Control of cellular responses to mechanical cues through YAP/TAZ regulation

Ishani Dasgupta and Dannel McCollum*

Department of Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, 01605

Running Title: Mechanical regulation of YAP/TAZ

*Address correspondence to: Dannel McCollum, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA, 01605. Tel.: (508) 856-8767. E-mail: dannel.mccollum@umassmed.edu

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Abstract
To perceive their three-dimensional environment, cells and tissues must be able to sense and interpret various physical forces like shear, tensile, and compression stress. These forces can be generated both internally and externally in response to physical properties, like substrate stiffness, cell contractility, and forces generated by adjacent cells. Mechanical cues have important roles in cell fate decisions regarding proliferation, survival, differentiation as well as the processes of tissue regeneration and wound repair (1). Aberrant remodeling of the extracellular space and/or defects in properly responding to mechanical cues likely contributes to various disease states such as fibrosis, muscle diseases, and cancer (2). Mechanotransduction involves the sensing and translation of mechanical forces into biochemical signals, like activation of specific genes and signaling cascades that enable cells to adapt to their physical environment. The signaling pathways involved in mechanical signaling are highly complex, but numerous studies have highlighted a central role for the Hippo pathway and other signaling networks in regulating the YAP and TAZ (YAP/TAZ) proteins to mediate the effects of mechanical stimuli on cellular behavior. How mechanical cues control YAP/TAZ has been poorly understood. However, rapid progress in the last few years is beginning to reveal a surprisingly diverse set of pathways for controlling YAP/TAZ. In this review we will focus on how mechanical perturbations are sensed through changes in the actin cytoskeleton, and mechanosensors at focal adhesions, adherens junctions, and the nuclear envelope to regulate YAP/TAZ.

Mechanical forces on cells.
Throughout this review we will be referring to the mechanical environment of a cell to describe both the variety of physical forces that can impinge upon cells as well as their cellular surroundings that can impact the internal and externally generated forces they experience. For example, the mechanical environment of a cell varies tremendously throughout the body depending on the tissue, the composition of the surrounding substrate and its stiffness, and the amount of space available. In addition, cells are affected by tension across the tissue, with some tissues such as the lung airways and blood vessels subjected to regular cyclic stretch due to breathing and the heart pumping blood through the circulatory system. Cells at fluid interfaces are also subjected to shear stress. Being able to respond to each of these stimuli can have important consequences in vivo. Studies from multiple labs have shown that the Hippo pathway and YAP/TAZ not only respond to mechanical stimuli such as cell density, cell area, tissue stretch, shear forces, and substrate stiffness, but are also important mediators of cellular responses to these stimuli (3-11).

What is the Hippo pathway?
The Hippo pathway was originally discovered in Drosophila melanogaster as a pathway that controlled cell proliferation and organ size control (12). Subsequent studies in mammalian cells showed that the core architecture and functions of the pathway are largely conserved in mammalian cells (13). The Hippo pathway’s primary function is to negatively regulate the activity of a pair of homologous transcriptional coactivators called YAP/TAZ. (Note that although distinct functions for YAP and TAZ have been observed (14-16), they are thought to be largely redundant and similarly regulated. Therefore we refer to them collectively as YAP/TAZ unless discussing specific experiments that were only done with one of the two proteins.) YAP/TAZ lack DNA binding domains and control transcription by association with the TEAD DNA binding proteins (17,18). In general YAP/TAZ promote cell proliferation, survival, and maintenance of stem cell fate. For example, YAP/TAZ are important for maintaining stem cell fate in various cell types including: embryonic, neural, bone, and liver stem cells, and their inactivation promotes differentiation of these stem cell types (19-22).

In fact, artificial expression of YAP/TAZ or inactivation of its inhibitors can revert differentiated cells back to a tissue-specific stem/progenitor cell state (22,23).

YAP/TAZ functions are opposed by the Hippo signaling pathway. The core Hippo pathway (Figure 1) consists of the multiple Ste20 family
kinases (MST1/2, TAO-family kinases (TAO), and several MAP4K members), which phosphorylate and activate the LATS1 and LATS2 (LATS1/2) kinases. Multiple accessory proteins such as SAV1, MOB1, and NF2 (also known as Merlin) promote LATS1/2 activation by upstream kinases. Once active LATS1/2 phosphorylates YAP/TAZ on multiple sites to promote its nuclear export (by creating a binding site for 14-3-3 proteins) and/or degradation through the ubiquitin proteasome system. The Hippo pathway has a large number of other components that influence pathway activity through the core regulators, with the exact mechanism of action remaining uncertain for many of them. It has also become clear that YAP/TAZ can be regulated independent of the Hippo pathway (i.e. in a manner that does not require LATS1/2) in response to mechanical cues (5,6). Various Hippo independent mechanisms for regulation of YAP/TAZ will be described later in the review. The relative importance of Hippo dependent and independent pathways may vary with cell type and stimulus.

YAP/TAZ are major mediators of mechanical signals
Mesenchymal stem cells and muscle stem cells (MSCs) will differentiate into distinct tissue types dependent on mechanical factors such as the area they have to attach to substrate, cytoskeletal tension, substrate stiffness, and shear stress (24-28). This behavior presumably reflects normal differences in mechanical environment that these cells encounter in different regions of the body since mesenchymal stem cells cultured on stiff matrices that mimic the collagenous bone are osteogenic whereas, on softer matrices they differentiate into other lineages such as adipocytes (24,25). The ability of substrate stiffness (5) or shear stress (9) to control cell type specific differentiation of MSCs depends on proper regulation of YAP/TAZ. For example, MSCs depleted for YAP/TAZ and grown on stiff substrates were unable to differentiate into osteogenic lineages and instead underwent adipogenic differentiation as if they were on a soft substrate (5).

Regulation of YAP/TAZ controls differentiation in a wide variety of other stem cell types (19-23). Whether mechanical factors play a role in control of cell fate by YAP/TAZ in these cell types is not known.

In endothelial cells, the Hippo pathway and YAP/TAZ play an important role in cell fate choices between survival and proliferation or cell death in response to shear stress or the area of cell substrate the cell has to attach to and spread onto. Human lung microvascular endothelial cells (HMVEC) grown on stiff substrates or large islands show cell spreading and proliferation, conversely when grown on soft matrices or small islands, they undergo apoptosis (29). YAP/TAZ knockdown in endothelial cells grown on large islands resulted in apoptosis, similar to the phenotype observed on soft islands, thus reinforcing the requirement of YAP/TAZ in the geometric control of cell survival (5). YAP/TAZ are also important for mechanical regulation of endothelial cell function in vivo. Studies in zebrafish endothelial cells showed that YAP translocates into the nucleus in response to flow/shear stress and fish lacking YAP show defects in vascular stability (30).

YAP/TAZ appear to not only control cell fate in response to mechanical stimuli, but they may also function to reinforce the cytoskeleton and contractile apparatus in response to mechanical stress. YAP is essential for generating actomyosin-mediated tissue tension that determines proper tissue shape in medaka fish (31). In mouse embryo fibroblast (MEFs), which display low numbers of stress fibers and do not spread or proliferate on soft substrates, cyclically stretching the cells caused them to spread out, form stress fibers, and proliferate (32). These effects were dependent on YAP/TAZ, showing that YAP/TAZ does not just control cell proliferation, but also drives reinforcement of the cytoskeleton in response to mechanical strain. Because tension stimulates YAP/TAZ activity these results suggest that YAP/TAZ could promote their own activity. How this positive feedback cycle is limited is not known, although it is possible that negative feedback could play a role (33) (34,35). One intriguing possibility is that loss of such negative feedback could lead to disease states.
such as cancer. Defects in normal cellular responses to mechanical signaling contributes to various disease states. For example, most normal (non-cancerous) cells display contact/density dependent inhibition of growth, where the cells stop proliferating when they reach a characteristic density (36). These restraints are commonly lost in transformed cells which lack normal density sensing causing hyperproliferation. YAP/TAZ activation causes cells to lose contact/density dependent inhibition of growth (3). Not surprisingly, YAP/TAZ are often found to be overexpressed or activated in cancers (37). How YAP/TAZ are activated is not entirely clear, as mutations in Hippo pathway genes are not commonly detected in cancer. Observations showing that YAP/TAZ are activated on stiff substrates (5) and tissue stiffness correlates with malignancy (2,38) suggest that the mechanical environment could be a major factor in YAP/TAZ activation during tumor progression.

**How are Mechanical stimuli sensed?**

In the following sections we will review progress made in understanding how cells sense mechanical cues and transduce those signals to control YAP/TAZ activity. Cellular structures such as cell-cell junctions, focal adhesions, and the nuclear membrane, sense and respond to internally and externally generated forces. In response to mechanical stress, these mechanoresponsive structures act both to strengthen the cell by enhancing the actin cytoskeleton to which they are connected, but also to regulate YAP/TAZ (Figure 1). In the following sections we will describe how the Hippo pathway appears to monitor levels of filamentous actin (F-actin) as an indirect readout for mechanical force. In addition, we will discuss how mechanoresponsive structures in the cell act through numerous signaling pathways to regulate YAP/TAZ.

**Actin cytoskeleton**

The actin cytoskeleton is known to be highly responsive to mechanical stresses experienced by the cell (5,39,40). Early studies in *Drosophila* and mammalian cells examining regulation of YAP/TAZ by mechanical cues identified an important role for the actin cytoskeleton (4-6,41-43), suggesting that by monitoring the actin cytoskeleton, the Hippo pathway and YAP/TAZ may be able to indirectly sense and respond to changes in the mechanical environment of the cell (Figure 2). These studies showed that manipulation of F-actin levels through mutation or knockdown of regulators of the actin cytoskeleton or treatment with F-actin inhibitory drugs had dramatic effects on Hippo signaling and YAP/TAZ activity. For example, knockdown of actin capping protein (CapZb) causes an increase in F-actin, nuclear YAP/TAZ, and YAP/TAZ target gene expression (5). In general, increasing F-actin levels promotes YAP/TAZ nuclear localization and loss of F-actin causes YAP/TAZ to accumulate in the cytoplasm. The sensitivity of YAP/TAZ to the status of the F-actin cytoskeleton has been harnessed not just for mechanical regulation but also for G-protein coupled receptor signaling, which modulates Hippo signaling by acting through Rho GTPase to increase F-actin levels (44-46) (Figure 2B).

Numerous questions remain regarding which F-actin containing structures are most important for controlling YAP/TAZ localization. Although one study suggests that actin bundles (stress fibers) are more important than branched actin networks generated by the Arp2/3 complex (5), the relative significance of various F-actin structures for YAP/TAZ regulation is not well established. The relative extent to which cells regulate YAP/TAZ by sensing total F-actin levels or cytoskeletal tension generated by F-actin structures remains uncertain. Many of the manipulations that increase F-actin likely also increase tension generated by the cell. Capping protein deletion in the mouse liver activates YAP/TAZ as observed in cultured cells and promotes proliferation and dedifferentiation similar to other studies where YAP/TAZ is activated in the liver (47). Interestingly, these cells also display enhanced contractility and stiffness, showing that the effects of deleting capping protein do not just affect F-actin levels, but they also affect tension generated by the cells, which is important for YAP regulation. Tension sensing pathways that regulate YAP/TAZ will be discussed in later sections.
Here we will review what is known about how F-actin controls YAP/TAZ regulation.

F-actin likely exerts at least some of its effects on YAP/TAZ independent of its effects on cytoskeletal tension since, at least for individual isolated cells, actin disruption is far more potent at inhibiting YAP nuclear localization than inhibiting myosin activity (48). The molecular pathways governing F-actin regulation of YAP/TAZ remain incompletely worked out. F-actin can influence YAP/TAZ activity through both Hippo pathway (LATS1/2) dependent (4,43,49) and independent mechanisms (5,6,48). What factors underlie which pathway predominates in a particular cell type or set of conditions is not well understood. LATS1/2 is activated by F-actin inhibition and the LATS1/2-MOB1 complex is important for YAP/TAZ regulation by F-actin (4,43,49). Regulation of LATS1/2 activation by upstream kinases in response to F-actin disruption appears to be redundantly distributed between several Ste20 family kinases such as MST1/2, MAP4K-family, and TAO (50-53). These kinases phosphorylate LATS1/2 at a site called the hydrophobic motif (HM), which then stimulates the kinase to autophosphorylate at the activation loop (AL) site to become fully active. STK25 was recently shown to directly phosphorylate LATS1/2 at the AL site to stimulate its activity when F-actin is disrupted (54). What remains unclear is whether the LATS1/2 upstream kinases are themselves regulated by F-actin or simply required for other mechanisms that promote LATS1/2 activation by loss of F-actin (Figure 2A). Independent of these upstream kinases, protein kinase A (PKA) can also stimulate LATS1/2 activation by F-actin perturbation (Figure 2A). PKA phosphorylates LATS1/2 at sites separate from the AL and HM motifs and enhances its activity (49). The relationship between PKA, F-actin, and LATS1/2 may be complex since a second study showed that PKA can influence LATS1/2 activity through regulation of the RHO GTPase (55). NF2 may have a role in promoting PKA phosphorylation of LATS1/2 since it can bind to both proteins (49). However, PKA can still activate LATS1/2 in NF2 null cells, suggesting that NF2 may function redundantly (49,55).

Various studies implicate NF2 in regulation of LATS1/2 and YAP/TAZ by F-actin. NF2 acts both in Drosophila (where it is known as Merlin) and mammalian cells to recruit LATS1/2 to the membrane where it can be activated by upstream kinases (56). The authors further showed that in Drosophila the interaction between NF2 and LATS1/2 is stimulated by F-actin disruption, suggesting that this could be a key mechanism for F-actin regulation of Hippo signaling in flies. Similar regulation in mammals has not been shown. In mammals, NF2 is known to be controlled by mechanical stimuli and angiometin proteins, which function in cell polarity and regulation of Hippo signaling by F-actin. At low cell density, phosphorylation on Ser518 by the p21 activated kinase (PAK) keeps NF2 in a closed conformation (57-60). NF2 Ser518 phosphorylation decreases at high cell density allowing NF2 to become active (61,62). Regulation of NF2 by phosphorylation appears to involve angiometins (there are 3 members called AMOT, AMOTL1, and AMOTL2). Angiometins regulate LATS1/2 and YAP/TAZ in response to changes in F-actin (63-65). Loss of NF2 phosphorylation allows binding of AMOT to NF2, which stimulates association of NF2 with LATS1/2 (66). It is not known in vivo if angiometins control NF2 regulation in response to changes in F-actin or cell density.

Angiometins have been shown to act through additional mechanisms to connect F-actin to regulation of LATS1/2 and YAP/TAZ (Figure 2A-B). Angiometins colocalize with (67,68) and bind to F-actin (63,69,70). Several studies identified angiometins as binding partners of LATS1/2 and YAP/TAZ (65,71-76). Angiometins are able to activate LATS1/2 and independently bind and sequester YAP/TAZ in the cytoplasm (71-76). AMOT has been proposed to directly sense F-actin levels in the cell to regulate Hippo signaling and YAP/TAZ activity because AMOT binding to F-actin impairs its ability to bind YAP and promote LATS1/2 activity (63-65). The AMOT binding site for F-actin is immediately flanked by L/PPxY motifs that can bind to the WW
domains of various proteins, including the Hippo pathway proteins SAV and YAP/TAZ. YAP (and possibly SAV) compete with F-actin for binding to AMOT (63,64). Binding to SAV and YAP through the L/PPxY sites is critical both for AMOT to act as a scaffold to promote LATS1/2 activation and YAP phosphorylation (65), and for it to bind and sequester YAP independently from LATS1/2 (63,72-76). These results are consistent with a model where high F-actin levels cause angiomotins to be bound to F-actin and unable to regulate YAP/TAZ (Figure 2B). When F-actin levels decrease, angiomotins are free to activate LATS1/2 (which inhibits YAP/TAZ), and to bind and sequester YAP/TAZ in the cytoplasm (Figure 2A). Consistent with this model, angiomotins can regulate Hippo signaling and YAP in response to mechanical or other stimuli that alter the F-actin cytoskeleton. For example, shear stress triggers formation of enhanced cortical actin bundles that sequester AMOT allowing YAP to translocate into the nucleus (30). AMOT binding to YAP was observed to be dramatically reduced when cells were subjected to shear. In uveal melanoma, oncogenic mutations in the heterotrimeric G protein α subunits, Gαq and Gα11, promote release of YAP from inhibitory binding to AMOT by stimulating F-actin polymerization. AMOT regulation by F-actin has also been implicated in down regulation of YAP to promote neuronal differentiation (77). Thus, angiomotins, in conjunction with NF2, appear to be significant players for control Hippo and YAP/TAZ activity in response to changes in F-actin levels.

**Nuclear Actin Sensing**

F-actin levels also contribute to YAP/TAZ regulation through a distinct mechanism in the cell nucleus (Figure 2, bottom). As in the cytoplasm, mechanical forces on the cell trigger actin polymerization in the nucleus (78,79), although the mechanisms are not well understood. A recent study (79) showed that the Arid1A protein (a component of the SWI/SNF chromatin remodeling complex) binds to and inhibits YAP/TAZ in the nucleus by blocking its interaction with the TEAD DNA binding protein. A WW-domain in YAP/TAZ binds to a PPxY motif in Arid1A. Reminiscent of YAP/TAZ regulation by F-actin and angiomotins, F-actin in the nucleus appears to compete with Arid1A-SWI/SNF for binding to YAP/TAZ. The exact mechanism of the competition is not known since the F-actin binding site(s) in the Arid1A-SWI/SNF complex has not been identified. Thus, mechanical stimuli induce F-actin polymerization in the nucleus causing Arid1A-SWI/SNF to associate with F-actin instead of YAP/TAZ, thereby freeing YAP/TAZ to activate transcription through association with the DNA binding factor TEAD. Arid1A-SWI/SNF do not affect YAP/TAZ nuclear localization, thus this pathway acts in conjunction with angiomotins, Hippo pathway, and other factors that influence YAP/TAZ nuclear localization. Angiomotins have also been reported in the nucleus (80), and it will be interesting in future studies to determine if they can regulate YAP/TAZ in the nucleus in response to changes in nuclear F-actin.

**Positive and negative regulation of YAP/TAZ at adherens junctions**

The actin cytoskeleton is closely connected to mechanical tension. Cells can generate tension autonomously through their actomyosin cytoskeleton. A significant part of the effect on YAP/TAZ caused by F-actin perturbation is likely due to disruption in tension. In epithelial tissues, with strong connections between cells, tension triggered by contractile forces of individual cells or stretch applied to the tissue is transmitted between cells by cell-cell junctions called adherens junctions. Tension sensing at adherens junctions governs tissue remodeling, wound healing, tissue elongation, cell extrusion and migration (81-83). Cellular responses to mechanical stress at adherens junctions are implicated in development, disease, organization and function of multicellular tissues. Central to adherens junctions are the cadherin family proteins which are the primary cell-cell adhesion receptors in adherent cells (84). Adherens junctions are comprised of transmembrane cadherin-catenin complexes and associated proteins which play a pivotal role in maintaining cell-cell contact and intercellular adhesion. They function to regulate cell-cell contact induced growth arrest known as contact inhibition of
proliferation, essential for tissue integrity and homeostasis (85-88). Cadherins interact with cadherins on adjacent cells with their extracellular domains. The cytoplasmic region of cadherins bind β-catenin. β-catenin binds α-catenin, which interacts with the actin cytoskeleton to connect the cadherin-catenin complex to the actin cytoskeletal network (89). This linkage is essential for maintaining the structure, stability and function of adherens junctions (90,91).

Several studies have shown that cadherin-mediated junctions play a role in mechanical force transmission across cell-cell contacts (92-96). Multiple studies also revealed that in absence of tension at cell-cell junctions, α-catenin is in an autoinhibited state but undergoes a tension-induced conformational change to assume an open active state, thereby transmitting tension dependent signals (97-99). Mechanical stress transduced by α-catenin can reinforce junctions. Purified α-catenin showed tension-induced vinculin binding (100), consistent with its ability to recruit vinculin to adherens junctions in response to actomyosin contractility in cells (97,101-103). Tension-dependent recruitment of vinculin by α-catenin promoted junctional actin assembly and E-cadherin accumulation, thereby reinforcing cell-cell junctions in response to stress (102).

Besides providing mechanical stability to tissues, numerous studies have connected junctional mechanotransduction to Hippo signaling (Figure 3). Mechanical strain applied to a dense monolayer of quiescent kidney epithelial (MDCK) cells was transduced to activate YAP and β-catenin mediated cell proliferation by E-cadherin (7). Studies in Drosophila also revealed that reduction of cytoskeletal tension at cell-cell junctions (as judged by the presence or absence of tension sensitive proteins at adherens junctions) disrupted wing growth, whereas increased cytoskeletal contractility promoted Yki (the Drosophila YAP/TAZ homolog) mediated cell proliferation (104). Cell density, like actin cytoskeletal contractility, was also reported to be an important determinant of mechanical tension at cell junctions, with tension decreasing as cell density increased in developing wing discs. Studies in both Drosophila and mammalian cells showed that the process of contact inhibition of proliferation involves modulation of Hippo signaling by tension dependent recruitment of LIM domain family proteins at adherens junctions. In Drosophila, localization of the LIM domain protein Jub (Ajuba, LIMD1, and WTIP in mammals) to adherens junctions was promoted by tension (104). Tension dependent localization of Jub to adherens junctions at low cell density triggered complex formation with Warts (the Drosophila LATS1/2 homolog), thereby impeding its activity, which increased Yki (YAP/TAZ) activation and cell proliferation. Conversely, under conditions of low tension, (either by inhibiting myosin II activity or at high cell density), Jub and Warts recruitment to junctions was impeded, resulting in Yki inactivation and decreased cell proliferation. Localization of Jub to adherens junctions is controlled by tension dependent recruitment by α-catenin (104-106). Interestingly α-catenin associates with both Vinculin (97) and Jub (106) via a tension-induced conformational change in α-catenin through distinct binding sites. This suggested a mechanism wherein cytoskeletal tension at junctions at low cell density triggered association between α-catenin and Jub, leading to an inhibitory recruitment of Warts and a concomitant increase in Yki activity and cell proliferation.

Two mammalian LIM domain proteins related to Jub in Drosophila called LIMD1 and TRIP6 are also implicated in tension-dependent regulation of Hippo signaling (107,108). LIMD1 is one of the three mammalian Jub homologs (LIMD1, AJUBA, and WTIP), and TRIP6 belongs to a related family of LIM domain proteins that also includes Zyxin and LPP. Similar to observations in Drosophila, LIMD1 and TRIP6 promote tension dependent recruitment and inhibition of LATS1/2 at adherens junctions as well as YAP/TAZ nuclear localization and activation (107,108). Like Jub, LIMD1, TRIP6 and LATS1/2 localization at adherens junctions was compromised at high cell density, likely due to decreased cytoskeletal tension, as evident from the impaired junctional localization of activated
myosin and vinculin (107,108). Simulation of epithelial mechanics (109-111) and experimental data obtained in Drosophila (110) are also consistent with reduced tension at cell-cell junctions at high cell density. Inhibition of YAP under high density was reduced by stimulating actin cytoskeletal tension (107) or physically stretching the cells (108). How LIMD1 and TRIP6 cause LATS1/2 inhibition is not entirely worked out, but in the case of TRIP6, tension promotes binding of TRIP6 to LATS1/2 and recruitment of LATS1/2 to adherens junctions. Binding of TRIP6 to LATS1/2 blocks association of LATS1/2 with its activator MOB1, providing a mechanism for LATS1/2 inhibition (108). It is also possible that adherens junction localization of LATS1/2 sequesters it away from its activators as has been proposed in Drosophila (112). The relationship between LIMD1, TRIP6, and other adherens junction proteins is not clear, but LIMD1 is in close proximity with α-catenin (107), and TRIP6 binds to vinculin and depends on vinculin for recruitment to adherens junctions (108). Combining evidence from Drosophila and mammalian cells suggests a model (Figure 3) where, at low cell density, tension activates α-catenin stimulating it to recruit LIMD1 and vinculin, which recruits TRIP6. Somehow both LIMD1 and TRIP6 are required for recruitment of LATS1/2. It will be important in future studies to determine how LIMD1 and TRIP6 function together to promote tension dependent inhibition of LATS1/2.

The studies discussed above describe how at low cell density tension-dependent signaling at adherens junctions promotes YAP/TAZ activation (Figure 3A). However, adherens junction signaling has also been identified as a primary negative regulator of YAP/TAZ and cell proliferation at high cell density (7,87,113,114). A variety of mechanisms have been identified for this inhibition that may vary with cell type, including both Hippo dependent (113) and independent mechanisms (87,114,115). In mammary epithelial cells at high density E-cadherin engagement was required for inhibition of cell proliferation and cytoplasmic localization of YAP in a manner that required the adherens junctions components α-catenin and β-catenin as well as core the Hippo pathway components NF2, LATS1/2, and Kibra (7,113). In contrast, in keratinocytes two reports show that α-catenin inhibits YAP activity through Hippo pathway independent mechanisms (87,114). Loss of α-catenin caused YAP/TAZ activation, cell proliferation, and squamous cell skin tumor formation in mice. The first study found that α-catenin bound to a complex of YAP and 14-3-3 protein to protect it from being dephosphorylated on LATS1/2 phosphorylation sites by the PP2A phosphatase, thereby inactivating YAP (114) (Figure 3B). The second study showed that α-catenin inhibits YAP through inhibition of SRC kinase (more on SRC inhibition of YAP below) (87). Interestingly, they observed that in α-catenin null cells at high cell density, β4-integrin localizes aberrantly to cell-cell junctions and recruits and activates SRC, which can then phosphorylate and activate YAP. The exact mechanism underlying each of these observations remains to be worked out.

It is unclear how adherens junctions switch from activation of YAP/TAZ to inhibition as they transit from low to high cell density. A general mechanism could be that at low density, mechanical strain sensed by the α-catenin tension sensor acts together with LIM domain proteins to promote YAP/TAZ activity (107,108) (Figure 3A). At high cell density these mechanisms are turned off (Figure 3B). Aside from loss of LATS1/2 inhibitors from adherens junctions at high cell density, what other mechanisms might trigger YAP/TAZ inhibition? One study suggests a role for remodeling of junctions and associated actin structures at high cell density in YAP/TAZ inhibition (116) (Figure 3B). This work showed that in polarized epithelial cells, actin organization shifts from mainly stress fibers at lower density to circumferential actin-myosin belts at higher density. Interestingly, this study found that the cortical actin-myosin belts in high density cells exerted tension that was required for NF2 to inhibit YAP/TAZ nuclear localization, which inhibited cell proliferation. The authors showed that while it was previously demonstrated that NF2 interacts with α-catenin and F-actin at low cell density to stabilize adherens junctions (117) (Figure 3A), at high cell density (Figure 3B),
increased tension in the circumferential actin-myosin belt caused NF2 to be released from the adherens junctions so that it could inhibit YAP/TAZ by promoting its nuclear export (116). How tension generated by the actomyosin belt at high cell density triggers NF2 release from adherens junctions is not known. Presumably the tension generated by the actomyosin belt at high density has different effects on YAP/TAZ compared to tension generated by stress fibers at lower cell density because of changes in junctional organization and/or differences in the junctional forces in each situation. This mechanism may be restricted to polarized epithelial cells that form actomyosin belts, as it was not observed in fibroblasts and non-polarized epithelia. Further research is clearly required to understand how cell-cell junctions remodel and interpret forces as they transit from low to high cell density.

Focal adhesions, substrate stiffness, and YAP/TAZ regulation

Just as cadherins mediate cell-cell interactions, integrins are transmembrane proteins that connect cells to their substrates. Integrin extracellular domains interact with the substrate and intracellular domains interact with various proteins to assemble complex structures called focal adhesions that connect to the actomyosin cytoskeleton and promote signaling (118). Focal adhesions are known to sense and respond to mechanical tension to promote cell spreading and migration based on substrate stiffness (119). Integrin engagement with extracellular substrate triggers activation of FAK and SRC tyrosine kinases (120). Stiffer substrates lead to more robust assembly of focal adhesions and stress fibers, increased activation of the FAK kinase, increased cell spreading, and increased YAP/TAZ activity in a manner that depends on the tension sensing focal adhesion protein talin (40,121). The mechanisms by which focal adhesions regulate the Hippo pathway and YAP/TAZ are highly complex (Figure 4) and, as with adherens junction signaling, may vary depending on cell type. In general, signaling from focal adhesions inhibits the Hippo pathway and promotes YAP/TAZ activity, resulting in increased proliferation and survival on stiffer substrates. Indeed, experiments comparing gene expression profiles of HEK293A cells on stiff versus soft substrates showed that the majority of the gene expression changes depended on YAP/TAZ and LATS1/2 (11), showing that Hippo signaling and YAP/TAZ plays a central role in transcriptional regulation in response to changes in substrate stiffness.

Focal adhesion signaling driven by the FAK and SRC tyrosine kinases regulates the Hippo pathway and YAP/TAZ at multiple levels. FAK and SRC have been shown to promote YAP nuclear localization and activity (122-125). The effects of substrate stiffness on YAP nuclear localization depend at least partly on FAK (122). FAK appears to act through multiple mechanisms. One study showed that FAK promotes association of protein phosphatase 1A (PP1A) with YAP to remove inhibitory phosphorylation on S397 (in mouse YAP) (123). FAK was also shown to promote YAP activity more directly by phosphorylating YAP on Y357 and MOB1 on Y26 (124) (Figure 4). Tyrosine phosphorylation of MOB1 inhibits its binding to LATS1/2. YAP phosphorylation on Y357 and other tyrosines by SRC family kinases promotes the ability of YAP to stimulate transcription, at least in part by enhancing interaction with TEAD (115,126,127). Like FAK, SRC can also directly regulate Hippo proteins (Figure 4). Cell adhesion triggers SRC phosphorylation of LATS1 (and to a lesser extent LATS2) to inhibit its activity (128). SRC also phosphorylates YAP on 3 sites (Y341/357/394) to enhance its activity (115,126). Although core Hippo pathway components have been detected at focal adhesions in crude preparations (129), they are not typically observed at focal adhesions. Thus, it is not clear where in the cell FAK and SRC phosphorylate Hippo components and YAP/TAZ and whether there are specific adaptor proteins that connect these kinases to Hippo pathway components.

FAK and SRC have also been shown to impinge upon Hippo signaling less directly through their downstream signaling pathways. For example, a study in breast epithelial cells (MCF10A) showed that cell adhesion to fibronectin coated substrates inhibited LATS1/2 activity through a FAK–SRC–PI3K–PDK1 pathway (130)
The exact manner by which PDK1 affects Hippo signaling is unclear, but it appears to have a role in assembly of a complex between the core kinases and scaffold proteins (131). This study also found that overexpression of constitutive active SRC in HEK293T cells induced tyrosine phosphorylation of MOB1, SAV, NF2, and LATS1, but not MST2, consistent with some of the studies cited above showing that SRC and FAK can act directly on Hippo pathway components. Integrin signaling has also been shown to regulate Hippo signaling through a RAC-PAK pathway whereby PAK phosphorylates NF2 which inhibits the ability of NF2 to activate LATS1/2 and inhibit YAP (129,132) (Figure 4).

A recent study (11) identified yet another mechanism for how focal adhesions transduce information about substrate stiffness to regulate Hippo signaling. This study focused on the ability of focal adhesions to transduce information about substrate stiffness to modulate the levels of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) (Figure 4, right side). On soft substrates (1kPa) PtdIns(4,5)P2 promotes Hippo signaling and YAP/TAZ inactivation. However, on relatively stiff substrates (40kPa) FAK is activated and reduces PtdIns(4,5)P2 levels by activating phospholipase C (PLC\(_1\)) (133,134). Meng and colleagues revealed a complex pathway that operates downstream of PtdIns(4,5)P2 on soft substrates. PtdIns(4,5)P2 appears to act through phospholipase D (PLD) by recruiting it to the membrane and activating it to produce phosphatidic acid (PA) (135). PA in turn recruits the PDZGEF2 protein which activates the small GTPase RAP2. RAP2 then promotes Hippo pathway signaling through two mechanisms. First RAP2 inhibits Rho. Rho is known to inhibit LATS1/2 and activate YAP/TAZ (5,43-46,55). The mechanism is not certain, but may involve the ability of Rho to promote F-actin assembly and to enhance cell contractility by activation of myosin. Second, RAP2 turns on the LATS1/2 activating MAP4K kinases (MAP4K4/6/7). RAP2 is clearly a major player in regulating YAP/TAZ in response to substrate stiffness because deletion of RAP2 in MCF10A and HEK293 cells prevented nuclear exclusion of YAP on soft substrates, and RAP2 deletion in mesenchymal cells impeded their differentiation into adipocytes when growth on soft substrates. However, it is clear that the RAP2 pathway is not the only one involved in regulating YAP/TAZ in response to substrate stiffness. RAP2 deleted cells still displayed some changes in YAP/TAZ regulated genes in response to substrate stiffness compared to LATS1/2 deleted cells which were largely unresponsive. Thus, one or more of the other focal adhesion pathways described above likely contribute. Although much has been learned about how focal adhesions control Hippo signaling and YAP/TAZ activity, major questions remain. For example, do all of these pathways operate simultaneously in cells? Do different cell types utilize different pathways, and if so why? Are their distinct types of substrate or ranges of substrate stiffness that regulate the relative activation and utilization of each focal adhesion signaling pathway that affects Hippo-YAP/TAZ signaling?

**Force dependent nuclear import of YAP**

Focal adhesions and stress fibers were recently shown to regulate YAP through yet another mechanism (136). Nuclear import of YAP triggered by cell spreading on stiff substrates was shown to depend on nuclear flattening as the cells spread. On soft substrates the cells and nucleus remain rounded and YAP resides in the cytoplasm. However, as substrate stiffness increases the cells become more spread, the nucleus flattens, and YAP is imported into the nucleus. Nuclear flattening and YAP nuclear import depended on focal adhesions, F-actin stress fibers, and the LINC (Linker of the Nucleoskeleton and Cytoskeleton) complexes in the nuclear membrane, with the stress fibers acting to connect focal adhesions to the LINC complex on the nucleus (136,137) (Figure 5). As described earlier in this review, focal adhesions and F-actin stress fibers could be affecting YAP through multiple mechanisms. However it was shown that deforming the nucleus using atomic force microscopy was sufficient to cause YAP to go into the nucleus even when focal adhesions and F-actin were disrupted (136). Although nuclear flattening was proposed to generally increase permeability of nuclear pores, YAP...
may be especially sensitive because it is relatively weakly folded and only just above the size threshold for passive nuclear import. It will be interesting to determine the in vivo contexts where this mechanism operates and how it integrates with other mechanisms for YAP regulation.

**YAP/TAZ regulation – why so complex?**

It has been clear for some time that YAP/TAZ are important mediators of the effects of mechanical stimuli on cells. One problem for the field has been, and will continue to be, that each one of the YAP/TAZ regulatory pathways likely affects multiple other YAP/TAZ pathways. For example, increased levels of F-actin also affect contractility and nuclear flattening. A major challenge will be to understand how the various YAP/TAZ regulatory pathways are integrated to generate cellular responses. One question that naturally arises is why is YAP/TAZ regulation so complicated? This may reflect the exceedingly complex array of mechanical environments that cells experience. Cells encounter extracellular matrices of varying composition and stiffness, variable amounts of space that they can occupy, as well as stretch and shear forces. In addition, different cell types have intrinsically different shapes and tissue organizations. Given this variability, it is not so surprising that YAP/TAZ regulation is so complex and, in all likelihood, even greater complexity will be revealed with additional research. Important challenges in the future will be to determine how cell type, mechanical environment, and tissue organization dictate which YAP/TAZ regulatory pathways are utilized and how they are coordinated. A further challenge will be to determine how YAP/TAZ control cellular responses to mechanical perturbations. Although huge progress has been made, there is clearly much left to learn about control and functions of YAP/TAZ.

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Figure Legends

Figure 1. Overview of YAP regulation by mechanical stimuli. Changes in the mechanical environment of the cell can control YAP activity through multiple Hippo dependent and independent mechanisms. The Hippo pathway acts to inhibit YAP nuclear localization. Several upstream Hippo pathway kinases like, MST1/2, MAP4K-family, and TAO phosphorylate LATS1/2 in the presence of its activator, MOB1. The active, phosphorylated form of LATS1/2 phosphorylates YAP, retaining it in the cytoplasm, thereby resulting in YAP inhibition. Tension sensed at focal adhesions, adherens junctions, and the nuclear envelope, as well as changes in F-actin levels in the cytoplasm and the nucleus controls YAP activity through both Hippo dependent and independent pathways. F-actin levels can be influenced by the mechanical environment through tension sensing at the adherens junctions, focal adhesions, and the nucleus. In turn, F-actin can affect tension experienced by the cell at each of these structures. In sum, increases in F-actin and tension inhibit Hippo signaling and promote YAP activity.

Figure 2. Changes in F-actin levels in response to mechanical stimuli regulates YAP activity. F-actin modulates YAP activity through both Hippo dependent and independent mechanisms. (A) In general, disassembly or loss of F-actin filaments results in LATS1/2 activation and a concomitant inhibition of YAP activity by various upstream pathways. Protein kinase A (PKA) phosphorylates and activates LATS1/2, thereby promoting Yap phosphorylation and inactivation. Other upstream kinases, like MAP4K-family and TAO activate LATS1/2, however, whether they are directly regulated by F-actin levels is not known. Additionally, angiomotins (AMOT) can directly sense F-actin levels to influence YAP activity. When actin levels are low, AMOT is free to bind SAV-MST1/2, LATS1/2, and YAP to promote LATS1/2 activation and inhibition of YAP by sequestering it in the cytoplasm. AMOT also binds NF2, to stimulate its ability to activate LATS1/2. AMOT can also bind directly to YAP, independent of Hippo signaling, to retain it in the cytoplasm. When nuclear F-actin levels are low, the Arid1A-SWI/SNF chromatin remodeling complex inhibits any nuclear YAP by blocking its interaction with the transcription factor TEAD. (B) Conversely, F-actin assembly mediated by GPCR signaling, shear stress, substrate stiffness, or other factors enables actin binding to AMOT. Thus, AMOT is no longer free to bind YAP, LATS1/2, SAV-MST1/2, which removes inhibitory signals to allow nuclear translocation of YAP, rendering it active. Nuclear F-actin assembly triggered by mechanical forces also promotes YAP activity. Arid1A-SWI/SNF associates with nuclear F-actin, allowing YAP to bind TEAD.

Figure 3. Regulation of YAP activity by adherens junctions signaling
(A) Under conditions of high mechanical tension (low density), cadherin mediated junctions function to trigger YAP activity. The α-catenin protein binds both β-catenin and actin stress fibers and is thus subject to pulling forces from neighboring cells and tension generated by the actin-myosin cytoskeleton. Tension causes α-catenin to undergo a conformational change, which increases its binding to LIMD1 and vinculin. LIMD1 promotes LATS1/2 recruitment to junctions and inhibition. Vinculin bound to α-catenin binds to F-actin and recruits TRIP6. TRIP6 competes with LATS1/2 activator MOB1 and binds LATS1/2 at adherens junctions, thereby inhibiting it. Together LIMD1 and TRIP6 inhibition of LATS1/2 allows YAP to translocate to the nucleus. (B) At high cell density, reduced tension possibly caused by loss of stress fibers causes α-catenin to revert to a closed conformation thereby impeding the junctional recruitment of LIMD1, vinculin, TRIP6 and LATS1/2, rendering LATS1/2 free to bind MOB1 and become active (phosphorylated) and inhibit YAP. Additionally, high cell densities promote circumferential actin belt contraction in some cell types, which releases Merlin bound to E-cadherin junctions. Merlin then enters the nucleus to drive the nuclear export of YAP. Once in the cytoplasm, YAP is phosphorylated by kinases, including LATS1/2. α-catenin inhibits YAP activity by binding to phosphorylated YAP, in a complex with 14-3-3 and protects it from dephosphorylation by PP2A phosphatase.
Figure 4. YAP regulation by focal adhesion signaling
Integrin engagement with extracellular matrix on stiff substrates triggers activation of focal adhesion signaling driven by FAK and SRC tyrosine kinases. FAK and SRC kinases modulate Hippo pathway and YAP activity in various ways. Both kinases function to activate YAP to promote cell proliferation on stiff substrates. FAK and SRC can directly phosphorylate YAP to promote its activity. In addition, FAK and SRK directly phosphorylate MOB1 and LATS1/2 respectively to inhibit their activity. They also inhibit LATS1/2 less directly through several signaling pathways. Integrin signaling through a FAK-SRC-PI3K-PDK1 pathway inhibits LATS1/2 activity, thereby enhancing YAP nuclear enrichment. FAK dependent activation of a RAC-PAK signaling cascade also ensures inhibition of LATS1/2 activity by phosphorylating and inhibiting NF2. Another mechanism by which focal adhesions regulate Hippo signaling is by modulating PIP2 levels. On stiff substrates, active FAK induces phospholipase C (PLC), which reduces PIP2. Reduction of PIP2 shuts down the signaling cascade that eventually leads to the activation of RAP2. RAP2 acts both to inhibit Rho, a known inhibitor of LATS1/2, and to activate LATS1/2 by activating MAP4K kinases. Thus, increasing substrate stiffness impedes RAP2 activation by inhibiting upstream PIP2, thereby promoting nuclear translocation and activation of YAP.

Figure 5. Model showing force dependent nuclear localization of YAP
(A) Cells grown on soft substrates are round and the nucleus is poorly coupled to the substrate. As a result, the nucleus maintains its round shape and YAP is retained in the cytoplasm because of the permeability barrier of the nuclear pores. (B) Increasing substrate stiffness causes enhanced coupling of the nucleus to the cell substrate due to increased connections between focal adhesions and LINC complexes in the nuclear membrane via actin stress fibers. This results in increased contractile forces on the nucleus causing it to flatten as the cell assumes a more spread-out morphology. Nuclear flattening causes the nuclear pores to be more permeable to YAP, resulting in increased levels of nuclear YAP.
Figure 2

A. Low F-actin levels

B. High F-actin levels

- GPCR
- Stiff substrate, shear stress
- Rho
- F-actin assembly
- AMOT
- LATS1/2
- SAV
- Nuclear F-actin assembly
- YAP
- TEAD
- Arid1A
- Swi/SNF
- Nucleus

- Actin
- PKA
- MAP4Ks, TAO
- NF2
- MST1/2
- SAV
- YAP
- LATS1/2
- P—P
Figure 5

A  Soft substrate

B  Stiff substrate
Control of cellular responses to mechanical cues through YAP/TAZ regulation
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