YAP Nuclear Localization in the Absence of Cell-Cell Contact Is Mediated by a Filamentous Actin-dependent, Myosin II- and Phospho-YAP-independent Pathway during Extracellular Matrix Mechano-sensing*

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Cell-cell contact inhibition and the mechanical environment of cells have both been shown to regulate YAP nuclear localization to modulate cell proliferation. Changes in cellular contractility by genetic, pharmacological, and matrix stiffness perturbations regulate YAP nuclear localization. However, because contractility and F-actin organization are interconnected cytoskeletal properties, it remains unclear which of these distinctly regulates YAP localization. Here we show that in the absence of cell-cell contact, actomyosin contractility suppresses YAP phosphorylation at Ser112, however, neither loss of contractility nor cell-cell contact, actomyosin contractility suppresses YAP phosphorylation at Ser112, however, neither loss of contractility nor increase in YAP phosphorylation is sufficient for its nuclear exclusion. We find that actin cytoskeletal integrity is essential for YAP nuclear localization, and can override phosphoregulation or contractility-mediated regulation of YAP nuclear localization. This actin-mediated regulation is conserved during mechanotransduction, as substrate compliance increased YAP phosphorylation and reduced cytoskeletal integrity leading to nuclear exclusion of both YAP and Ser(P)112-YAP. These data provide evidence for two actin-mediated pathways for YAP regulation; one in which actomyosin contractility regulates YAP phosphorylation, and a second that involves cytoskeletal integrity-mediated regulation of YAP nuclear localization independent of contractility. We suggest that in non-contact inhibited cells, this latter mechanism may be important in low stiffness regimes, such as may be encountered in physiological environments.

Nuclear localization of the transcriptional co-activator YAP (and its ortholog TAZ) and activation of the TEAD family transcription factors promote cell proliferation, differentiation, stem cell fate, and organ size regulation (1–3). Deficiencies in YAP regulation lead to developmental defects (4) and tumor formation (5). YAP nuclear localization is regulated by a broad range of biochemical and mechanical cues that include serum soluble cues (6), cell-cell contact mediated by adhesions junctions (AJs) (7), and cell mechanosensing of extracellular matrix (ECM) physical properties or stretch (8–10). These upstream inputs converge on the regulation of the core components of the Hippo signaling pathway to regulate YAP nuclear localization. Genetic studies in Drosophila and cell biological studies in culture have identified the epistatic relationship of the core components of the Hippo signaling pathway (3). Upstream signals activate Mst1/2 (Hippo kinase in Drosophila), which in turn activates Lats1/2 kinases (Wts in Drosophila), and Lats phosphorylates YAP (Yorkie in Drosophila) on serine 112, which induces it to form a complex with 14-3-3 and retains it in the cytoplasm (1, 11–13). In the absence of the activation of Hippo signaling, YAP is imported into the nucleus where it promotes TEAD-mediated transcription. However, the interplay between the inputs of serum factors, cell-cell contact, and cell mechanosensing in regulation of YAP nuclear localization is not well understood.

Hippo signaling-mediated regulation of YAP via cell-cell contact has been well characterized (14). YAP is localized to the nucleus and active in cells growing at low density, but becomes cytoplasmic in confluent cultures, and thus underlies the classic paradigm of contact inhibition of proliferation (12). YAP Ser112 phosphorylation increases during contact inhibition, and overexpression of non-phosphorylatable YAP delays proliferation arrest, allowing cells to reach higher densities than controls (12). The role of cadherin- and catenin-mediated cell-cell AJs in this process is being elucidated (14). In Drosophila, a-catenin and the LIM protein Ajuba form a complex at AJs and recruit Wts/Lats1/2 kinase to regulate YAP phosphorylation, cytoplasmic retention, and tissue growth (15). Hippo signaling regulation via AJs occurs during mouse embryonic development, in which the protein angiomotin forms a complex with Lats1/2 at the AJs to activate Hippo signaling and thus retain YAP in the cytoplasm (7).

On the other hand, cellular mechanosensation-mediated regulation of YAP localization is thought to be distinct from the

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3 The abbreviations used are: AJ, adherens junction; ECM, extracellular matrix; MEF, mouse embryonic fibroblast; DMSO, dimethyl sulfoxide; Pa, pascal; ANOVA, analysis of variance; Lat-A, latrunculin A.

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Cell junctional complexes that regulate the Hippo cascade (16). Cell spreading on large patches of ECM, cell attachment to stiff or stretched ECM (8, 10), or fluid shear stress (17) all induce YAP activation and nuclear localization in a range of cell types. These pathways may be Hippo independent or dominant, as LATS1/2 inactivation cannot rescue YAP inhibition in cells with reduced mechanical stress (8). Although mechanical regulation of YAP localization depends on cell attachment, it is not dependent on integrin engagement (18), but rather thought to be dependent on the maintenance of tension in an intact, contractile actomyosin cytoskeleton. Indeed, pharmacological inhibition of actin assembly or myosin II ATPase blocks YAP nuclear translocation in cells on stiff or large ECMs (8).

Although the initial evidence that YAP is regulated by the cytoskeleton came from studies of mechanosensation, more recent evidence suggests that cells need mechanical tension to sustain YAP transcriptional activities and/or nuclear localization, irrespective of the inducing input (16). In contact inhibited Drosophila epithelial tissue, myosin II contractility regulates the formation of the Wts kinase complex at AJs to suppress Hippo signaling, and in the absence of contractility this complex is disrupted and Hippo signaling is activated (15). Furthermore, in dense cultures in vitro, YAP nuclear localization is rescued by ECM stretching (9, 10). Mechanoregulation of YAP in the absence of cell-cell contact has also been shown to be dependent on ECM stiffness sensation, myosin II motor activity, and cell spreading area (8, 19).

Thus, the mechanisms by which the actomyosin cytoskeleton regulates YAP localization and phosphorylation and their relationship to cell-cell contact remain major unanswered questions in the field. Although mechanosensation is well known to alter myosin II activity (20, 21), it is not known how mechanosensation might affect F-actin levels in cells. Furthermore, recent evidence suggests that actin filaments, rather than myosin II activity, may be the key regulator of YAP. Two elegant studies in Drosophila showed that Hippo signaling reduces cellular F-actin (22), whereas increasing F-actin by loss of capping protein can suppress Hippo signaling (23). In addition, LATS1/2-independent regulation of YAP can be achieved in cells cultured on soft, small matrices or at high cell density by promotion of actin assembly via depletion of the actin depolymerizing factor Cofilin, or the filament capping proteins capZ or gelsolin (10). Further complicating these findings, it has also been shown that myosin inhibition by blebbistatin only partially affects YAP nuclear localization, yet it was predominantly dependent on the actin-binding protein angiomotin (24).

To resolve the role of myosin contractility and F-actin in regulation of YAP nuclear localization, we utilized breast epithelial cells as a contact-independent model system, and mouse embryonic fibroblasts (MEF) as a contact-independent model system. We found that in the absence of cell-cell contacts in either epithelial or fibroblast cells, YAP localizes to the nucleus even in the absence of actomyosin contractility. Furthermore, actomyosin contractility suppresses YAP phosphorylation at Ser112, and when contractility is inhibited, even phosphorylated YAP localized to the nucleus. Although contractility and phosphorylation is dispensable for YAP nuclear localization, we find that nuclear localization of YAP or phosphorylated YAP is strictly dependent on the abundance of F-actin filaments. This actin-dependent regulation is also conserved during mechanotransduction when cells are grown on soft substrates, as would be expected to occur physiologically.

Experimental Procedures

Cell Culture, Treatments, and Transfections—MEFs (kindly provided by Mary C. Beckerle, Huntsman Cancer Institute, Salt Lake City, UT) were cultured in DMEM (Invitrogen) supplemented with 100 μg/ml of penicillin/streptomycin, 2 mM l-glutamine, and 10% FBS. MCF10A mammary epithelial cells (ATCC, Manassas, VA) were cultured in mammary epithelium basal medium (Lonza, catalogue number cc-3151) supplemented with bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, and hydrocortisone from mammary epithelium basal medium Single Quots kit (Lonza, catalogue number cc-4136), 100 ng/ml of cholera toxin, 100 μg/ml of penicillin/streptomycin, and 10% FBS. For immunofluorescence assays under sparse culture conditions, ~3,500 cell/cm² were plated on coverslips coated with 10 μg/ml of fibronectin or 10 μg/ml of poly-L-lysine, as noted, and allowed to spread overnight. For dense culture assays, ~4 × 10⁵ cells/cm² were seeded on fibronectin-coated coverslips and cultured at 37 °C in 5% CO₂ for 24 h. Cells were treated with blebbistatin (Toronto Research Chemicals), latrunculin-A (Invitrogen), Y-27632 (Sigma), manganese chloride, or DMSO vehicle control at the indicated concentrations for 2 h. Cell culture and drug treatments were performed at 37 °C in 5% CO₂.

For ECM stiffness-dependent assays, quinone-activated polyacrylamide/bisacrylamide hydrogels on coverslips or 10-cm Petri dishes were purchased from (Matrigen, CA) or were made by hand (55.0 and 0.7 kPa) in which the gel surface was cross-linked with Sulfo-SANPAH reactive amines by UV exposure. Hydrogels were coupled with 10 μg/ml of fibronectin at room temperature for 1 h and equilibrated with media prior to cell plating. For immunofluorescence, ~3,500 cell/cm² were plated on coverslip hydrogels, for biochemical assays (whole cell lysis, F/G-actin separation) cells were plated on Petri dish hydrogels and grown overnight to ~60% confluence.

The FLAG-YAP and FLAG-YAP-8SA (including the following mutations: S121A, S109A, S127A, S128A, S131A, S163A, S164A, and S381A) expression vectors consisting of the cDNA encoding human YAP fused to the COOH terminus of FLAG (obtained from Kun-Liang Guan, University of California, San Diego), which were also used in Ref. 12. The YAP1-GFP expression vector consisting of the cDNA encoding mouse YAP fused to the COOH terminus of FLAG was purchased from Genecopoeia (catalogue number EX-Mm21312-M98). YAP[S112A] mutant was generated on the YAP1-GFP construct using QuikChange site-directed mutagenesis (Agilent Technologies). mApple-β-actin construct was kindly provided by M. Davidson (Florida State University, Tallahassee, FL). MEFs were transfected with these constructs (Nucleofector solution-V, Lonza) with DNA oligos at 18 h prior to fixation. FLAG-YAP constructs were transfected (Effectene, Qiagen) and cultured for 24 h then seeded on fibronectin-coated coverslips for another ~16 h prior to fixation.
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**Immunofluorescence**—For immunolocalizing YAP and FLAG-YAP, cells on fibronectin-coated coverslips or on polyacrylamide/bisacrylamide hydrogels were fixed with 4% paraformaldehyde (Electron Microscopy Science) in cytoskeleton buffer (CB; 20 mM PIPES, pH 6.8, 138 mM KCl, 3 mM MgCl₂, and 2 mM EGTA). Cells were permeabilized with 0.5% Triton X-100 in CB for 5 min followed by a 10-min wash with 0.1 M glycine to quench free aldehydes. Next, cells were washed with TBST (Tris-buffered saline, 0.1% Tween 20) two times for 5 min each and blocked for 1 h with blocking buffer (TBST and 2% BSA). Cells were incubated with anti-mouse YAP (Santa Cruz Biotechnology, Inc., sc-101199) in blocking buffer at 1:200 dilution for 2 h at room temperature or anti-rat FLAG (BioLegend number 637301) at 1:200 overnight at 4 °C and washed three times for 5 min each with TBST. Coverslips were then incubated with fluorophore-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) at 1:500 in blocking buffer for 1 h at room temperature. F-actin was stained with Alexa Fluor 488 or 561 phalloidin (Invitrogen) at 1:500 dilution along with the secondary antibodies. Coverslips were washed again with TBST three times for 5 min each, and DNA was stained with 1.4 μM DAPI (Sigma) with the second wash. Finally, coverslips were mounted on glass slides with mounting media (DAKO) and sealed with nail polish.

Cells expressing YAP1-GFP and mApple-actin were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in CB, and washed with 0.1 μM glycine. After a wash with PBS containing 0.5% Triton X-100 for 10 min. Permeabilized cells were washed with 0.1 mM glycine in CB for 10 min, then washed with PBS two times for 5 min each, and rinsed with PBS with 0.1% Triton X-100. After blocking with antibody dilution solution (PBS, 0.1% Triton X-100 and 2% BSA) for 1 h, F-actin was stained with Alexa Fluor 488 phalloidin at 1:500 dilution and G-actin with deoxyribonuclease-I conjugated with Alexa Fluor 594 (Invitrogen) at 5 μg/ml concentration for 1 h at room temperature. Coverslips were washed three times with PBS, 5 min each, and mounted on a glass slide with mounting media (DAKO) and sealed with nail polish.

**Microscopy and Image Analysis**—Immunolabeled samples were imaged by spinning disc confocal microscopy with an Eclipse Ti microscope body (Nikon) equipped with a Yokogawa spinning disc scanning unit as described in Ref. 26. Samples on coverslips were imaged with a 60× 1.49 NA/oil objective and on hydrogels with 60× 1.2 NA/water objective. Images were acquired with a cooled-CCD camera (HQ2 or Myo from Photometrics). For immunostained cells, Z-stacks of images were acquired for each channel, and the middle confocal slice was chosen from the images of the nucleus detected in the DAPI channel. On the corresponding slice in the YAP channel, the average fluorescence intensity in the nucleus and just outside the nucleus (cytoplasm) was measured to determine the nuclear/cytoplasmic ratio. For cells expressing YAP1-GFP and mApple-actin, to reduce image photobleaching, instead of Z-stacks, a single image at the middle nuclear plane (determined in the DAPI channel) was acquired in the GFP channel, then the microscope was focused to the coverslip surface for acquiring the image in the mApple channel.

For F- to G-actin ratio measurements, confocal Z-stacks of images were acquired for each channel with a 400-nm step size using a stage piece Z-stepper (Mad City Labs) integrated with an XYZ automated stage and controller (Applied Scientific Instruments). Integrated fluorescence intensity was measured on the background-subtracted sum projections for each channel and ratioed to give the F:G-actin ratio. Image analysis was performed using Imagej software.

**Nuclear/Cytoplasmic Fractionation**—Nuclear/cytoplasmic fractionation was performed essentially according to Dignam et al. (27) but with the following modifications. All buffers used were kept on ice and centrifugations were done at 4 °C with soft braking. MEFs were grown on fibronectin-coated 15-cm culture dish to ~60% confluence. After a single wash with PBS, cells were scraped with PBS (containing 1 mM DTT and 1× protease inhibitor) and harvested by centrifugation at 1000 × g for 15 min. The cell pellet was gently resuspended with five times the volume of pellet with buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 1× protease inhibitor) and incubated on ice for 15 min, followed by homogenization (Wheaton). Cell lysis was checked by trypan blue staining of the nucleus after every 20 strokes. The cell lysate was spun at 1000 × g for 5 min to collect the pellet as the nuclear fraction and the supernatant as the cytoplasmic fraction. The nuclear fraction was washed by centrifugation two times with buffer A (1000 × g for 5 min each), resuspended with buffer A to a similar volume as the cytoplasmic fraction, and sonicated. Both fractions were boiled with sample buffer keeping an identical final volume. Each fraction was immunoblotted for YAP, Ser(112)-YAP, and histone H3.

**Fractionation of F- and G-actin by Triton Solubilization**—Determination of the fraction of F- and G-actin in cells was done following the method of Rasmussen et al. (28) with modifications. MEFs were grown on fibronectin-coated 10-cm culture dishes to ~60% confluence. Cells were washed briefly with cold PBS, and lysed with 600 μl of actin stabilization buffer (0.1 M PIPES, pH 6.9, 30% glycerol, 5% DMSO, 1 mM MgSO₄, 1 mM EGTA, 1% Triton X-100, 1 mM ATP, and 1× protease inhibitor) for 10 min at room temperature. Cells were collected by scraping, followed by centrifugation at 90,000 × g for 30 min at 4 °C to separate detergent-insoluble (F-actin) and soluble (G-actin) fractions. An equal volume of 2× sample buffer was added to the soluble fraction and 1× sample buffer to the insoluble pellet to make both fractions 1× of identical volume. Samples were boiled for 10 min, and an equal volume was loaded for immunoblotting actin.

Determination of F- to G-actin from cells grown on hydrogels or dense culture was performed according to Cramer et al. (25) with modifications. Cells were grown to ~60% confluence...
on fibronectin-coated hydrogels or to 100% confluence for dense culture in 10-cm culture dishes. Cells were washed with PBS and incubated with actin stabilization buffer for 10 min. The Triton-soluble G-actin was then collected upon cell lysis by pipetting up the solution from the dish at room temperature. After a brief wash with actin stabilization buffer, the Triton-insoluble F-actin was dissolved directly on the hydrogel surface by adding boiling F-actin solubilization buffer (0.06 M Tris, pH 6.8, 2% SDS (w/v), 1% β-mercaptoethanol (v/v)). The F-actin fraction was collected from the dish by carefully scraping and pipetting the solution. Both the Triton-soluble and insoluble fractions were concentrated by Speed-Vac and made to an identical volume with actin-stabilizing buffer. Sample buffer was added to each fraction to the identical volume and boiled for 10 min and immunoblotted for actin. For experiments to compare the F:G ratio in dense culture and sparse cultures, an extra pair of culture dishes at the two plating densities was used to count cells, and the sample loading volume was adjusted accordingly to keep the cell number similar between dense and sparse conditions.

**Western Blot**—For immunoblotting whole cell extracts, the same number of MEFs grown and treated with the indicated drugs for 2 h were washed with PBS and directly lysed with boiling 1× sample buffer (Quality Biological, Inc.) supplemented with 5% β-mercaptoethanol. Samples were collected by scraping, sonicated, and boiled for 10 min. Protein samples from whole cell lysis, nucleus/cytoplasmic, or F/G-actin fractions were separated by SDS-PAGE and electrotransferred to Immobilon-FL (Millipore) membrane for immunoblotting. Membranes were blocked for 1 h at room temperature with blocking solution (3% skim milk in TBST), incubated with primary antibodies overnight at 4 °C in blocking solution. After reacting with primary antibodies, membranes were washed three times for 5 min each with TBST and incubated with fluorescent-conjugated secondary antibodies for 1 h 30 min at room temperature. Secondary antibodies used at 1:5,000 dilutions in blocking solutions are: anti-rabbit IRDye 680RD and anti-mouse IRDye 800CW (LI-COR, Inc.). Membranes were washed three times for 5 min each with TBST and imaged with the Odyssey infrared imaging system (LI-COR, Inc.) and analyzed with ImageJ software. Integrated fluorescence intensity for each of the Western blot bands was measured from the background-subtracted images. Primary antibodies used for Western blots were: anti-mouse actin, clone C4 (1:8,000, Millipore number MAB1501), anti-mouse Yap (1:1,000, Santa Cruz Biotechnology number sc-101199), anti-rabbit phospho-YAP (Ser127) (1:1,000, Cell Signaling number 4911), and anti-rabbit Histone H3 (1:5,000, Cell Signaling number 9715).

**Statistical Analysis**—Statistical comparison done with the Student’s t test between a pair of datasets. One-way ANOVA tests were performed on group of data containing three or more data sets, p values from ANOVA tests are listed in Table 1.

**Results**

In the Absence of Cell-Cell Contact, Nuclear Localization of Yap Requires F-actin Cytoskeletal Integrity, but Not ROCK-mediated Myosin II Contractility—We sought to examine the interplay between cytoskeletal integrity, contractility, and cell contact inhibition in regulation of Yap nuclear localization and phosphorylation. We utilized MFC-10A epithelial cells that normally form tissues, and MEFs that exhibit contact inhibition of motility (29), but also function and proliferate as individual cells. We first examined the role of cytoskeletal integrity and contractility in Yap localization in confluent cultures by immunolocalizing Yap and staining actin filaments (F-actin) with fluorescent phalloidin in cells treated with pharmacological inhibitors. In confluent cultures of either MEFs or MCF10A cells plated on fibronectin, F-actin formed a band around the cell periphery at cell-cell contacts, and Yap was excluded from the nucleus (Fig. 1, A and B). To test if Yap localization was affected by cytoskeletal integrity in confluent culture, cells were treated with either vehicle (DMSO) control or 2 μM latrunculin-A (Lat-A) for 2 h to depolymerize F-actin. This showed that treatment with Lat-A disrupted the peripheral F-actin band, which was replaced by a series of F-actin punctae, and Yap retained in the cytoplasm, YAP was strongly localized to the nucleus, similar to the effects of confluence (Fig. 1, C and E), indicating that Yap cytoplasmic retention requires cell-cell contact. Treatment of sparse cultures of both cell types with Lat-A showed that disruption of F-actin caused loss of nuclear Yap and its retention in the cytoplasm, similar to the effects of confluence (Fig. 1, C and E). Inhibition of contractility with blebbistatin or Y-27632 induced loss of stress fibers and promoted lamellipodia as expected (Fig. 1, C and E). However, surprisingly, in sparse cells with contraction inhibited, Yap remained partially localized to the nucleus.
in both MEFs and MCF10A cells (Fig. 1, C and E). Quantification of the nuclear:cytoplasmic ratio of YAP in immunofluorescence images confirmed these observations (Fig. 1, D and F). Together, these results indicate that in both fibroblasts and epithelial cells, YAP nuclear localization is primarily regulated by cell-cell contact. Although in the absence of cell-cell contact nuclear localization of YAP requires F-actin cytoskeletal integrity, but not ROCK-mediated myosin II contractility.

In Sparse Cells, Nuclear Localization of YAP Is Promoted by F-actin, and Is Independent of Integrin Signaling, Myosin II Contractility, and Is Not Fully Dependent on Ser112 Phosphorylation—We next sought to understand the cytoskeletal mechanisms regulating YAP localization in the absence of cell-cell contact. As results were similar in fibroblasts and epithelial cells, subsequent studies were done on MEFs, because these were more easily maintained in sparse culture. Because disruption of cytoskeletal integrity by Lat-A causes dissolution of focal adhesions, this suggests that effects of Lat-A on YAP localization could be due to changes in integrin signaling from focal adhesions. In addition, integrins have been shown to regulate Hippo signaling in MCF-10A cells under serum starvation (30).

To test the role of integrin in mediating actin-dependent regulation of Yap in sparsely plated cells in the presence of serum, we cultured cells in serum-containing media and blocked integrin engagement by culturing MEFs on poly-L-lysine-coated coverslips (31) and compared YAP localization to that in cells grown on the integrin ligand fibronectin. This showed that on poly-L-lysine YAP was localized to the nucleus, whereas disrupting actin cytoskeleton by Lat-A excluded YAP from the nucleus under both plating conditions (Fig. 2, A, B, and D).

Next, we constitutively activated integrin by treatment of cells with Mn2+ (1 mM) (32). This showed that manganese treatment failed to suppress YAP nuclear exclusion by actin cytoskeletal disruption (Fig. 2, C and D). Together, these results suggest that
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in sparse cells in the presence of serum, Yap nuclear localization is independent of integrin signaling but dependent on cytoskeletal integrity.

The canonical pathway for regulation of Yap localization is through phosphoregulation of Ser112, which promotes Yap nuclear exclusion and retention in the cytoplasm when phosphorylated, and nuclear localization when dephosphorylated (11, 12, 33, 34). To determine the effects of cytoskeletal perturbations on Yap phosphorylation, we treated sparse MEFs with cytoskeletal inhibitors followed by Western blot analysis of total Yap and Yap phosphorylated on Ser112 (Ser(P)112-Yap). This showed that compared with control, both Lat-A (18) and blebbistatin treatment increased Ser(P)112-Yap (Fig. 3A). This is in agreement with effects of blebbistatin on Yap phosphorylation reported in cancer-associated fibroblasts (35) and in mammary epithelial cell (18).

Because our findings above showed that a substantial portion of Yap localized to the nucleus during myosin II inhibition in sparse cells, this suggested that phosphorylated Yap could localize in the nucleus when contractility is inhibited. To test this, we co-immunolocalized Yap and Ser(P)112-Yap in sparse MEFs under cytoskeletal perturbations. This showed that even in vehicle control, about half the Ser(P)112-Yap was localized to the nucleus (nuclear:cytoplasmic ratio of 1) (Fig. 3, B and C), in agreement with Wada et al. (19). However, Ser(P)112-Yap was significantly excluded from the nucleus when the actin cytoskeleton was depolymerized with Lat-A (Fig. 3, B and C). In contrast, treatment of cells with Y-27632 or a range of concentrations of blebbistatin had no effect on the nuclear:cytoplasmic ratio of Ser(P)112-Yap as compared with control (Fig. 3, B and C). Results from immunofluorescence assays were further supported by biochemical fractionation of the nucleus and cytoplasm from sparse MEFs. This showed in control cells that although Yap and Ser(P)112-Yap were concentrated in the nucleus, both were also present in the cytoplasm at low levels (Fig. 3D). Depolymerizing actin filaments with Lat-A decreased both Yap and Ser(P)112-Yap in the nuclear fraction relative to the cytoplasm (Fig. 3D). In contrast, in cells treated with blebbistatin and Y-27632, although the cytoplasmic fraction of Yap and Ser(P)112-Yap increased relative to control, there was still a substantial proportion of Ser(P)112-Yap in the nucleus (Fig. 3D). Furthermore, considerable Ser(P)112-Yap was present in the nuclear fraction across a range of blebbistatin doses (Fig. 3D). Taken together, these data show that neither lack of contractility nor Yap phosphorylation at Ser112 are sufficient for Yap nuclear exclusion in sparse cells, but loss of F-actin is sufficient for nuclear exclusion of both Yap and Ser(P)112-Yap. Together our results indicate that in the absence of cell-cell contact, nuclear localization of Yap is promoted by F-actin cytoskeletal integrity, independent of integrin activation and ligand binding, myosin II contractility, or Ser112-Yap phosphorylation.

Mechanosensation of Stiff ECM Promotes Nuclear Localization of Yap and Ser(P)112-Yap in an F-actin-dependent Manner—Mechanosensation of ECM stiffness is known to modulate myosin II activity (21) and Yap nuclear localization (8) such that soft ECMs down-regulate myosin II activity, increase Yap phosphorylation, and retain Yap in the cytoplasm, whereas stiff ECMs promote myosin II activity, Yap dephosphorylation, and drive Yap into the nucleus (9, 36). Given our observation that Ser(P)112-Yap can reside in the nucleus during myosin II inhibition in sparse cells, we first sought to determine the effects of ECM compliance on Yap localization and phosphorylation, and then determine the role of F-actin cytoskeletal integrity in these effects. We plated cells sparsely on ECMs made of polyacrylamide substrates of defined stiffness with fibronectin coupled to their surface, and performed immunofluorescence and Western blot analysis of Yap localization and phosphorylation, and analysis of F-actin integrity. In agreement with previous reports (8, 9), in MEFs grown on stiff substrates (50 kPa), cells were well spread and exhibited stress fibers and strong nuclear localization of Yap, whereas on soft substrates (0.5 kPa), cells were poorly spread and Yap was excluded from the nucleus (Fig. 4, A and B). Western blot analysis showed that, consistent with previous studies (9), Ser(P)112-Yap was higher in cells on soft compared with that in cells on stiff ECMs (Fig. 4, C and D). Phospho-immunolocalization showed that in cells on soft ECMs, both Yap and Ser(P)112-Yap were significantly excluded from the nucleus (Fig. 4, E and F). Together, these results show that ECM compliance and myosin II inhibition both promote Yap phosphorylation at Ser112, but show differential effects on Yap and Ser(P)112-Yap localization in sparse MEFs. Our results further show that the effects of substrate compliance on both Yap and cell morphology more closely resemble the effects of actin depolymerization (Figs. 1C and 4A) than they do the effects of myosin II inhibition (Fig. 1C), possibly implicating F-actin as a potential contractility-independent regulator of Yap during mechanosensation.

Whether stiffness sensing or cell-cell contacts modulate F-actin integrity to regulate Yap localization is not known. We thus sought to test whether F-actin regulates Yap nuclear localization independent of contractility during mechanosensation or cell-cell contact. To determine the effects of ECM compliance or cell-cell contact on F-actin integrity, we used either biochemical fractionation or localization-based (25) assays to quantify the F-actin to globular-actin (G-actin) ratio (F:G) in cells. We first validated the assays by determining the F:G ratio in cells treated with Lat-A. Fractionation of the detergent-soluble G-actin from the insoluble F-actin or direct staining of F-actin by fluorescent phalloidin and G-actin by fluorescent deoxyribonuclease-I (25) in individual cells both showed that Lat-A treatment significantly reduced the F:G ratio compared with vehicle control (Fig. 5, A–D), as expected. In contrast, treatment of cells with blebbistatin to inhibit myosin II contractility had no effect on the F:G ratio (Fig. 5, A–D). We then determined how substrate compliance affected the F:G-actin ratio in cells plated sparsely on ECMs of different stiffness. Surprisingly, both detergent fractionation and localization-based assays showed that the F:G-actin ratio in cells grown on soft substrates was significantly lower compared with cells grown on stiff substrates (Fig. 5, E–H). This shows that ECM mechanosensing regulates both contractility (20, 21) and F-actin integrity. Together with our aforementioned results, this shows that the high F:G-actin ratio correlates with Yap nuclear localization, but contractility and Yap phosphoryla-
tion do not. This further suggests that F-actin may be responsi-
ble for YAP nuclear localization in blebbistatin-treated cells.

To test the hypothesis that F-actin regulates YAP localization when myosin II is inhibited, we used a high dose of blebbistatin (100 μM) for 2 h to completely inhibit myosin contractility (37), then subsequently depolymerized actin filaments with Lat-A.

Similar to 50 μM blebbistatin treatment, YAP was still partially localized to the nucleus even in the presence of 100 μM blebbistatin (Fig. 5, I and J). Importantly, however, Lat-A mediated actin depolymerization in blebbistatin-treated cells caused YAP to be excluded from the nucleus (Fig. 5, I and J). Coupled with the results from low-stiffness substrates, these results sug-

FIGURE 3. In sparse cells, nuclear localization of YAP is promoted by F-actin independent of myosin II contractility and is not fully dependent on Ser112 phosphorylation. A, left, representative Western blots of whole cell lysates from MEFs grown on fibronectin-coated culture dishes and treated with DMSO, Lat-A (2 μM), and blebbistatin (50 μM) for 2 h and probed with antibodies to YAP or YAP phosphorylated on Ser112 (pS112-YAP). Right, quantification represents the ratio of integrated band intensity between Ser(P)112-YAP and YAP from background subtracted images and normalized with respect to DMSO control ratio. B, maximum intensity projections of confocal Z-stack immunofluorescence images of sparsely plated MEFs treated with the indicated agents for 2 h. YAP was immunostained with a-YAP (red), α-Ser(P)112-YAP (green), and DNA with DAPI (blue). Note that Ser(P)112-YAP was better detected with methanol fixation (used here), whereas YAP was better detected with paraformaldehyde fixation (used in Fig. 1). C, quantification of the nuclear/cytoplasmic ratio of pS112-YAP in the middle confocal slice of the nucleus. D, representative Western blots of the nucleus (Nuc) and cytoplasmic (Cyt) fractions from MEFs grown on fibronectin-coated culture dishes with the indicated treatments for 2 h for the indicated proteins and probed with antibodies to YAP, Ser(P)112-YAP, and Histone H3. Scale bars 30 μm. Error bars are S.E. Statistical comparison done with Student’s t test between pair of samples connected with lines on the column plots: *, p value < 0.05; **, p value < 0.005; ns, p value > 0.05. One-way ANOVA tests were performed on group of data containing three or more data sets, p values from ANOVA tests are listed in Table 1.
gest that F-actin is responsible for YAP nuclear localization in cells under low contractility conditions.

To test whether cell-cell contact-mediated inhibition of YAP nuclear localization also occurs through actin disassembly, we compared the actin assembly state in dense and sparse cultures. This showed that the F:G-actin ratio was not effected by cell confluence (Fig. 5, K and L), suggesting that signaling from adherens junctions during contact inhibition dominates over cytoskeletal integrity in regulation of YAP nuclear localization. However, it is also possible that changes in F-actin architecture in dense culture compared with sparse culture (Fig. 1, A–C and E) may contribute to YAP regulation. Together, these data support the notion that in the absence of cell-cell contact, F-actin, and not myosin II contraction, is the critical regulator of YAP nuclear localization during mechanosensation.

Two Pathways Regulate YAP Localization in Sparse Cells: an F-actin-dependent, Phosphorylation-independent Pathway, and a Myosin II- and Phosphorylation-dependent Pathway—Our results suggest that F-actin can override the canonical phoshoregulation of YAP localization to promote nuclear localization of phospho-YAP. We sought to test this by assaying the effects of cytoskeletal perturbation on constitutively active mutants of tagged YAP constructs (FLAG-YAPwt, GFP-YAP1wt) that cannot be regulated by Lats1/2 kinase by using FLAG-YAP8SA that has all the known Lats phosphorylation sites mutated to alanines, or GFP-YAP1S112A, which carries a mutation at the Lats phosphorylation site that has been shown to be the most potent regulator of YAP nuclear localization (12, 38). To first validate the functionality of the YAP constructs, we examined their localization in MEFs where phosphorylation by Lats kinase is known to be key for YAP nuclear exclusion (12, 39). This showed under sparse culture conditions where YAP phosphorylation is known to be low (12), both the FLAG-tagged and GFP-tagged YAP wild-type constructs and both FLAG-YAP8SA and GFP-YAP1S112A localized to the nucleus (Fig. 6, A, I, and K), as expected. In dense culture conditions where YAP is known to become phosphorylated (12), FLAG-YAPwt and GFP-YAP1wt were reduced in the nucleus, whereas FLAG-YAP8SA and GFP-YAP1S112A were significantly enriched in the nucleus (Fig. 6, D and F–H). Thus, tagged YAP constructs

FIGURE 4. Mechanosensation of soft ECM promotes nuclear exclusion of YAP and phospho-Ser112-YAP. A–F, MEFs were grown on fibronectin-coupled polyacrylamide hydrogels of 50.0 (stiff) and 0.5 kPa (soft) stiffness. A, maximum intensity projections of confocal Z-stack immunofluorescence images of YAP (red), phalloidin staining of F-actin (green), and DAPI staining of DNA (blue). B, quantification of the nuclear/cytoplasmic ratio of YAP in the middle confocal slice of the nucleus. C, representative Western blots from whole cell lysates of MEFs grown on fibronectin-coated soft and stiff substrates and probed with antibodies to YAP and YAP phosphorylated on Ser112 (pS112-YAP). D, quantification of the ratio of integrated band intensity between Ser(P)112-YAP and YAP from background-subtracted Western blot images and normalized with respect to 50.0 kPa. E, maximum intensity projections of confocal Z-stack immunofluorescence images of sparsely plated MEFs grown on fibronectin-coated stiff and soft substrates and fixed with methanol and immunostained to detect YAP (red), Ser(P)112-YAP (green), and stained with DAPI for DNA (blue). F, quantification of the nuclear/cytoplasmic ratio of Ser(P)112-YAP in the middle confocal slice of the nucleus. Scale bars 30 μm. Error bars are S.E., Statistical comparison done with Student’s t test: *, p value < 0.05; **, p value < 0.005.
localize in a manner that is consistent with that described for Lats-mediated phosphoregulation of endogenous YAP in sparse and dense culture.

We next examined the localization of the phosphomutants in response to cytoskeletal perturbations. This showed that in sparse culture, treatment with Lat-A to depolymerize F-actin induced nuclear exclusion of all of the constructs (Fig. 6, B, C, and I-L), significantly decreasing the nuclear:cytoplasmic ratio of the YAP signal, albeit to a lesser extent for the 8SA mutant relative to the S112A mutant or for the 8SA mutant relative to the wild-types (Fig. 6, A-C and K-L). In dense culture, although actin depolymerization did not affect FLAG-YAPwt localization, this treatment surprisingly caused exclusion of FLAG-YAP8SA from the nucleus (Fig. 6, D–F). Together, these results support the notion of a phosphorylation-independent but actin cytoskeleton-dependent pathway for YAP nuclear exclusion, even in contact-inhibited cells.

We then determined the role of YAP Ser112 phosphorylation in myosin-dependent regulation of YAP localization. We found that inhibition of myosin II caused GFP-YAPIwt to behave similarly to that of the endogenous protein with a partial decrease in the nuclear localization compared with untreated cells (Figs. 6, I and J, and 1, C and D). However, the non-phosphorylatable GFP-YAPIS112A remained strongly localized to the nucleus,
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with no significant difference in nuclear:cytoplasmic signal ratio compared with the same construct in controls (Fig. 6, K and L). These data, along with endogenous Yap data, suggest that in the absence of contact inhibition, two distinct pathways act to regulate Yap localization in MEFs, an F-actin-mediated pathway that is Ser(P)112-Yap-independent, and a myosin II-mediated pathway that requires phosphoryregation of Ser112.

ECM Compliance-mediated Regulation of Yap Nuclear Localization Occurs via an F-actin-dependent, Phosphorylation-independent Pathway—We then examined the role of Ser112 phosphorylation in F-actin-dependent nuclear localization of Yap during mechanosensation in MEFs. We expressed either YapP112A-GFP or YapP1112A-GFP in sparse cells grown on stiff and soft ECMS. This showed that on stiff ECMS YapP112A-GFP or YapP1112A-GFP constructs all localized to the nucleus. In contrast, on soft ECMS cells formed small clusters and could not spread effectively, and YapP112A-GFP or YapP1112A-GFP constructs were excluded from the nucleus, and exhibited a significantly reduced nuclear:cytoplasmic localization ratio (Fig. 7, A–D). Together with our observation of reduced F-actin content in cells plated on soft ECMS, this shows ECM compliance-mediated regulation of Yap nuclear localization occurs through an F-actin-dependent but phosho-Ser112-independent pathway.

Discussion

Our study shows that in both mouse embryonic fibroblasts and in human mammary epithelial cells, contact inhibition promotes nuclear exclusion of Yap, overriding actomyosin cytoskeletal regulation of Yap. However, in the absence of cell-cell contact, integrity of the actin filament cytoskeleton becomes the dominant regulator of Yap nuclear localization, independent of ROCK-mediated myosin II contractility. A detailed characterization in MEFs revealed that actomyosin contractility suppresses Yap phosphorylation at Ser112, yet in the absence of cell-cell contact and contractility, Ser112 phosphorylation is overridden by actin cytoskeletal integrity leading to nuclear localization of both phospho- and non-phospho-Yap. These data along with Yap phosphomutant data show the existence of two mechanisms for cytoskeleton-mediated Yap regulation; one actomyosin contractility-mediated regulation of Yap phosphorylation, and a second cytoskeletal integrity-mediated dominant mechanism that overrides phosphoryregation. This actin-mediated Yap regulation is also conserved during mechanotransduction, where we found Yap nuclear localization through ECM stiffness sensation occurs through an F-actin-dependent but phosho-Ser112-dependent manner.

Hippo signaling-mediated Yap phosphorylation and nuclear exclusion have been well documented in animal tissue and in vitro dense cell culture systems (12, 40–43). Phosphorylation-mediated Yap nuclear exclusion is largely dependent on cell-cell AJ, as Yap is sequestered at the AJ by α-catenin and in vitro assay showed Ser(P)112-Yap binding to 14–3-3 also depends on α-catenin (40). In the absence of α-catenin, this complex breaks apart and Yap gets into the nucleus independent of Hippo signaling (40). In sparse culture conditions there is no AJ, possibly leading to the disassembly of the α-catenin-Yap and 14–3-3-Ser(P)112-Yap-α-catenin complex, which could provide a possible mechanism for the observed Yap and Ser(P)112-Yap localization in the nucleus independent of contractility. In addition, a recent report showed that even under activation of the Hippo signaling cascade and Yap phosphorylation (Ser112), Yap localizes to the nucleus of intestinal epithelial cells via the Src family kinase Yes, causing more cell proliferation (44). This also suggests the existence of additional mechanisms in regulation of Yap nuclear localization other than phosphorylation of the Ser112 residue.

Our data provides a step forward in understanding actin-mediated Yap nuclear localization beyond the requirement of actomyosin contractility and Ser112 phosphorylation of Yap. A possible molecular player for such actin-mediated Yap regulation is through angiomotin-like proteins (24). Angiomotins bind the WW domain of Yap independent of its phosphorylation at Ser112 residue, and overexpression of angiomotin could sequester constitutively active Yap (YAP1S112A) in the cytoplasm (45). Angiomotins competitively bind to both F-actin and Yap, and overexpression of an actin binding mutant of angiomotin sequesters more Yap in the cytoplasm than overexpression of wild-type protein (24). This mechanism could underlie our observations that a high F:G-actin ratio in control and myosin-inhibited cells was associated with both Yap and...
Ser(P)112-YAP nuclear localization. A high level of F-actin could sequester angiomotins, leaving YAP and Ser(P)112-YAP free to be imported into the nucleus. In support to this notion, it was also reported that knocking down angiomotins significantly rescued YAP nuclear localization in blebbistatin-treated cells (24). In support of our findings of an F-actin-dependent pathway of YAP nuclear localization, it was shown that knocking down the actin depolymerization factor coflin, which is known to increase the F:G-actin ratio (28), rescued YAP transcriptional activity on soft substrates (10). Promoting more F-actin by knocking down coflin or capping protein in animal tissue was also found to increase YAP nuclear localization and tissue growth (22, 23). In agreement with our findings, it was also reported that overexpressing actin increases F-actin, promoting cell spreading and thus overriding the small cell shape phenotype seen with cells plated on soft substrates (46). F-actin-mediated YAP regulation could have a physiologically important role as it was shown that promoting F-actin polymerization increased ovarian follicle growth mediated by YAP (47).

Our study suggests a model where in cells at low density, YAP is nuclear and has low levels of phosphorylation because of the lack of adherens junctions and the high level of myosin II contractile activity. Also at low cell density, F-actin structures promote YAP nuclear localization. Inhibition of myosin II contractility in cells in sparse culture increases YAP phosphorylation, but not sufficiently to trigger cytoplasmic accumulation of YAP. However, F-actin disruption can trigger cytoplasmic localization of YAP. At high cell density, YAP is highly phosphorylated and out of the nucleus, presumably because of signaling from adherens junctions. The observation that non-phosphorylatable YAP localizes to the nucleus in cells at high density is consistent with an essential role for phosphorylation in YAP nuclear exclusion under these conditions. One question that remains is why myosin II inhibition in cells at low density triggers YAP phosphorylation, but not nuclear exclusion, whereas at high cell density, YAP phosphorylation appears to effectively promote YAP nuclear exclusion. One possibility is that YAP is much more highly phosphorylated on Ser112 and...
other LATS sites in cells at high density compared with cells at low density treated with myosin inhibitors. This prediction awaits further experimentation.

Contractility- and phosphorylation (Ser^{112})-independent localization of YAP in the nucleus, especially in the absence of cell-cell contact, may reflect a cellular adaptation for cancer cells. Cancer cells during metastasis often lose cell-cell contact and migrate through tissues of different stiffness. Having contractility and phosphorylation-independent nuclear localization of YAP might promote cancer cell proliferation and maintenance in soft tissue.

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