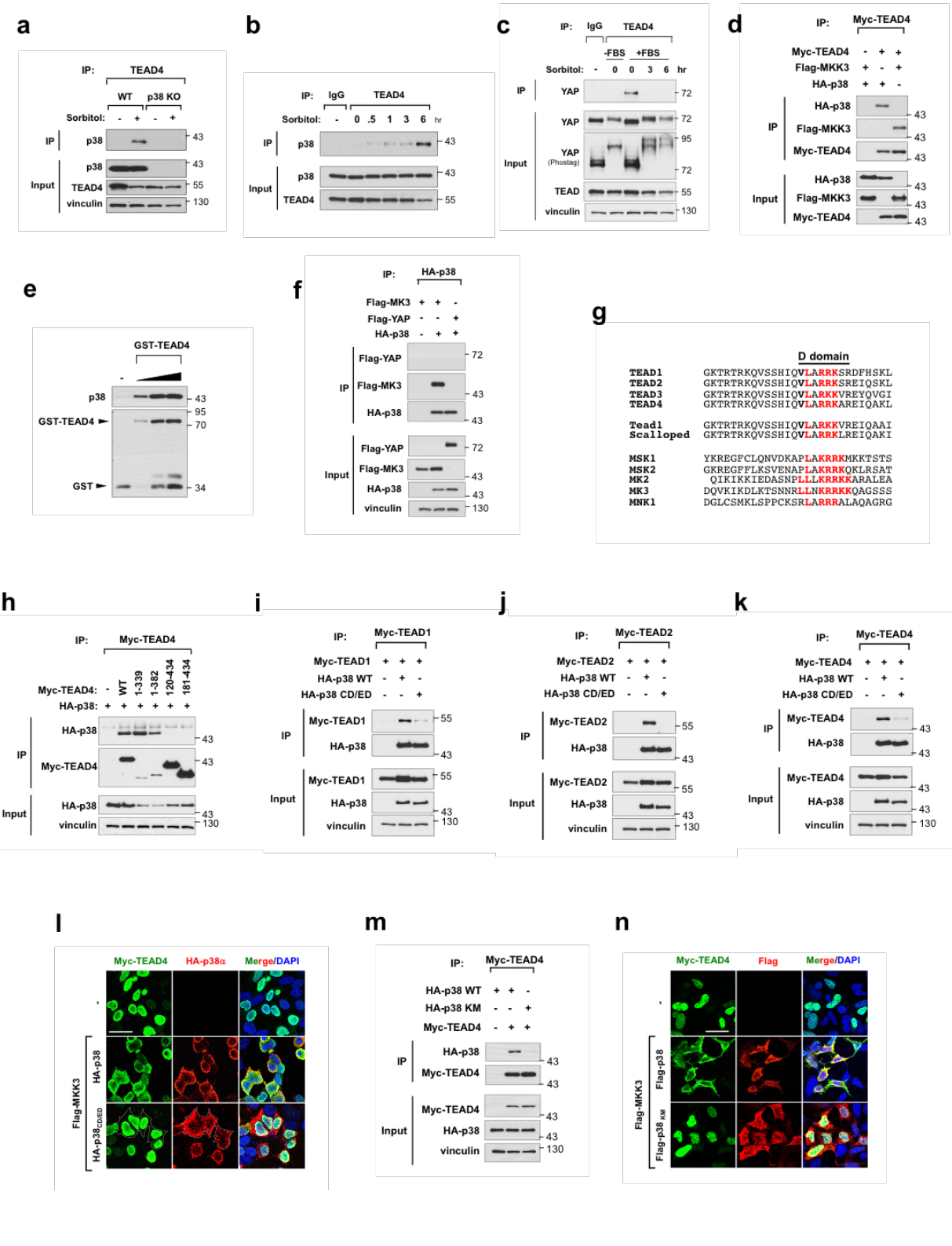
NaCl: 0 .5 1 6 hr
p-p38
p-MK2
k

Figure 2.2: p38 mediates stress-induced TEAD cytoplasmic translocation via protein-protein interaction.

a, Detection of osmotic stress-induced TEAD4 and p38 interaction by immunoprecipitation (IP) assay. IP and WB denote immunoprecipitation and Western blot, respectively. **b,** Immunoprecipitation showing time course of sorbitol-induced TEAD-p38 interaction. **c,** TEAD immunoprecipitation shows osmotic stress ablates TEAD-YAP interaction. **d,** TEAD interacts with p38 and MKK3 as shown by immunoprecipitation. **e,** *In vitro* binding assay using bacterially-purified proteins shows a direct interaction between p38 and TEAD. **f,** p38 does not interact with YAP. **g,** Sequence alignment of TEAD with canonical p38 substrates. Putative D domain (red) is conserved in N-terminus of all TEAD isoforms. **h,** The TEAD D domain is required for interaction with p38. p38 binds TEAD4 C-terminal truncation constructs (1-339 and 1-382), but not N-terminal truncations (120-434 or 181-434) in an immunoprecipitation assay. **i-k,** The p38 CD/ED docking motif is required for interaction with TEAD. p38 WT, but not p38 CD/ED mutant co-immunoprecipitates with TEAD1 (g), TEAD2 (h), and TEAD4 (i). **l,** TEAD-p38 interaction mediates TEAD cytoplasmic translocation. Immunofluorescence staining shows TEAD cytoplasmic translocation occurs in cells transfected with p38 WT, but not CD/ED mutant. **m,** Effect of p38 kinase activity on p38-TEAD binding. Immunoprecipitation assay shows TEAD binds p38 WT but not kinase-dead mutant, p38KM. **n,** Effect of p38 kinase activity on TEAD cytoplasmic translocation. Immunofluorescence shows ectopic expression of p38 WT, but not kinase-dead mutant p38 KM, induces TEAD4 cytoplasmic translocation. Scale bars in l and n are 20μm.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Input p38 43
TEAD4 55
vinculin 130
b
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Sorbitol: - 0 .5 1 3 6 hr
IP p38 43
Input p38 43
TEAD4 55
c
IP: IgG TEAD4
-FBS +FBS
Sorbitol: - 0 0 3 6 hr
IP YAP 72
Input YAP 72
YAP (Phostag) 95 72
TEAD 55
vinculin 130
d
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Myc-TEAD4 - + +
Flag-MKK3 + - +
HA-p38 + + -
IP HA-p38 43
Flag-MKK3 43
Myc-TEAD4 43
Input HA-p38 43
Flag-MKK3 43
Myc-TEAD4 43
e
GST-TEAD4
p38 43
GST-TEAD4 95 70
GST 34
f
IP: HA-p38
Flag-MK3 + + -
Flag-YAP - - +
HA-p38 - + +
IP Flag-YAP 72
Flag-MK3 43
HA-p38 43
Input Flag-YAP 72
Flag-MK3 43
HA-p38 43
vinculin 130
g
D domain
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Tead1 GKTRTRKQVSSHIQVLARKKVREIQAAI
Scalloped GKTRTRKQVSSHIQVLARRKLREIQAKI
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MSK2 GKREGFFLKSVENAPLAKRRKQKLRSAT
MK2 QIKIKKIEDASNPLLLKRRKKARALEA
MK3 DQVKIKDLKTSNNRLLNKRRKKQAGSSS
MNK1 DGLCSMKLSPPCKSRLARRRALAQAGRG
h
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Myc-TEAD4: - WT 1-339 1-382 120-434 181-434
HA-p38: + + + + + +
IP HA-p38 43
Myc-TEAD4 43
Input HA-p38 43
vinculin 130
i
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HA-p38 WT - + -
HA-p38 CD/ED - - +
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HA-p38 43
Input Myc-TEAD1 55
HA-p38 43
vinculin 130
j
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Myc-TEAD2 + + +
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HA-p38 CD/ED - - +
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HA-p38 43
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HA-p38 43
vinculin 130
k
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HA-p38 CD/ED - - +
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Input Myc-TEAD4 43
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vinculin 130
l
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Flag-MKK3
HA-p38
HA-p38CD/ED
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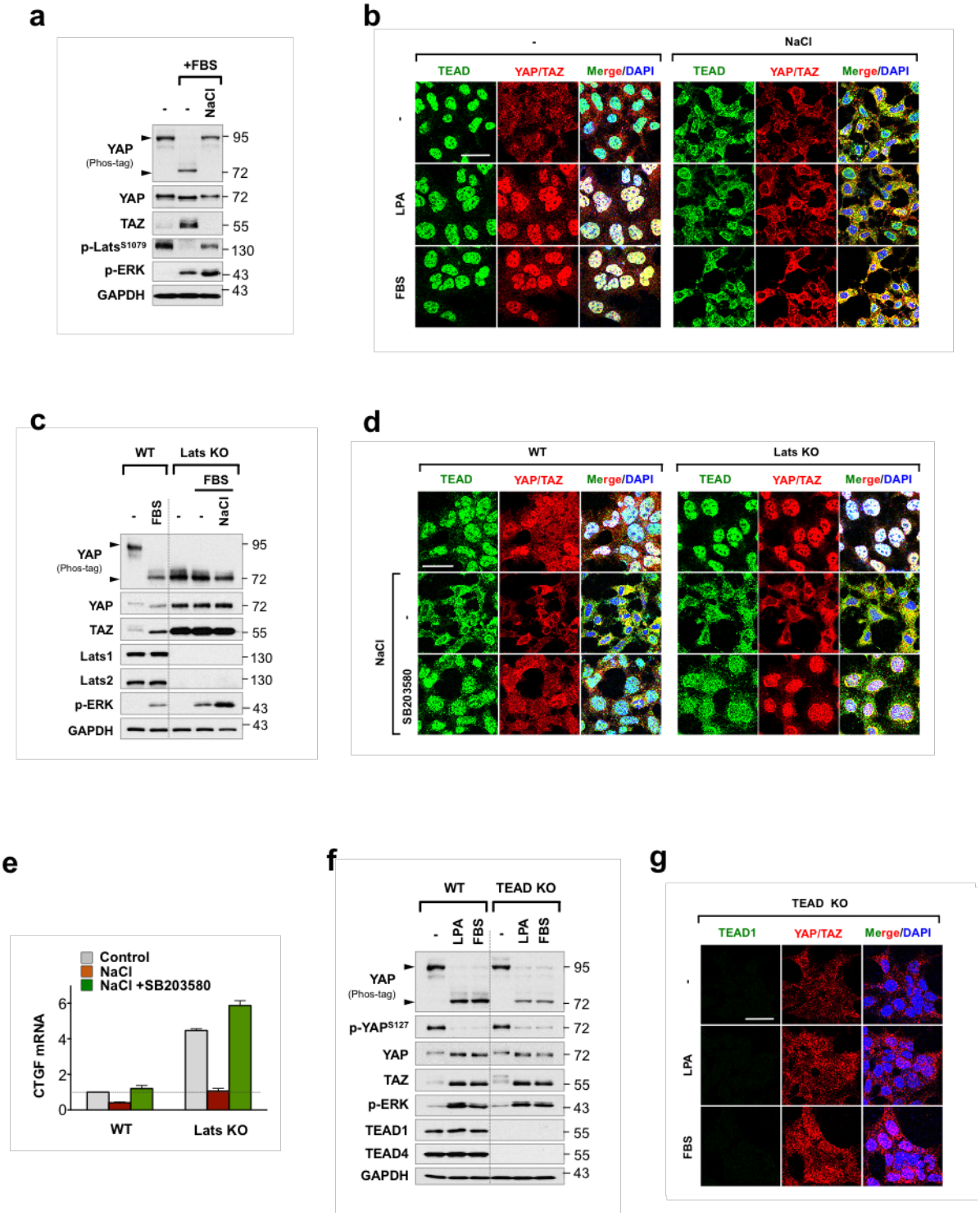
Input Myc-TEAD4 43
HA-p38 43
vinculin 130
n
Myc-TEAD4 Flag Merge/DAPI
Flag-MKK3
Flag-p38
Flag-p38KM

Figure 2.3: TEAD cytoplasmic translocation prevents YAP activation.

a, b, Effect of osmotic stress on serum-, and LPA-induced YAP activation. Osmotic stress blocks serum and LPA-induced YAP dephosphorylation as shown by Western blot (a, lower arrow), and nuclear translocation as shown by immunofluorescence staining (b). **c, d,** Stress-induced TEAD and YAP cytoplasmic translocation is p38-dependent, but Hippo-independent. YAP is constitutively dephosphorylated in Lats KO cells as indicated by YAP phostag gel (c). Immunofluorescence shows stress induces YAP and TEAD cytoplasmic translocation in the Lats KO cells, which is blocked by SB203580 treatment (d). **e,** Quantification of CTGF mRNA by qRT-PCR in WT and Lats KO cells stimulated with osmotic stress with or without SB203580 treatment. Data are presented as mean ± s.e.m. from *n=3* independent experiments. **f, g,** Detection of YAP/TAZ localization by immunofluorescence staining in TEAD KO cells stimulated with LPA or serum. Western blotting indicates YAP dephosphorylation by LPA or serum stimulation is intact in TEAD KO cells (f), whereas immunofluorescence shows YAP nuclear accumulation is impaired (g). Scale bars in b, d, and g are 20μm.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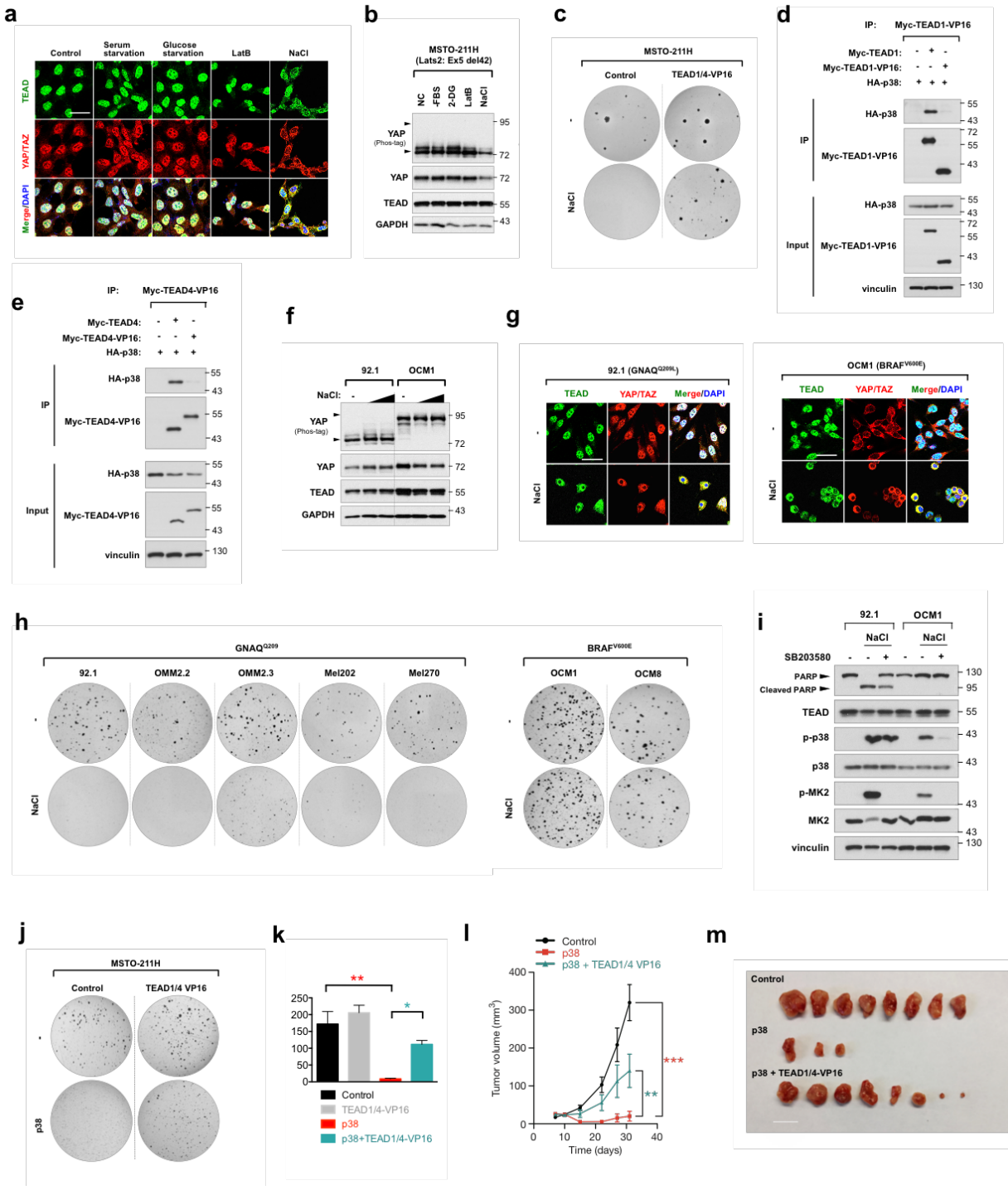
YAP (Phos-tag)
YAP
TAZ
p-Lats^{S1079}
p-ERK
GAPDH
95
72
72
55
130
43
43
b
-
NaCl
TEAD
YAP/TAZ
Merge/DAPI
LPA
FBS
c
WT
Lats KO
FBS
- FBS - - NaCl
YAP (Phos-tag)
YAP
TAZ
Lats1
Lats2
p-ERK
GAPDH
95
72
72
55
130
130
43
43
d
WT
Lats KO
TEAD
YAP/TAZ
Merge/DAPI
NaCl
SB203580
e
Control
NaCl
NaCl +SB203580
CTGF mRNA
0
2
4
6
WT
Lats KO
f
WT
TEAD KO
- LPA FBS - LPA FBS
YAP (Phos-tag)
p-YAP^{S127}
YAP
TAZ
p-ERK
TEAD1
TEAD4
GAPDH
95
72
72
72
55
43
55
55
43
g
TEAD KO
TEAD1
YAP/TAZ
Merge/DAPI
LPA
FBS

Figure 2.4: TEAD inhibition restricts YAP-driven cancer cell growth.

a, b, Effect of osmotic stress on TEAD and YAP cytoplasmic sequestration in MSTO-211H mesothelioma cells. Note that unlike HEK293A (Fig. 1a), serum starvation, glucose starvation, and latruculin B did not induce YAP cytoplasmic localization because of Lats2 mutation in MSTO-211H cells. Only NaCl treatment elicited TEAD and YAP cytoplasmic translocation as detected by immunofluorescence (a) despite constitutive YAP dephosphorylation as detected by Western blot (b). **c,** Stress-induced TEAD inhibition suppresses anchorage independent growth. Osmotic stress inhibited colony formation in control, but not TEAD1/4-VP16 expressing MSTO-211H cells. **d, e,** Immunoprecipitation shows p38 cannot bind TEAD1-VP16 (d) or TEAD4-VP16 (e). **f,** Western blotting of YAP phosphorylation status in UM cell lines 92.1 and OCM1. **g,** Immunostaining of TEAD and YAP/TAZ in UM cell lines 92.1 and OCM1 upon osmotic stress. Note that YAP displays cytoplasmic staining under normal condition in OCM1 cells. Scale bars in a and g are 20μm. **h,** Differential effect of TEAD inhibition on anchorage-independent growth of GNAQ-mutant and BRAF-mutant UM cells. Stress-induced TEAD inhibition ablated colony formation in all GNAQ-mutant cell lines, whereas BRAF-mutant cell growth was insensitive. **i,** Western blot for PARP cleavage in 92.1 and OCM1 cells. NaCl stimulation induces apoptosis in 92.1 but not OCM1. Pretreatment with p38 inhibitor rescues cells from osmotic stress-induced apoptosis. **j,** TEAD1/4 -VP16 rescues p38-induced inhibition of colony formation of MSTO-211H cells. **k,** Quantification of (j). *n=3* biological replicates. Data are presented as mean ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; p values were determined using one-way ANOVA followed by Tukey's multiple comparison test. **l,** TEAD1/4-VP16 rescues p38-induced inhibition of MSTO-211H *in vivo* tumor xenograft growth. Nude mice were injected with control, p38, or p38 + TEAD1/4-VP16 expressing MSTO-211H cells and tumor growth was measured at the indicated times. Data are presented as mean ± s.e.m. *n = 4* mice per group. $^{**}p < 0.01$; $^{***}p < 0.001$; p values were determined using two-way ANOVA. **m,** Nude mice were injected with control, p38, or p38 + TEAD1/4-VP16 expressing MSTO-211H cells and tumors were harvested after 4 weeks. Only three tumors developed in the p38 group. Scale bar, 10mm.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Serum starvation
Glucose starvation
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NaCl
TEAD
YAP/TAZ
Merge/DAPI
b
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NC
-FBS
2-DG
LatB
NaCl
YAP (Phos-tag)
YAP
TEAD
GAPDH
c
MSTO-211H
Control
TEAD1/4-VP16
NaCl
d
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Myc-TEAD1:
Myc-TEAD1-VP16:
HA-p38:
IP
HA-p38
Myc-TEAD1-VP16
Input
vinculin
e
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Myc-TEAD4:
Myc-TEAD4-VP16:
HA-p38:
IP
HA-p38
Myc-TEAD4-VP16
Input
vinculin
f
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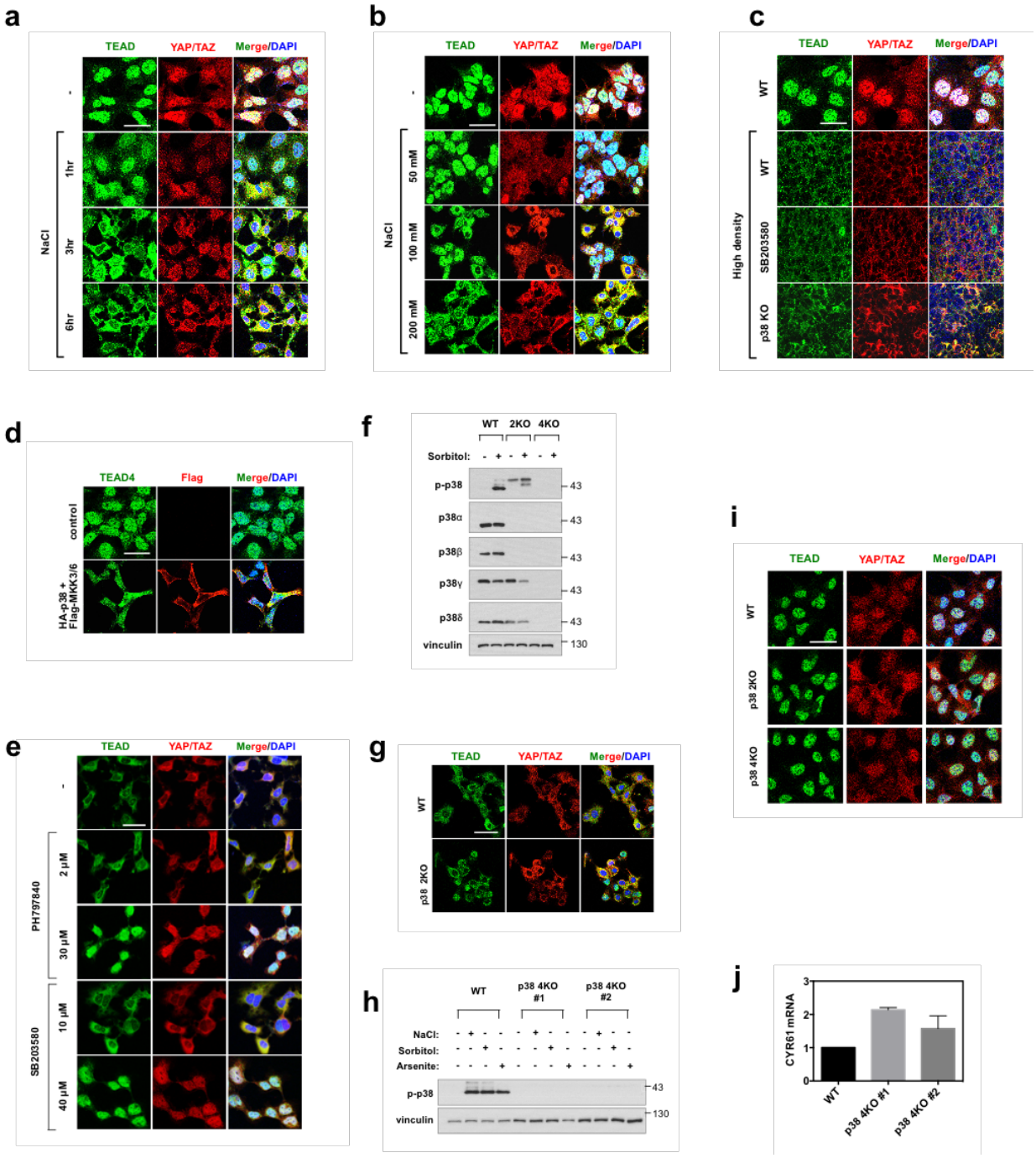
OCM1
NaCl:
YAP (Phos-tag)
YAP
TEAD
GAPDH
g
92.1 (GNAQ Q209L)
OCM1 (BRAF V600E)
TEAD
YAP/TAZ
Merge/DAPI
NaCl
h
GNAQ Q209
92.1
OMM2.2
OMM2.3
Mel202
Mel270
BRAF V600E
OCM1
OCM8
NaCl
i
92.1
OCM1
NaCl
SB203580
PARP
Cleaved PARP
TEAD
p-p38
p38
p-MK2
MK2
vinculin
j
MSTO-211H
Control
TEAD1/4 VP16
p38
k
Control
TEAD1/4-VP16
p38
p38+TEAD1/4-VP16
l
Control
p38
p38 + TEAD1/4 VP16
Tumor volume (mm3)
Time (days)
m
Control
p38
p38 + TEAD1/4-VP16

Figure S2.1: Osmotic stress induces TEAD cytoplasmic translocation.

a, Time course of osmotic stress-induced TEAD cytoplasmic translocation. HEK293A cells were treated with NaCl for 0, 1, 3, or 6 hours and stained for immunofluorescence. **b,** Dose response for NaCl-induced TEAD cytoplasmic translocation. HEK293A cells were stimulated with different concentrations of NaCl for 6 hr and stained for immunofluorescence. TEAD cytoplasmic translocation occurs from100mM NaCl. **c,** High cell density-induced TEAD cytoplasmic localization is p38 independent. Immunofluorescence shows inhibition or KO of p38 upon high cell density has no effect on TEAD cytoplasmic translocation. **d,** Cells ectopically expressing MKK3/p38 were stained for immunofluorescence. MKK3/p38 promotes TEAD4 cytoplasmic translocation. **e,** Dose response for p38 inhibitor treatment. HEK293A cells were pre-treated with different doses of p38 inhibitors as indicated, followed by NaCl stimulation and stained for immunofluorescence. **f,** Western blot of p38α/β knockout cells show upregulation of p38δ/γ isoforms. **g,** p38 2KO cells were stained for immunofluorescence. Deletion of p38α/β isoforms is not sufficient to inhibit TEAD cytoplasmic translocation. **h,** Immunoblotting for p-p38 in p38 4KO cells shows impaired p38 activity under various p38 activating stimuli (200 mM NaCl, 500 μM sorbitol, 500 μM arsenite). **i,j** p38 4KO shows no effect on TEAD localization (i) or target gene expression, as measured by qRT-PCR (j) under basal conditions. Scale bars in a-e, g, and i are 20μm. Data are presented as mean ± s.e.m. from *n=3* independent experiments.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TEAD
YAP/TAZ
Merge/DAPI
-
NaCl
1hr
3hr
6hr
b
TEAD
YAP/TAZ
Merge/DAPI
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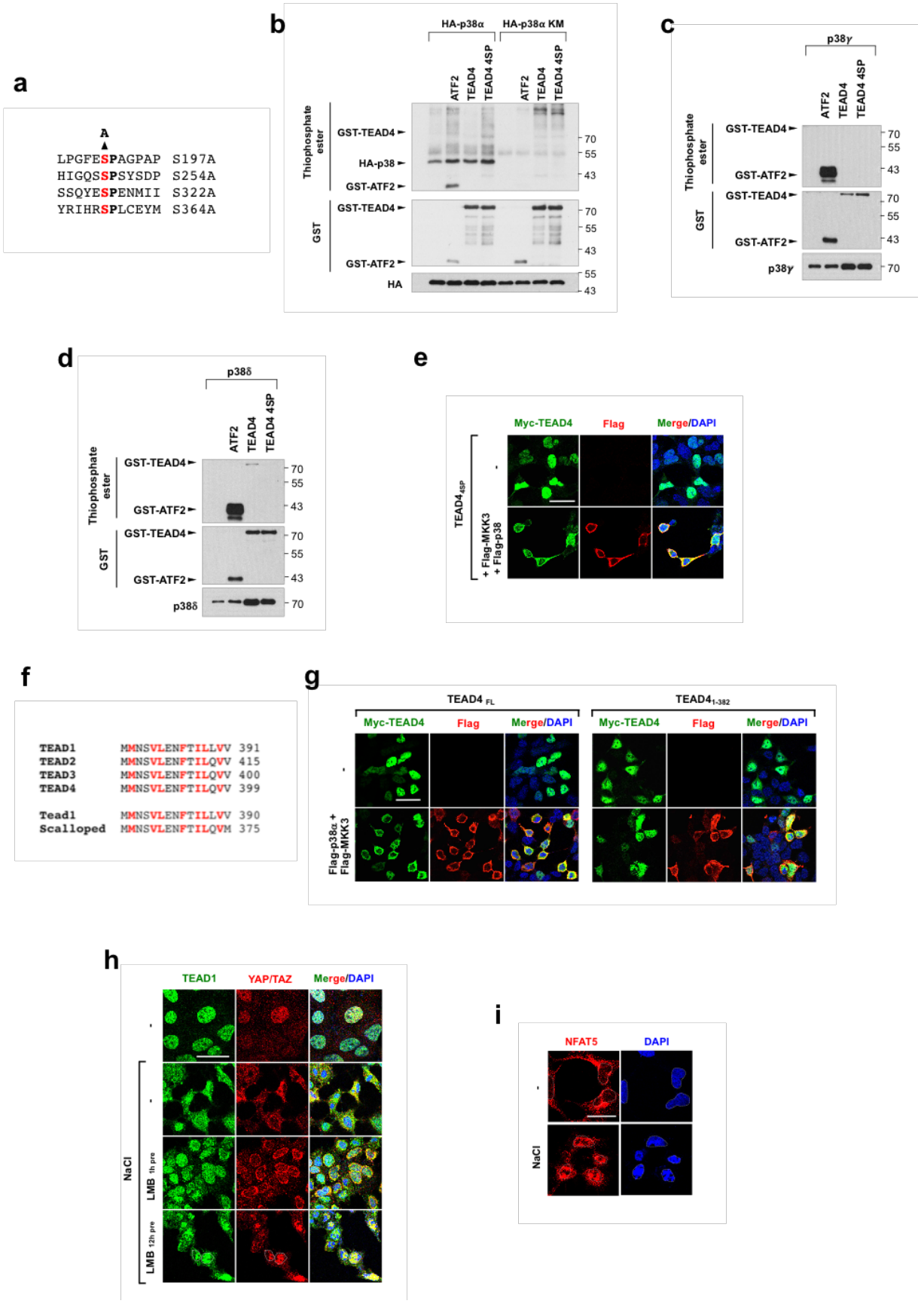
50 mM
100 mM
200 mM
c
TEAD
YAP/TAZ
Merge/DAPI
WT
High density
WT
SB203580
p38 KO
d
TEAD4
Flag
Merge/DAPI
control
HA-p38 +
Flag-MKK3/6
f
WT
2KO
4KO
Sorbitol:
p-p38
p38α
p38β
p38γ
p38δ
vinculin
43
130
i
TEAD
YAP/TAZ
Merge/DAPI
WT
p38 2KO
p38 4KO
e
TEAD
YAP/TAZ
Merge/DAPI
-
PH797840
2 μM
30 μM
SB203580
10 μM
40 μM
g
TEAD
YAP/TAZ
Merge/DAPI
WT
p38 2KO
h
WT
p38 4KO #1
p38 4KO #2
NaCl:
Sorbitol:
Arsenite:
p-p38
vinculin
43
130
j
CYR61 mRNA
0
1
2
3
WT
p38 4KO #1
p38 4KO #2

Figure S2.2: Phosphorylation of TEAD by p38 is not required for cytoplasmic translocation.

a, Sequence of TEAD4-4SP construct harboring mutations in putative p38 phosphorylation sites. **b-d,** TEAD is a poor substrate for p38 phosphorylation. *In vitro* kinase assay for p38α (b), p38γ (c), and p38δ (d) using TEAD as a substrate. No phosphorylation or weak phosphorylation was detected in TEAD4 WT, which was further ablated in TEAD4-4SP, whereas ATF2 was effectively phosphorylated by p38. **e,** Detection of TEAD4-4SP cytoplasmic translocation by p38 using immunofluorescence. **f,** Sequence of putative TEAD nuclear export signal. **g,** TEAD cytoplasmic translocation requires a nuclear export signal. Immunofluorescence shows truncation of TEAD disrupting a putative nuclear export signal in TEAD (1-382) mutant inhibits CRM1-dependent TEAD nuclear export compared to full length TEAD. **h,** TEAD cytoplasmic translocation is a CRM1-dependent process. HEK293A cells were pretreated with LMB for the indicated times followed by NaCl stimulation and staining for immunofluorescence. Pretreatment with LMB inhibits TEAD cytoplasmic translocation. **i,** Immunofluorescence of NFAT5 nuclear translocation upon NaCl stimulation. Scale bars in e and g-i are 20μm.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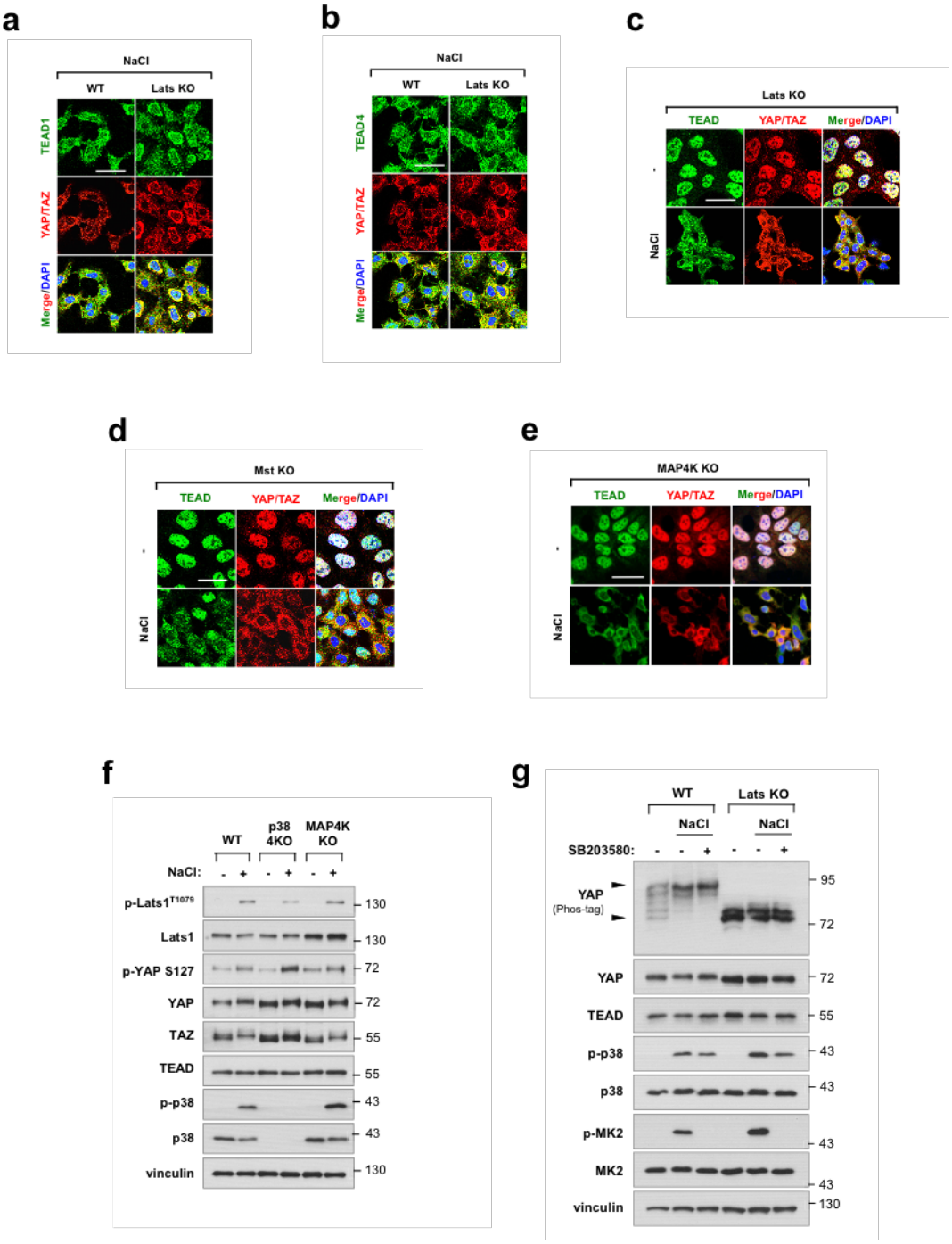
HA-p38α KM
ATF2
TEAD4
TEAD4 4SP
Thiophosphate ester
GST
GST-TEAD4
HA-p38
GST-ATF2
HA
70
55
43
c
p38γ
ATF2
TEAD4
TEAD4 4SP
Thiophosphate ester
GST
GST-TEAD4
GST-ATF2
p38γ
d
p38δ
ATF2
TEAD4
TEAD4 4SP
Thiophosphate ester
GST
GST-TEAD4
GST-ATF2
p38δ
e
Myc-TEAD4
Flag
Merge/DAPI
TEAD4 4SP
-
+ Flag-MKK3
+ Flag-p38
f
TEAD1 MMNSVLENFTILLVV 391
TEAD2 MMNSVLENFTILQVV 415
TEAD3 MMNSVLENFTILQVV 400
TEAD4 MMNSVLENFTILQVV 399
Tead1 MMNSVLENFTILLVV 390
Scalloped MMNSVLENFTILQVM 375
g
TEAD4 FL
TEAD4 1-382
Myc-TEAD4
Flag
Merge/DAPI
-
Flag-p38α +
Flag-MKK3
h
TEAD1
YAP/TAZ
Merge/DAPI
-
-
NaCl
LMB 1h pre
LMB 12h pre
i
NFAT5
DAPI
-
NaCl

Figure S2.3: Stress-induced TEAD cytoplasmic translocation is independent of Hippo pathway.

a, b, Stress-induced TEAD cytoplasmic translocation is independent of Lats1/2. WT and Lats KO cells were stimulated with NaCl and stained with anti-TEAD1 (a) or anti-TEAD4 (b) antibody for immunofluorescence. **c, d, e,** Stress-induced TEAD cytoplasmic translocation is independent of Hippo core kinases and MAP4K. Lats KO (c), Mst KO (d), and MAP4K KO (e) cells were treated with NaCl and stained for immunofluorescence. **f,** Western blot showing MAP4K and p38 are independent branches of the MAPK pathway. **g,** Western blot showing stress-activated p38 does not affect YAP phosphorylation status. Scale bars in a-e are 20μm.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WT
Lats KO
TEAD1
YAP/TAZ
Merge/DAPI
b
NaCl
WT
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TEAD4
YAP/TAZ
Merge/DAPI
c
Lats KO
TEAD
YAP/TAZ
Merge/DAPI
-
NaCl
d
Mst KO
TEAD
YAP/TAZ
Merge/DAPI
-
NaCl
e
MAP4K KO
TEAD
YAP/TAZ
Merge/DAPI
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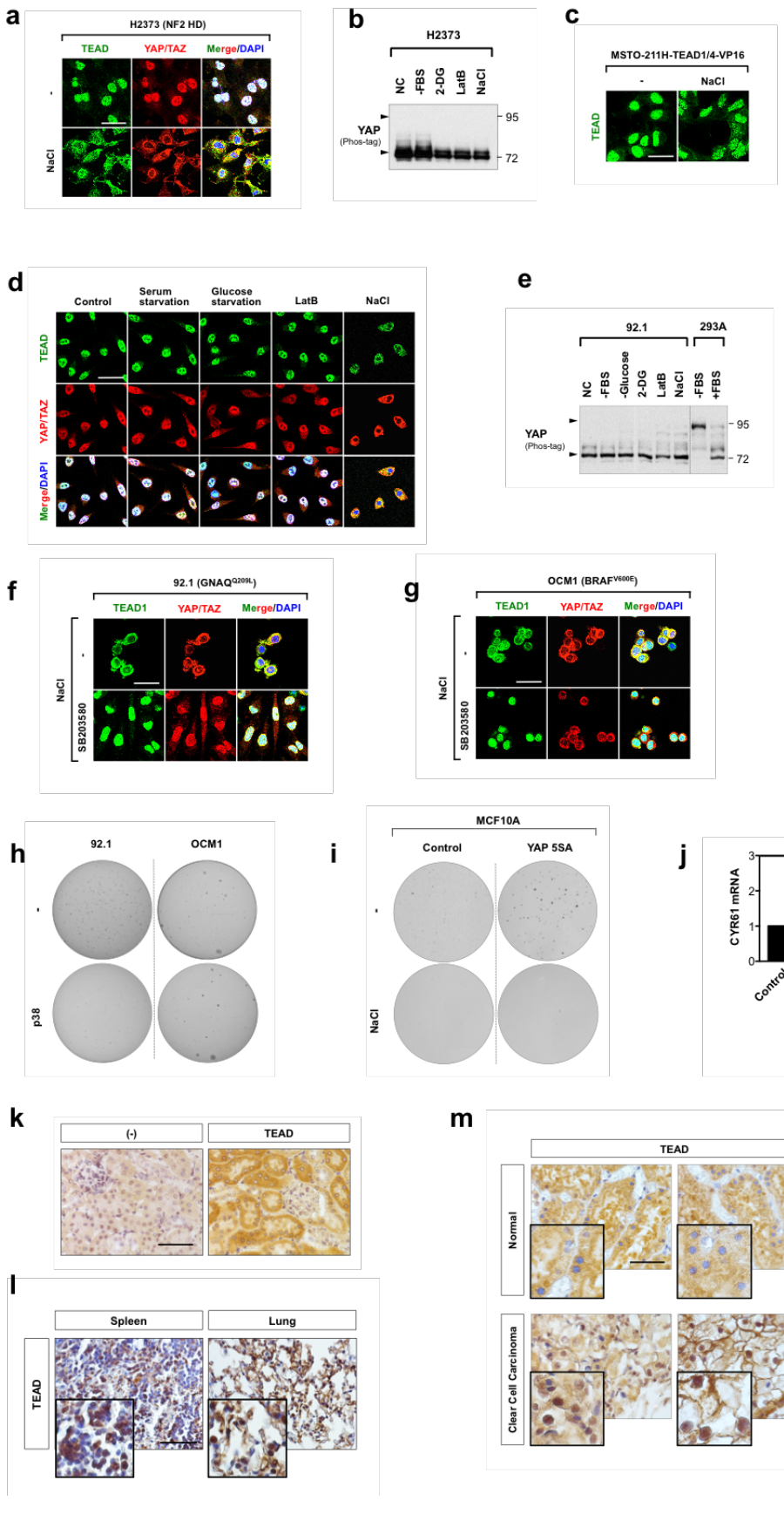
YAP
TAZ
TEAD
p-p38
p38
vinculin
130
130
72
72
55
55
43
43
130
g
WT
Lats KO
NaCl
NaCl
SB203580: - - + - - +
YAP
(Phos-tag)
YAP
TEAD
p-p38
p38
p-MK2
MK2
vinculin
95
72
72
55
43
43
43
43
130

Figure S2.4: Stress-induced TEAD inhibition uncouples YAP localization and dephosphorylation in YAP-driven cancer cells.

a, b, Stress-induced TEAD and YAP/TAZ cytoplasmic translocation in H2373 mesothelioma cells, which have homozygous deletion of NF2. Immunofluorescence showing NaCl stimulation induces TEAD and subsequent YAP/TAZ cytoplasmic sequestration (a), despite constitutive dephosphorylation of YAP as shown by western blot (b). NC, normal condition. **c,** Nuclear localization of TEAD-VP16 in the presence of osmotic stress. MSTO-211H cells stably expressing TEAD1/4-VP16 construct were treated with NaCl and stained for immunofluorescence. **d, e,** Stress promotes TEAD and YAP cytoplasmic sequestration in YAP-driven uveal melanoma cells. 92.1 cells were treated with YAP-inhibiting stimuli as in Fig. 1a, b. NaCl treatment elicits TEAD and YAP cytoplasmic translocation shown by immunofluorescence (d), despite constitutive dephosphorylation of YAP shown by western blot (e). **f, g,** p38 mediates stress-induced TEAD cytoplasmic translocation in UM cell lines. Immunofluorescence shows treatment with SB203580 blocks NaCl-induced cytoplasmic translocation of TEAD in 92.1 (f) and OCM1 (g). **h,** p38 expression inhibits colony formation of GNAQ-mutant 92.1 cells but not BRAF-mutant OCM1 cells. **i,** Colony growth assay showing osmotic stress inhibits anchorage independent growth of YAP-5SA transformed MCF10A. **j,** Expression of p38 reduces target gene expression induced by hyperactive YAP as measured by qRT-PCR. Target gene expression is rescued by constitutively active TEAD. Data are presented as mean from $n=2$ independent experiments. **k-m,** Immunohistochemistry staining of TEAD. Negative control staining for pan-TEAD antibody (left) and normal kidney tissue staining with pan-TEAD (right) (k). Nuclear staining of TEAD detected in mouse spleen and lung tissues (l). Cytoplasmic staining of TEAD is detected in tubule cells of normal kidney while nuclear staining is detected in renal clear cell carcinomas derived from transformed tubule cells (m). Scale bars in a, c-d, and f-g are 20μm. Scale bars in k-m are 50 μm.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H2373 (NF2 HD)
TEAD
YAP/TAZ
Merge/DAPI
-
NaCl
b
H2373
NC
-FBS
2-DG
LatB
NaCl
YAP
(Phos-tag)
95
72
c
MSTO-211H-TEAD1/4-VP16
-
NaCl
TEAD
d
Control
Serum starvation
Glucose starvation
LatB
NaCl
TEAD
YAP/TAZ
Merge/DAPI
e
92.1
293A
NC
-FBS
-Glucose
2-DG
LatB
NaCl
-FBS
+FBS
YAP
(Phos-tag)
95
72
f
92.1 (GNAQ Q209L)
TEAD1
YAP/TAZ
Merge/DAPI
-
NaCl
SB203580
g
OCM1 (BRAF V600E)
TEAD1
YAP/TAZ
Merge/DAPI
-
NaCl
SB203580
h
92.1
OCM1
-
p38
i
MCF10A
Control
YAP 5SA
-
NaCl
j
CYR61 mRNA
3
2
1
0
Control
p38
p38 + TEAD1/4-VP16
k
(-)
TEAD
l
Spleen
Lung
TEAD
m
TEAD
Normal
Clear Cell Carcinoma

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Chapter 3: Regulation of TEAD by cell-cell contact and cell-matrix interaction.

3.1 Introduction

The Hippo pathway, originally discovered in *Drosophila*, is an evolutionarily conserved signaling pathway that regulates cell proliferation, differentiation, tissue homeostasis, and organ size. In mammals, the core components of the Hippo pathway are comprised of serine/threonine kinases Mammalian STE20-like kinases (MST1/2) and Large Tumor Suppressor kinases (LATS1/2), as well as effector proteins Yes-associated protein (YAP) and Transcriptional Activator with PDZ binding domain (TAZ). The Hippo pathway is activated upon phosphorylation of MST1/2, which can interact with scaffold protein, Salvador Homolog 1 (SAV1). SAV1 functions to bridge MST1/2 to LATS1/2 to increase MST1/2 kinase activity (Callus et al 2006, Wu et al 2003). In addition, MST1/2 phosphorylates Mob1 Homolog (MOB1), which upon phosphorylation binds the auto-inhibitory region of LATS1/2 to further enhance LATS1/2 activation(Praskova et al 2008). Activation of LATS1/2 results in direct phosphorylation of YAP/TAZ and its inactivation. Once phosphorylated YAP/TAZ is sequestered in the cytoplasm by 14-3-3 binding or ubiquinated and targeted for degradation (Hao et al 2008, Lei et al 2008, Liu et al 2010, Zhao et al 2010, Zhao et al 2007). Inactivation of the Hippo pathway results in activation of YAP/TAZ, which translocates to the nucleus, binds TEAD, and induces target gene expression (Lai et al 2011, Zhao et al 2008). YAP/TAZ does not contain a DNA binding domain and thus requires binding to transcription factors, such as TEAD to initiate transcription (Zhao et al 2008). Recently, studies have revealed several extracellular signaling pathways as upstream regulators

of Hippo signaling. These upstream regulatory pathways include G-Coupled Protein Receptors (GPCRs), Wnt, TGFβ, EGF, and Notch (Hansen et al 2015). Additionally, Hippo signaling is known to be regulated by cell density, cellular stress, and mechanical and cytoskeletal cues (Meng et al 2016).

The TEAD family of transcription factors is largely responsible for mediating the effects of Hippo signaling. TEAD contains a TEA/ATTS DNA-binding domain in the N-terminus and a C-terminal domain that recruits transactivation partners. Without transactivating binding partners YAP/TAZ, TEAD is not known to have transcriptional activity. Without YAP/TAZ accumulation in the nucleus, TEAD binds to Vestigial Like Family Member 4 (VGLL4), repressing target gene expression (Jiao et al 2017, Jiao et al 2014, Zhang et al 2014a). Previous reports have shown changes in TEAD subcellular localization in the context of embryonic lineage differentiation as well as in response to environmental stresses such as osmotic stress and high cell density(Home et al 2012, Lin et al 2017). Additionally, an alternatively spliced isoform of TEAD4, one lacking the N-terminal DNA binding domain has been found to localize to both the cytoplasm and the nucleus and act as a dominant negative form to suppress YAP/TAZ activity(Qi et al 2016). Thus, subcellular localization of TEAD may be an additional means of regulating Hippo signaling output.

Due to its role in cell proliferation, survival, and differentiation, the Hippo pathway has been shown to be involved in organ regeneration and tissue repair(Moya & Halder 2018). After induction of liver injury through partial hepatectomy, there is an increased

YAP nuclear localization and upregulation of YAP target gene expression in regenerating hepatocytes (Grijalva et al 2014). Increase in YAP activity is also seen in dextran sodium sulfate (DSS) induced injury models in intestinal epithelium. After initial loss of YAP levels during the injury phase, YAP levels in the regenerating intestinal epithelium increase significantly, exceeding levels seen in a homeostatic, uninjured intestinal epithelium (Barry et al 2013, Cai et al 2010, Gregorieff et al 2015). In the skin, YAP/TAZ are localized to the nucleus after wound healing and knockdown of YAP/TAZ delays wound closure (Elbediwy et al 2016, Lee et al 2014). The role of TEAD in regeneration of these tissues has not been well studied. Thus, investigating whether TEAD plays a role in tissue regeneration and wound healing, in both YAP-dependent and -independent capacities, may prove beneficial for regenerative medicine.

The functions of the Hippo pathway, though advantageous in regenerative medicine, prove detrimental when dysregulated or unrestricted. Dramatic *in vivo* overgrowth phenotypes have been observed in loss of function mutations in the Hippo core kinases as well as overexpression of YAP/TAZ. These observations suggest the importance of Hippo signaling in the development of cancer. Components of the transcriptional unit of Hippo signaling, namely the YAP/TAZ-TEAD complex, are commonly seen to be amplified or dysregulated despite absence of loss of function mutations in Hippo core kinases (Harvey et al 2013, Johnson & Halder 2014). Recent studies have emphasized the role of YAP/TAZ hyperactivity in various cancers. Elevated YAP levels and increased nuclear localization has been reported in a majority of solid tumors including lung, liver, breast, colon, skin, and ovary (Moroishi et al 2015).

In KRAS driven models of pancreatic ductal adenocarcinoma, YAP is essential for cancer progression and tumor recurrence in the absence of KRAS, suggesting the possible synergistic effects of targeting YAP in combination with drugs targeting KRAS pathways (Kapoor et al 2014, Shao et al 2014, Zhang et al 2014b). In uveal melanoma, driven by $G\alpha_q$ and $G\alpha_{11}$ mutations, targeting YAP using the YAP/TAZ inhibitor, verteporfin, successfully inhibits tumor growth (Feng et al 2014, Yu et al 2014). Additionally, YAP/TAZ has been shown to be required for loss of APC-induced crypt hyperplasia in mice and, concordantly, is a clinical predictor of colorectal cancer progression (Azzolin et al 2014, Wang et al 2013, Wang et al 2017). TAZ is overexpressed in 20% of human breast cancers and has been shown to play a role in invasion, migration, tumorigenesis, and chemoresistance (Chan et al 2008, Cordenonsi et al 2011, Lai et al 2011).

TEADs have also been implicated in several solid tumors. TEADs are known to regulate mesothelin, a gene that serves as a cancer biomarker due to its overexpression in many tumors (Hucl et al 2007). Furthermore, high TEAD expression levels are seen in prostate and breast cancers and is an indicator of poor clinical outcome (Han et al 2008, Knight et al 2008, Pobbati & Hong 2013, Richardson et al 2006). Upregulation of TEAD has also been reported to be play a role in driving epithelial to mesenchymal transition (EMT) as well as conferring resistance to apoptosis (Diepenbruck et al 2014, Landin Malt et al 2012). Recently, TEADs have also been reported to drive the transcriptional program responsible for increased invasiveness and resistance to MAPK inhibition in melanomas (Verfaillie et al 2015).

The Hippo pathway is a growth control pathway and plays fundamental roles in cell proliferation, organ size control, stem cell function and differentiation, tissue homeostasis and regeneration, and tumor suppression. As many of these properties are impaired or deregulated in cancers, the Hippo pathway has become an attractive target in the development of new cancer therapeutics. However, the Hippo pathway has proven itself difficult to target as upstream Hippo core kinases cannot be traditionally targeted with kinase inhibitors due to their role as tumor suppressors. Additionally, since YAP/TAZ has no intrinsic enzymatic activity, development of small molecules to regulate its activity is challenging. The YAP/TAZ-TEAD complex is extremely important for oncogenic activity as YAP/TAZ has no DNA binding ability, and TEAD is reported to be transcriptionally inactive without YAP/TAZ binding. Thus, design of small molecules, such as verteporfin, for the treatment of YAP/TAZ overexpressing tumors has focused on interrupting this protein-protein interaction. Understanding the mechanisms of TEAD regulation, independent of YAP/TAZ binding, will provide valuable insights for the development of new cancer therapeutics and may also aid in therapeutics promoting regeneration of injured tissues.

3.2 Results

TEAD localization is regulated during wound healing and tissue regeneration.

Though modulating YAP/TAZ nuclear cytoplasmic translocation has long been a means of controlling Hippo signaling output, understanding mechanisms of TEAD translocation may provide new indications for YAP/TAZ dysregulated diseases. As

such, we set out to identify stimuli in which promoted TEAD cytoplasmic localization. We found that high cell density was able to drive TEAD cytoplasmic localization (Fig 2.1b and Fig 3.1a). High cell density driven TEAD cytoplasmic translocation was conserved across varying cell types, though at varying degrees (Fig 3.1a). Because post-translational modifications play important roles in regulating protein localization and activity, we sought to determine whether TEAD translocation was due to post-translational modifications. Using Phosphosite, a curated and compiled database of published mass spectrometry (MS) results, we made point mutations at potential sites of post-translational modification in TEAD. However, of the sites that were mutated, none affected TEAD localization under basal conditions to recapitulate the effects of high cell density (Supplementary Fig 3.1a). However, it is possible that there are other potential post-translational modification sites that have not yet been identified.

After induction of injury to the confluent cell monolayer using an in-vitro scratch assay, TEAD was translocated from the cytoplasm to the nucleus. In HEK293A cells, the greatest effects on TEAD translocation induced by wounding was seen at one hour and decreased with time. After 7 hours, only cells at the leading edge of the wound retained nuclear TEAD (Fig 3.1b). The effects of wounding on TEAD translocation is also seen in HaCaT cells, albeit with different kinetics (Supplementary Fig 3.2a). TEAD nuclear accumulation may occur in two ways, through translocation or through synthesis of new proteins. Using cyclohexamide, an inhibitor of new protein synthesis, we observed that TEAD nuclear accumulation was still intact, indicating that nuclear localization of TEAD in cells after induction of injury is due to protein translocation and

not protein synthesis (Fig 3.1c). Because the cells at the leading edge of the wound retain nuclear TEAD while cells several rows back from the wound lose nuclear TEAD, we suspected that perhaps loss of cell-cell contacts at the leading edge promotes TEAD nuclear accumulation. Cell-cell contact is sensed through cell-cell junctions, namely adherens junctions and desmosomes as well as tight junctions. Cell-cell junctions are known to affect intracellular signaling and regulate proliferation, differentiation, and tissue homeostasis (Garcia et al 2018). Because HEK293A cells do not possess tight junctions, we only utilized cells deficient in core components of adherens junctions and desmosomes to investigate whether cell-cell contact plays a role in injury induced TEAD nuclear translocation. Cells deficient in cell-cell junction components α-catenin (CTNNA1), β-catenin (CTNNB1), Desmoplakin (DSP), Plakiglobin (JUP) and Angiomotin (AMOT) did alter TEAD nuclear accumulation (Supplementary Fig. 3.3a). Though these cell-cell junction components did not affect TEAD translocation, further work will be needed to validate whether or not cell-cell junctions play a role.

Signaling through Focal Adhesion Kinase (FAK) has been shown to play important roles in wound healing, regeneration, and cancer (Owen et al 2011, Sieg et al 1999, Sulzmaier et al 2014, Wong et al 2014) and thus may play a role in mediated TEAD nuclear translocation upon injury of the cell monolayer. However, inhibition of FAK with two separate inhibitors (PF573228 and PF431396) did not inhibit TEAD translocation. In addition, treatment of the cells to inhibit Src signaling downstream of FAK using Dasatinib, did not prevent TEAD translocation. Finally, inhibition of ROCK with Y27632 to inhibit actin stress fiber assembly and focal adhesion assembly also did

not inhibit TEAD nuclear accumulation (Supplementary Fig 3.3b). The processes involved in wound repair, such as cell proliferation and cell migration, often depend on rearrangement of the cytoskeleton. For this reason, we inhibited cytoskeleton rearrangement of the cells with either Nocodazole or Latrunculin B to observe the role of the cytoskeleton on TEAD translocation. Disruption of microtubules with Nocodazole or actin assembly with Latrunculin B showed no effects on TEAD translocation (Supplementary Fig. 3.3c). The composition and availability of extracellular matrix proteins also plays a role in wound repair (Chester & Brown 2017), however seeding cells on either laminin or fibronectin did not affect TEAD nuclear translocation as compared to cells seeded on poly-lysine (Supplementary Fig 3.3d). This suggests that TEAD nuclear translocation upon injury is not specific to a particular extracellular matrix composition. To determine the proliferative potential of the cells after injury, cells were stained with Ki67, a marker for cell proliferation. As expected, in a confluent cell monolayer, few cells are in active phases of the cell cycle and the positive Ki67 staining correlates with nuclear TEAD. However, upon injury, when TEAD translocation occurs, cells shift to active phases of the cell cycle (Fig 3.1d) suggesting that TEAD is necessary during wound healing to drive cell proliferation and wound closure. Accordingly, TEAD also seems to play a role in wound repair *in vivo*. In a DSS intestinal injury induced model, TEAD nuclear translocation is seen at day 3 of DSS administration and remains nuclear at day 7 as compared to the uninjured, normal control (Fig 3.1e). Additionally, in DSS induced colitis-associated cancer (CAC), TEAD is nuclear in the cancerous tissue as compared to the normal control in which TEAD is

largely cytoplasmic (Fig. 3.1e). Thus, TEAD plays a role in *in vivo* tissue repair and regeneration and may also be advantageous to the growth and development of cancers.

TEAD translocation is regulated by extracellular matrix proteins.

Though laminin and fibronectin did not show differences in injury induced TEAD translocation, these extracellular matrix proteins did play a role on TEAD nuclear accumulation in a confluent, undisrupted cell monolayer. Cells seeded on poly-lysine showed cytoplasmic staining of TEAD at high cell density, however, cells seeded on either laminin or fibronectin showed an inhibition of TEAD cytoplasmic translocation (Fig 3.2a, b). This suggests that the composition of the extracellular matrix can regulate TEAD subcellular localization. The intensity of TEAD nuclear localization is dependent on the amount of available fibronectin. Increasing concentration of fibronectin correlated with an increase in the number of cells with nuclear TEAD (Fig 3.2b, c). Furthermore, the nuclear accumulation of TEAD promoted by fibronectin is mediated through FAK. TEAD nuclear localization induced by fibronectin can be inhibited through inhibition of FAK. Use of FAK inhibitors PF573228 and PF431396 reversed nuclear TEAD levels to that of cells seeded on poly-lysine (Fig. 3.2b, c). Similar results were seen in HaCaT cells (Supplementary Fig. 3.4a). Src is known to form a complex with FAK to initiate signaling cascades resulting in cell motility, cell proliferation, and cell survival (Mitra & Schlaepfer 2006). In the context of TEAD translocation under high cell density, we also found Src to play a role in FAK-mediated TEAD nuclear localization. TEAD nuclear localization induced by fibronectin was inhibited by use of a Src inhibitor, Dasatinib, suggesting that the Src-FAK complex is necessary for fibronectin induced

TEAD nuclear accumulation (Fig. 3.2c, d). We observed that the morphology of cells seeded on fibronectin differed from cells seeded on poly-lysine, the cells were more capable of spreading and were therefore slightly larger. Thus, to investigate whether cell size and shape were playing a role in TEAD translocation we utilized a Polydimethylsiloxane (PDMS) stretch system. Cells were seeded onto a poly-lysine PDMS membrane and stretched to recapitulate the spreading and larger cell size of cells seeded on fibronectin. We observed that stretching of the cells did not induce TEAD nuclear localization (Supplementary Fig 3.5a), indicating that the larger shape and size may not play a role in TEAD localization.

3.3 Discussion

The Hippo pathway plays important roles in tissue growth, regeneration, and homeostasis. The functional processes involved, such as cell proliferation, migration, and survival, may often be dysregulated and lead to development of cancers. Thus, elucidating the mechanisms that control the Hippo pathway and fine tune these biological processes is important for human malignancies. Most studies have focused on the upstream regulation of the Hippo pathway and numerous extracellular signals have been found to modulate Hippo pathway activity. In addition, studies have shown several pathways in the cell that cross-talk with the Hippo pathway and fine-tune cellular response to environmental signals (Hansen et al 2015). Few mutations have been found in upstream Hippo components yet YAP/TAZ remains dysregulated in many cancers (Harvey et al 2013, Plouffe et al 2015), thus investigating Hippo kinase

independent mechanisms of YAP regulation will be valuable. Studying downstream transcriptional regulation of the Hippo pathway through regulation of TEAD will provide critical insight into modulation of Hippo processes.

The finding that TEAD localization can be modulated by injury and extracellular matrix composition indicates that TEAD may be a plausible target for future therapeutics in the fields of regenerative medicine and cancer. The FAK-Src complex has been implicated in cancers and elucidation of the mechanism of TEAD regulation by FAK-Src will provide further therapeutic insight. Because these findings are preliminary, further mechanistic work and evaluation of the biological function of TEAD translocation will need to be completed. However, these data suggest that TEAD translocation can be used to regulate the signaling of YAP/TAZ driven cancers as well as other signaling pathways that cross-talk with the Hippo pathway.

3.4 Experimental Procedures

Cell Culture

All cell lines were maintained at 37°C with 5% CO2. HEK293A, HaCaT, and MEF cells were cultured in DMEM (Invitrogen, 11965118) containing 10% FBS (Gibco, 10437028) and 50 μg/ml penicillin/streptomycin (Invitrogen, 15140122). MCF10A cells were cultured in DMEM-F12 supplemented with 5% horse serum (Invitrogen, 26050088), 20 ng/ml EGF (Peprotech, AF-100-15), 0.5 μg/ml hydrocortisone (Sigma, H4001-25G), 100 ng/ml cholera toxin (Sigma, C8052-2MG), and 10 μg/ml insulin (Sigma, I1882-100MG). The following compounds were used to treat cells: Disruption of

F-actin (latrunculin B, 0.2µg/ml), disruption of microtubules (Nocodazole, 200ng/mL), Src inhibition (dasatinib, 5µM), FAK inhibition (PF573228, 10µM) (PF431396, 10µM), inhibition of protein synthesis (Cyclohexamide, 100µg/mL, 3hr pre-treatment), ROCK inhibitor (Y27632 5µM). No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. The cell lines were not authenticated.

Immunofluorescence microscopy

Cells were seeded in 12 well plates on coverslips 1 days prior to experimentation. Coverslips were pretreated with Poly-L-ornithine solution (Sigma, P4957) diluted 1:20, laminin (10µg/ml), or fibronectin (10µg/ml) overnight at 37°C, with a quick phosphate-buffered saline (PBS) wash prior to cell seeding. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, 2280) for 15 min followed by permeabilization with 0.1% Triton-X for 5 mins. Cells were blocked in 3% BSA for 1 hr and incubated overnight at 4°C in primary antibodies diluted in 3% BSA. Secondary antibodies were diluted in 3% BSA and incubated for 1 hr. Slides were mounted with prolong gold antifade reagent with DAPI (Invitrogen, P36931). Each image is a single *Z* section at the same cellular level. Images were captured with a Nikon Eclipse T*i* confocal microscope. Images depicted in figures were exported from NIS elements imaging software.

In Vitro Scratch Assay

Cells were plated at high density $1x10^6$ cells/well in a 12-well plate 1 day before induction of injury. To induce the wound, coverslips were removed from the well and cells were scratched and scraped from the coverslip using a clean, sterile razor. After scratch, coverslips were submerged in PBS once to wash away cellular debris and replaced back into the original well.

Antibodies

The following antibodies were purchased from Cell Signaling and used at the indicated dilution for immunohistochemistry and immunofluorescence: pan-TEAD (13295, 1:1000), Myc tag (2276, 1:1000). The following antibodies were purchased from Santa Cruz Biotechnology and used at the indicated dilution for immunofluorescence: YAP (sc-101199, 1:1000). TEAD1 (610923, 1:1000) was purchased from BD.

Mutagenesis

TEAD site-directed mutagenesis was carried out using Q5 Hot Start High Fidelity DNA Polymerase from New England Biolabs (M0494) per manufacturer protocol.

Immunohistochemistry

Tissues were subject to heat induced antigen retrieval using 10mM sodium citrate buffer followed by 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Sections were incubated overnight at 4°C with pan-TEAD antibody and detected using Vectastain elite ABC kit and DAB Peroxidase Substrate kit (Vector Laboratories) as per

manufacturer protocol. Intestinal injury slides were provided by from Dr. Wendong Huang, City of Hope.

3.5 Acknowledgements

Chapter 3, in full, is unpublished material; Lin, K.C., Guan, K.L. The dissertation author was the primary investigator and author of this material.

Figure 3.1: TEAD localization is regulated during wound healing and tissue regeneration.

a, Immunofluorescence of TEAD in various cell lines at high cell density. In several cell lines, TEAD translocates to the cytoplasm upon high cell density. **b,** Using an in vitro scratch assay, injury was induced in a confluent cell monolayer. Immunofluorescent staining of TEAD shows the kinetics of TEAD translocation at the indicated time points. **c,** cells were pre-treated with CHX before scratch. Cells were fixed 1hr after scratch and stained for TEAD to observe localization. **d,** Cells were scratched and fixed after 1hr. Cells were then stained for proliferative marker, Ki67 and TEAD. Cells that have nuclear TEAD correlate with cells in active phases of the cell cycle. Scale bars in a-d are 20μm.

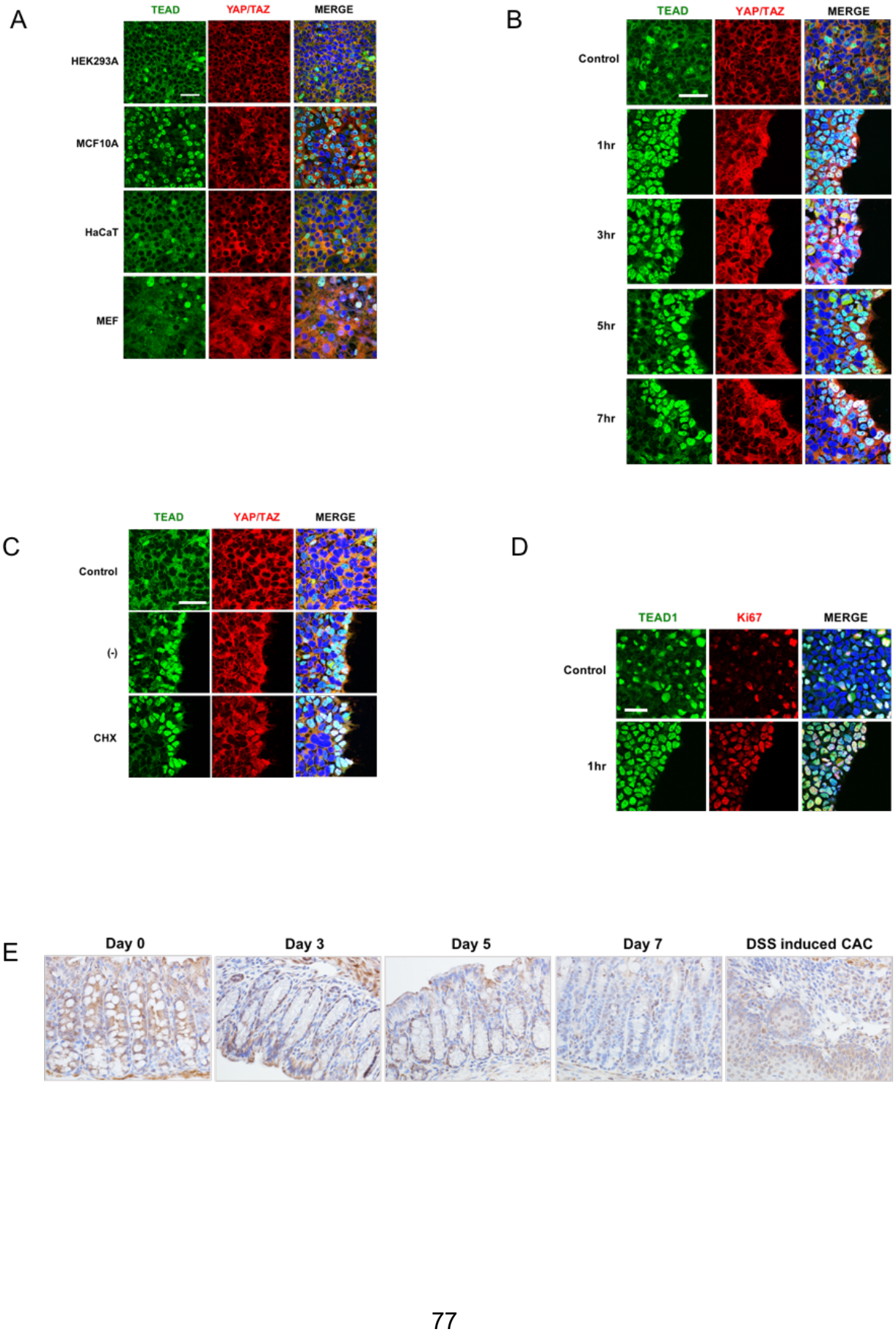
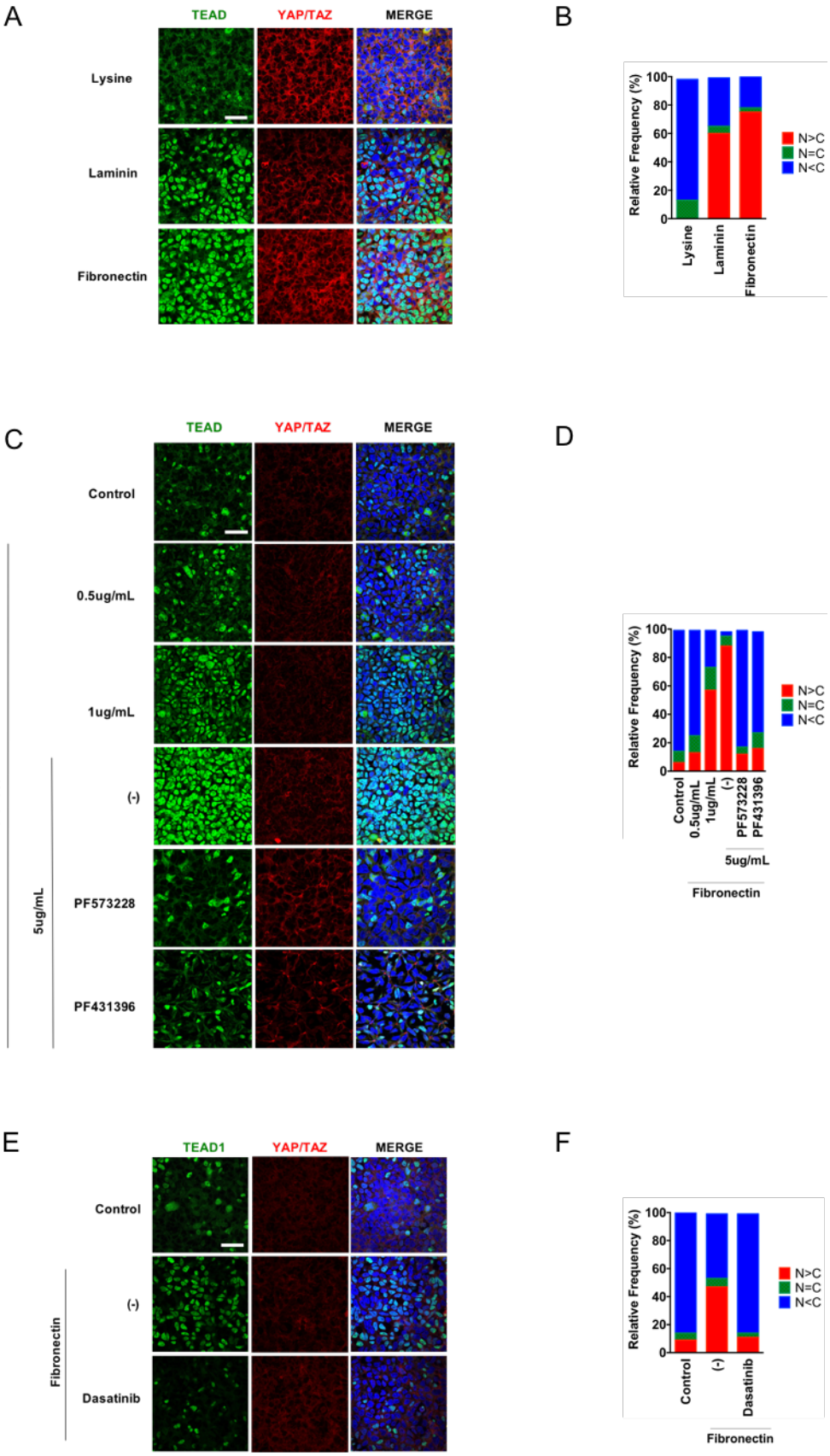

A
TEAD
YAP/TAZ
MERGE
HEK293A
MCF10A
HaCaT
MEF
B
TEAD
YAP/TAZ
MERGE
Control
1hr
3hr
5hr
7hr
C
TEAD
YAP/TAZ
MERGE
Control
(-)
CHX
D
TEAD1
Ki67
MERGE
Control
1hr
E
Day 0
Day 3
Day 5
Day 7
DSS induced CAC

Figure 3.2: FAK-Src signaling regulates TEAD nuclear translocation.

a, HEK293A cells were seeded onto coverslips coated with the indicated extracellular matrix proteins. Immunofluorescent staining of TEAD shows differential TEAD localization in the presence of extracellular matrix proteins. **b,** Quantification of panel a. **c,** Cells were seeded on increasing doses of fibronectin in the presence or absence of FAK inhibitors, PF573228 and PF431396. Treatment with FAK inhibitors can reverse fibronectin induced TEAD nuclear accumulation. **d,** Quantification of panel c. **e,** Cells were seeded on fibronectin with or without Src inhibitor, Dasatinib. Treatment with Dasatinib blocked the effects of TEAD nuclear localization by fibronectin. **f,** Quantification of panel e. Scale bars in a, c, and e are 20μm.


A
TEAD
YAP/TAZ
MERGE
Lysine
Laminin
Fibronectin
B
Relative Frequency (%)
100
80
60
40
20
0
N>C
N=C
N<C
Lysine
Laminin
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C
TEAD
YAP/TAZ
MERGE
Control
0.5ug/mL
1ug/mL
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PF573228
PF431396
Fibronectin
5ug/mL
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Relative Frequency (%)
N>C
N=C
N<C
Control
0.5ug/mL
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PF573228
PF431396
5ug/mL
Fibronectin
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TEAD1
YAP/TAZ
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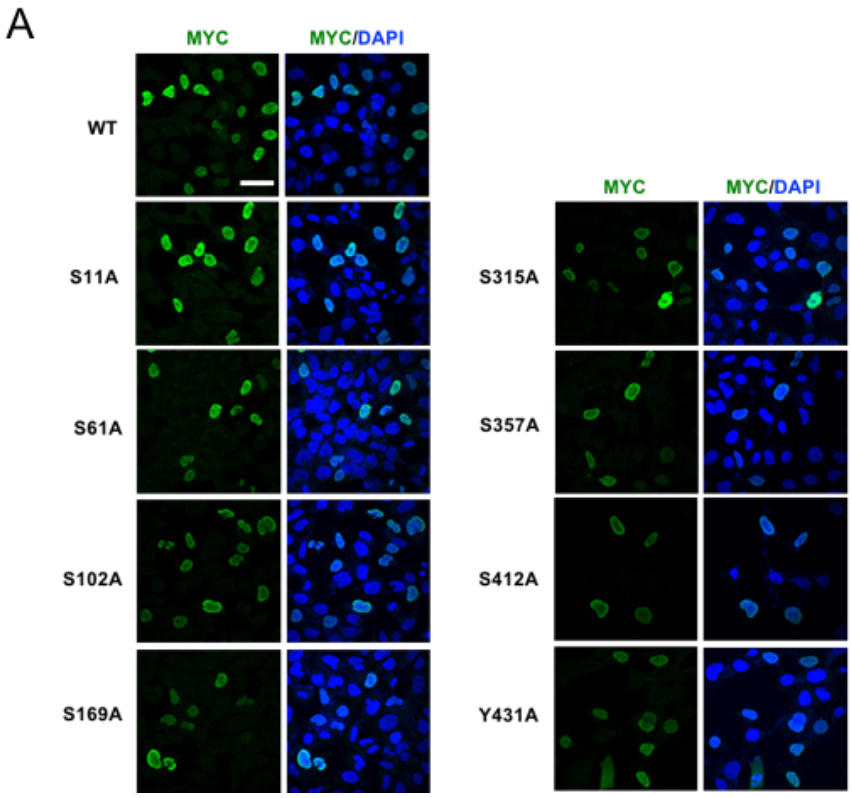

Figure S3.1: Mass Spectrometry identified putative post-translational modifications do not effect TEAD translocation.

a, TEAD1-4 were aligned and conserved residues that were potential post-translational modification sites were mutated on Myc-TEAD1. None of the mutated sites altered Myc-TEAD1 localization. Scale bar is 20μm.

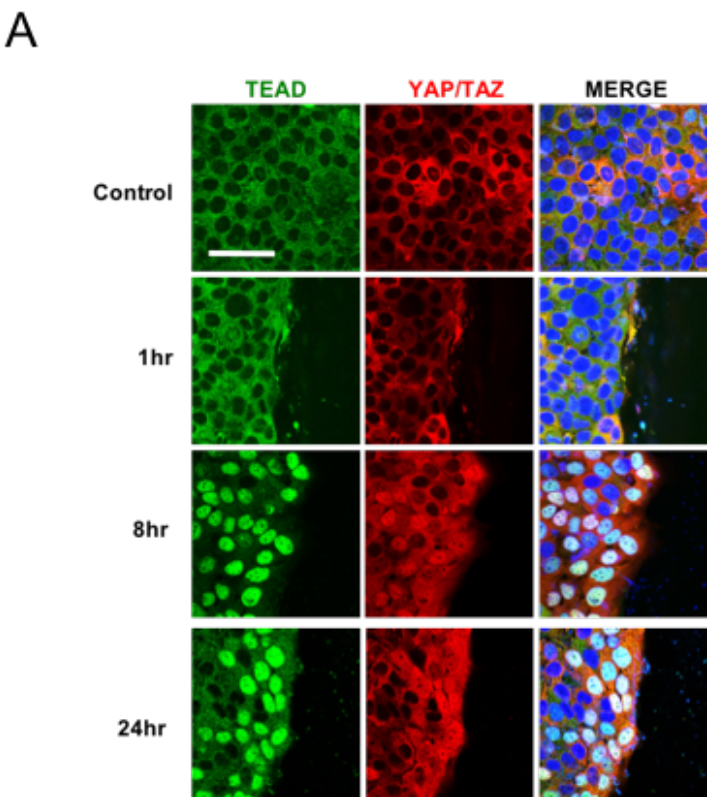

Figure S3.2: TEAD nuclear translocation occurs in HaCaT cells.

a, A scratch was introduced in a confluent monolayer of HaCaT cells and then stained for TEAD. TEAD nuclear translocation occurs in response to injury similar to HEK293A cells, however the kinetics of translocation differ. Scale bar is 20μm.

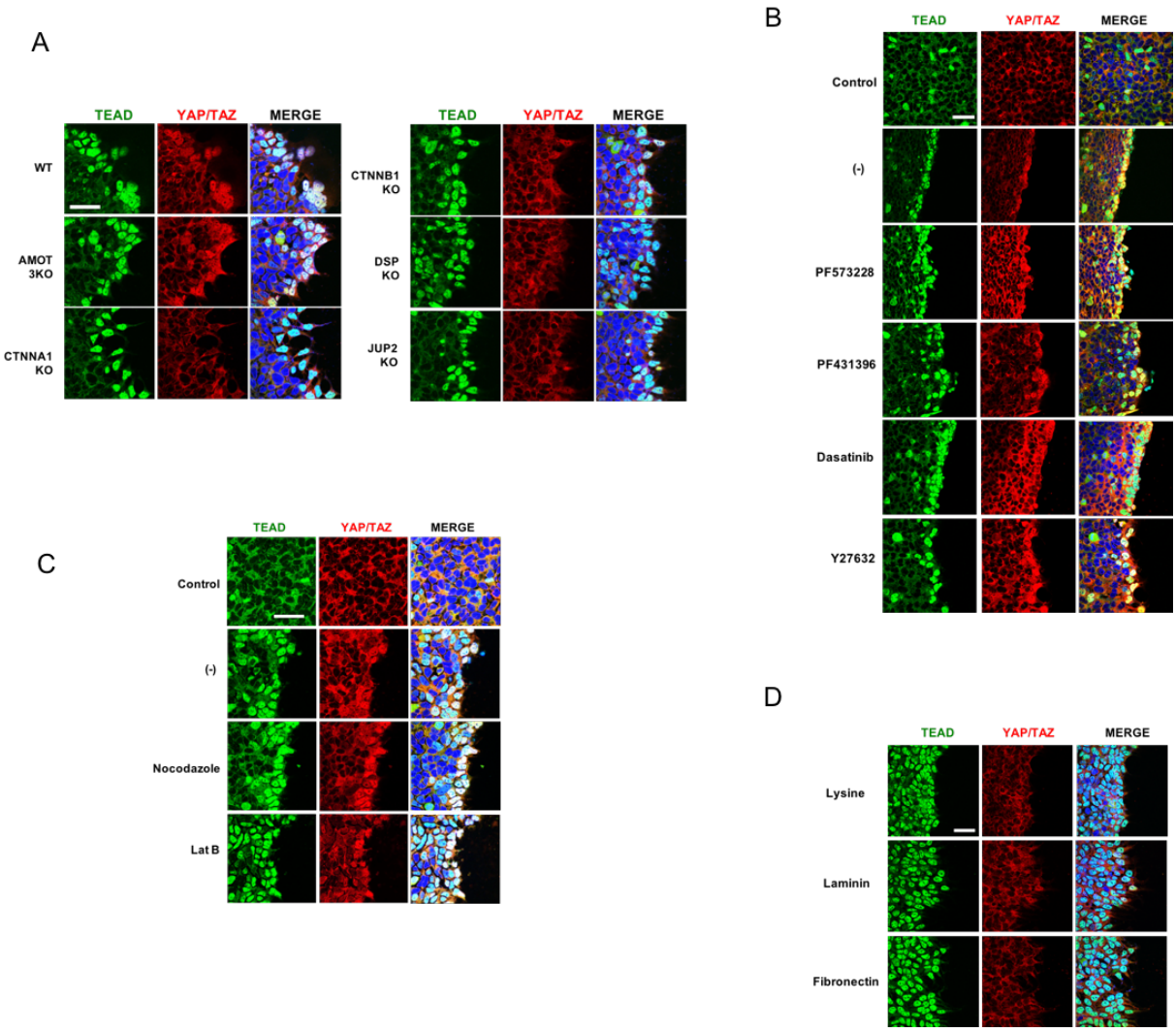

Figure S3.3: Cell-cell junction, cytoskeletal rearrangements, and extracellular matrix components do not play a role in TEAD translocation.

a, HEK293A cells with knockout of adherens junction and desmosome components were stained for TEAD after induction of injury. Loss of cell-cell junction components did not alter TEAD localization. **b,** FAK, Src, and ROCK were inhibited in HEK293A cells upon induction of injury using various inhibitors. Inhibition of FAK, Src, and ROCK did not inhibit TEAD nuclear localization upon injury. **c,** Rearrangement of the cytoskeleton was inhibited by inhibiting microtubule formation and actin assembly using Nocodazole and Latruculin B, respectively. Inhibition of cytoskeletal rearrangement did not inhibit TEAD nuclear localization. **d,** Cells were seeded on lysine, laminin, or fibronectin and then subjected to wounding. Extracellular matrix proteins do not effect TEAD localization upon injury. Scale bars in a-d are 20μm.

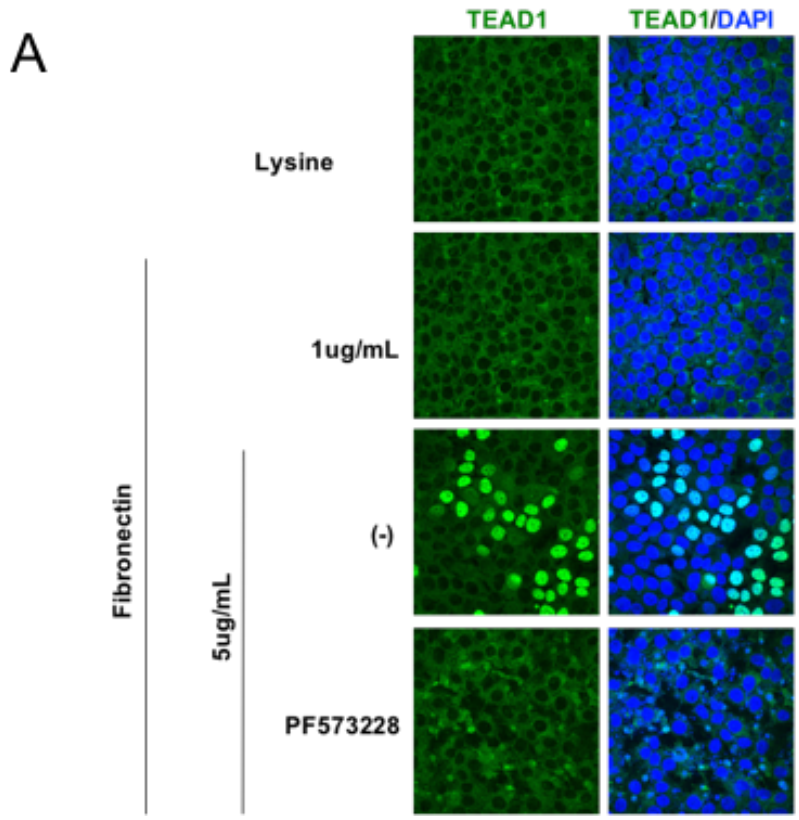

Figure S3.4: Fibronectin induces TEAD nuclear localization in HaCaT cells through FAK.

a, Cells were seeded on increasing doses of fibronectin in the presence or absence of FAK inhibitor, PF573228. Treatment with FAK inhibitor reverses fibronectin induced TEAD nuclear accumulation.

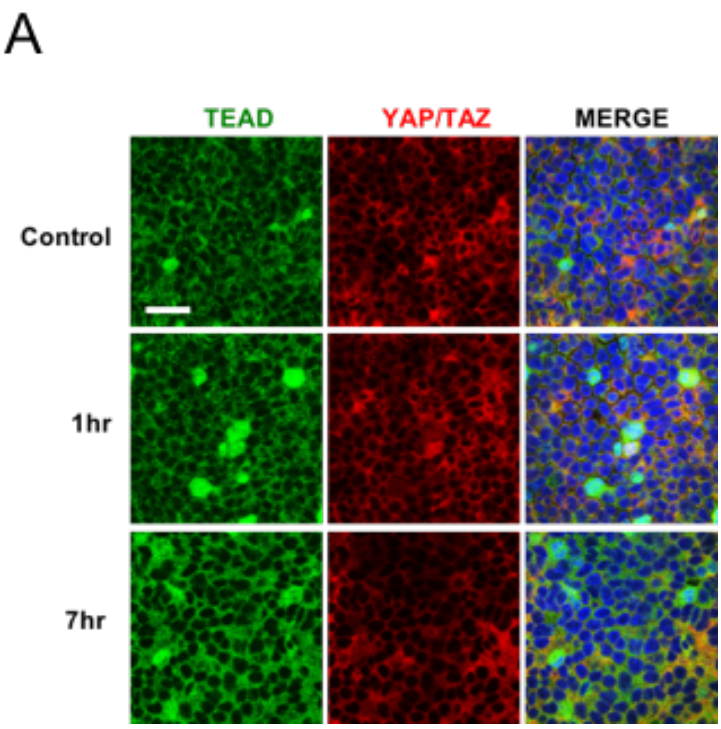

Figure S3.5: Cell size and shape does not mediate TEAD nuclear translocation.

a, Cells were seeded on PDMS coated with lysine and allowed to attach overnight. The membrane was then stretched to increase cell area by 10% for either 1 hr or 7 hrs. Increasing cell area did not promote TEAD nuclear localization. Scale bar is 20μm

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Chapter 4: Conclusion

4.1 Conclusion and future directions

The Hippo pathway has garnered great interest as a master regulator of cell growth, proliferation, and homeostasis. The Hippo pathway coordinates single cell growth to growth of tissues for organ homeostasis in multicellular, complex organisms. Studies have shown the Hippo pathway to be indispensable in development, stem cell function, and tissue regeneration(Johnson & Halder 2014, Mo et al 2014). The key components of the pathway are comprised of serine/threonine kinases Mammalian STE20-like kinases (MST1/2), Large Tumor Suppressor kinases (LATS1/2), and transcription co-activators Yes-associated protein (YAP) and its paralog Transcriptional Activator with PDZ binding domain (TAZ). Activated MST1/2, in a heterodimeric complex with their adaptor protein Salvador Homolog (SAV1), phosphorylates LATS1/2 and their adaptor protein Mob1 homolog (MOB1)(Callus et al 2006, Praskova et al 2008, Tapon et al 2002, Wu et al 2003) (Fig.1). Recently, it has also been shown that members of the MAP4K4 family also phosphorylate and activate LATS(Meng et al 2015, Zheng et al 2015), thus being considered as a core component of the Hippo pathway. Inactivation of YAP/TAZ via phosphorylation by LATS1/2 results in YAP/TAZ cytoplasmic sequestration by binding to 14-3-3 or targeting of YAP/TAZ for ubiquitination and degradation (Hao et al 2008, Liu et al 2010, Zhao et al 2010, Zhao et al 2007). When the Hippo pathway is inactive, YAP/TAZ are dephosphorylated and accumulate in the nucleus, where they bind to transcription factors to regulate target gene expression. The TEAD family of transcription factors are the main binding partners of YAP/TAZ(Vassilev et al 2001, Zhao et al 2008). When YAP/TAZ are activated and

translocated to the nucleus, binding to TEAD drives expression of a wide range of target genes such as CTGF and Cyr61(Lai et al 2011, Zhao et al 2008). In the past decade, a slew of studies has expanded our understanding of the Hippo pathway to include an array of upstream signals that modulate YAP/TAZ outputs as well as its diverse biological functions.

In comparison to YAP/TAZ regulation, few studies have elucidated mechanisms regulating TEAD transcriptional activity that is independent of YAP/TAZ binding. Studies have suggested that TEAD is phosphorylated and that this phosphorylation plays a role in regulating the DNA binding ability of TEAD (Gupta et al 2000, Jiang et al 2001). Additionally, it has been found that S-palmitoylation of TEAD regulates TEAD protein stability and transcriptional activity (Chan et al 2016, Noland et al 2016). Functionally, palmitoylation of TEAD stabilizes YAP/TAZ binding as palmitoylation deficient TEAD mutants show substantially decreased YAP/TAZ binding and diminished transcriptional activity (Chan et al 2016). It has also been shown that TEAD localization is important for embryonic differentiation. TEAD4 was shown to regulate a transcriptional program necessary for driving trophectoderm differentiation (Home et al 2012). Cells with nuclear localization of TEAD4 differentiate into the trophectoderm lineage whereas cells with cytoplasmic TEAD4 differentiate into the inner cell mass lineage (Home et al 2012). In drosophila, Hippo (Hpo), the homolog of MST1/2, promotes cytoplasmic translocation of Scalloped (Sd), the homolog of TEAD (Cagliero et al 2013).

Much of the work on the role of Hippo in cancer has focused on YAP/TAZ dependent mechanisms of tumorigenesis. Perhaps it is unsurprising that mutations in Hippo core components are relatively rare(Harvey et al 2013) as regulation of YAP/TAZ

can be carried out through a diverse network of upstream signals and regulators, several of which are commonly mutated in cancers(Hansen et al 2015). With such a broad array of regulators, modulating YAP/TAZ signaling by targeting upstream regulators is challenging. Thus, understanding mechanisms regulating the TEAD transcription factor family may prove beneficial in identifying ways of inhibiting YAP/TAZ activity.

Regulation of Hippo pathway transcription factor TEAD by p38 MAPK-induced cytoplasmic translocation

Many extracellular signals are known to regulate YAP/TAZ localization, however, signals that regulate TEAD localization have not been well studied. Under most conditions, including conditions that regulate YAP/TAZ translocation, TEAD is exclusively localized to the nucleus. However, under conditions of high cell density and osmotic stress, TEAD is translocated to the cytoplasm (Fig 2.1a, b). We have made several important observations of TEAD translocation as follows.

Firstly, under osmotic stress, TEAD cytoplasmic translocation is mediated by p38. Osmotic stress induced TEAD cytoplasmic translocation is reversed by p38 inhibitors and is ablated in p38 KO cells (Fig. 2.1c, e). Overexpression of p38 and its upstream activating kinase MKK3 is sufficient to drive TEAD cytoplasmic translocation (Fig. 2.1d).

Secondly, TEAD cytoplasmic translocation is mediated by direct binding to p38. TEAD and p38 are shown to bind directly using immunoprecipitation assays (Fig. 2.2 a-e). p38 substrates have a conserved binding sequence known as the D domain. This D

domain is recognized by the CD/ED site on p38(Cargnello & Roux 2011, Tanoue et al 2000, Tanoue et al 2001). Deletion of the D domain on TEAD or mutation of the CD/ED site on p38 abolishes TEAD-p38 binding as well as TEAD cytoplasmic translocation (Fig. 2.2 h-n).

Thirdly, TEAD cytoplasmic translocation prevents YAP activation. TEAD cytoplasmic translocation can override YAP activating signals. In LATS1/2 KO cells, in which YAP is constitutively phosphorylated, activated, and accumulated in the nucleus, osmotic stress induces TEAD cytoplasmic translocation, and subsequently, YAP cytoplasmic translocation (Fig. 2.3c, d). Though YAP remains constitutively dephosphorylated with the addition of osmotic stress, it is unable to accumulate in the nucleus due to absence of TEAD (Fig. 2.3c, d). The role of TEAD in retaining YAP in the nucleus is further validated in TEAD KO cells. In TEAD KO cells, YAP phosphorylation is similar to that in wild-type cells, becoming phosphorylated under serum starvation and dephosphorylated with either LPA or serum stimulation (Fig. 2.3f). However, despite a normal phosphorylation response, YAP is unable to localize to the nucleus in TEAD KO cells, even in the YAP stimulating conditions (Fig. 2.3g). We conclude that the presence of TEAD in the nucleus is required to retain active YAP in the nucleus. Therefore, by inhibiting TEAD activity by modulating localization, we may be able to surpass all YAP activating signals.

Lastly, we tested the effect of TEAD cytoplasmic translocation on YAP-driven cancer cell lines and found that TEAD cytoplasmic translocation restricts YAP-driven cancer cell growth. TEAD cytoplasmic translocation induced by osmotic stress preferentially restricted cell growth as compared to YAP-independent cancers (Fig.

2.4h). Furthermore, overexpression of p38 in the YAP-driven mesothelioma cell line MSTO-211H inhibited cell growth in vitro and in vivo (Fig. 2.4j-m). Furthermore, the inhibited growth by p38 overexpression could be rescued by constitutively active TEAD (Fig. 2.4j-m).

Therefore, our study provides the first observation that TEAD localization can be dynamically regulated by osmotic stress. Furthermore, inducing TEAD cytoplasmic translocation can override YAP activating signals. YAP/TAZ is commonly activated in cancers despite the absence of mutations in upstream (Harvey et al 2013), thus finding a therapeutic target in YAP driven cancers is challenging. In addition, several signaling networks converge on YAP/TAZ regulation, and targeting one upstream regulator may not sufficiently inhibit YAP/TAZ activity. Thus, inhibiting YAP/TAZ downstream signaling, such as TEAD activity, may prove therapeutically beneficial in cancers with amplification of either YAP/TAZ or TEAD. Future studies elucidating Hippo-independent mechanisms of TEAD regulation will provide greater insight on how to better treat YAP/TAZ or TEAD driven cancers.

Regulation of TEAD by cell-cell contact and cell-matrix interaction.

In our initial screen for signals promoting TEAD cytoplasmic translocation, we identified two signals capable of inducing TEAD cytoplasmic localization. We observed that osmotic stress and high cell density induced TEAD cytoplasmic localization. In regards to osmotic stress, we discovered that p38 mediated TEAD translocation. We next attempted to elucidate the mechanism of TEAD translocation under high cell density.

Firstly, we observed that under high cell density, TEAD is translocated to the cytoplasm, however, after loss of cell-cell contacts by induction of a wound to the cell monolayer, TEAD accumulated in the nucleus (Fig. 3.1a, b). Furthermore, nuclear translocation of TEAD correlated with cells reinitiating and entering active phases of the cell cycle (Fig. 3.1d). TEAD nuclear translocation in response to injury was also observed in vivo in a DSS induced injury model. After induction of injury TEAD translocated to the nucleus of intestinal cells (Fig. 3.1e). In a DSS induced CAC model, TEAD is also found to be nuclear in the cancer tissue (Fig. 3.1e).

In addition, we observed that the composition of the extracellular matrix also can impact TEAD translocation. Presence of laminin or fibronectin inhibited high cell density induced TEAD cytoplasmic translocation (Fig. 3.2a, b). TEAD nuclear accumulation in the presence of fibronectin was due to FAK/Src signaling (Fig. 3.2c-f).

This work is the first observation that FAK/Src signaling in response to the presence of fibronectin can regulate TEAD localization. Though preliminary, the data suggests that TEAD localization plays a role in wound repair and tissue regeneration. It is known that upon tissue injury, matrix proteins such as fibronectin are critical in tissue repair(Chester & Brown 2017). Thus, our observations that TEAD is nuclear after injury in vitro and in vivo as well as in the presence of fibronectin, suggest that TEAD plays a critical role in regulating the transcriptional program needed for successful regeneration. The presence of TEAD in the DSS induced CAC also suggests that cells may take advantage of the transcriptional program of TEAD for cancer development and progression.

Further studies will be needed in order to carefully investigate the mechanism by which FAK/Src regulates TEAD translocation, what the biological role of TEAD is in tissue regeneration and whether all nuclear transcriptional programs driven by TEAD are YAP-dependent. In addition, it would be interesting to examine whether TEAD has significant biological functions in the cytoplasm.

4.2 Acknowledgements

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