Calmodulin activates the Hippo signaling pathway by promoting LATS1 kinase–mediated inhibitory phosphorylation of the transcriptional coactivator YAP

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The Hippo signaling pathway regulates tissue growth and cell fate, and its dysregulation can induce tumorigenesis. When Hippo is activated by cell–cell contact, extracellular signals, or cell polarity among others, the large tumor suppressor 1 (LATS1) kinase catalyzes inhibitory phosphorylation of the transcriptional coactivator Yes-associated protein (YAP) to maintain YAP in the cytoplasm or promote its degradation. Separately, calmodulin is a Ca^{2+}-dependent protein that modulates the activity of target proteins and regulates several signaling cascades; however, its potential role in the Hippo pathway has not been identified. Here, using diverse experimental approaches, including in vitro binding analyses, kinase assays, RT–PCR, and confocal microscopy, we reveal that calmodulin promotes Hippo signaling. We show that purified YAP and LATS1 bind directly to calmodulin and form a Ca^{2+}-dependent ternary complex in vitro. Importantly, Ca^{2+}/calmodulin directly stimulated the activity of LATS1 kinase. In cultured mammalian cells, we demonstrated that endogenous YAP and LATS1 coimmunoprecipitate with endogenous calmodulin. In cells with activated Hippo signaling, we show that calmodulin antagonism significantly (i) decreases YAP phosphorylation, (ii) increases expression of two Hippo target genes (connective tissue growth factor [CTGF] and cysteine-rich angiogenic inducer 61 [CYR61]) that regulate cell proliferation and tumor progression, and (iii) enhances the interaction of YAP with its major transcription factor, thereby facilitating transcription of target genes. Collectively, our data demonstrate that calmodulin activates the Hippo kinase cascade and inhibits YAP activity via a direct interaction with LATS1 and YAP, thereby uncovering previously unidentified crosstalk between the Ca^{2+}/calmodulin and Hippo signaling pathways.

The Hippo pathway is a highly conserved signaling network that governs organ size and tissue homeostasis in higher-order vertebrates by restricting cell proliferation and promoting apoptosis (1, 2). First identified in the fruit fly Drosophila melanogaster (3), this pathway consists of a serine/threonine kinase cascade and a downstream transcriptional module. In the core kinase cascade, when Hippo is activated, mammalian Ste20-like kinases 1 and 2 catalyze phosphorylation of large tumor suppressor 1 and 2 (LATS1/2) kinases (4, 5). Once phosphorylated, LATS1/2 become active and in turn catalyze phosphorylation of the transcriptional coactivators Yes-associated protein (YAP) and WW domain–containing transcription regulator protein 1 (also known as transcriptional coactivator with PDZ-binding motif [TAZ]) (6–9). Phosphorylation of YAP and TAZ on Ser^{127} and Ser^{89}, respectively, triggers their association with 14-3-3 proteins, which leads to their retention in the cytoplasm (7, 9). In contrast, when Hippo is inactive, unphosphorylated YAP and TAZ are translocated to the nucleus where they exert cotranscriptional activity by binding to transcription factors. The transcriptional enhanced associate domain (TEAD) family members are the major transcription factors to which YAP and TAZ bind, leading to expression of Hippo target genes involved in cell growth, proliferation, migration, and survival (10, 11). In addition to regulating their subcellular localization, LATS1/2-mediated phosphorylation of YAP and TAZ, on Ser^{381} and Ser^{306}, respectively, triggers their ubiquitination and subsequent degradation (12). Diverse signals, including mechanical cues, cell polarity, cell adhesion, extracellular soluble factors, and cellular stresses, modulate activation of the Hippo pathway (13). Because of its key role in metazoan physiology and development, it is not surprising that dysregulation of the Hippo network may have severe consequences, including organomegaly and tumorigenesis, because of cell hyperproliferation and inhibition of apoptosis (14).

Ca^{2+} is a fundamental intracellular messenger that regulates a broad range of cellular functions. The effects of Ca^{2+} are conveyed via several Ca^{2+}-binding proteins (15). The highly conserved and ubiquitous protein calmodulin is an archetypal example of Ca^{2+}-binding protein. Binding of Ca^{2+} to its four Ca^{2+}-binding sites induces a conformational change in calmodulin, facilitating association with numerous proteins (16). Through binding to its structurally and functionally diverse targets, calmodulin modulates their activity, thereby participating in a broad range of cellular processes, including cell cycle progression, cell proliferation, cyclic nucleotide

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metabolism, glycogen metabolism, cytoskeletal arrangement, and smooth muscle contraction (17).

Calmodulin has been documented to regulate in a Ca^{2+}-dependent manner several major signaling pathways, including the mitogen-activated protein kinase (18, 19) and PI3K/protein kinase B networks (20, 21). Nevertheless, despite reports that Ca^{2+} signaling influences the Hippo network (22), the possible involvement of calmodulin in modulating Hippo had not been investigated. Therefore, we tested the hypothesis that calmodulin regulates Hippo signaling. We observed that YAP and LATS1 interact with calmodulin both in cells and *in vitro*. Importantly, these interactions have functional consequences. *In vitro*, Ca^{2+}/calmodulin directly stimulates LATS1 kinase activity. In cells, the highly selective cell-permeable calmodulin antagonist CGS9343B significantly reduces Hippo activation mediated by cell–cell contact or serum starvation. Altogether, our data identify for the first time a communication between the Ca^{2+}/calmodulin and Hippo pathways and reveal that calmodulin is a previously unrecognized regulator of Hippo signaling.

Results

Calmodulin interacts with YAP and LATS1 in cells

Reports that Ca^{2+} signaling modulates Hippo (22) prompted us to investigate whether the Ca^{2+}-binding protein calmodulin, which regulates several signaling networks (18–21), influences Hippo activation. To do so, we first assessed whether calmodulin associates with the Hippo transcriptional coactivator YAP. Analysis was performed by immunoprecipitation. In order to stabilize transient protein–protein interactions, HeLa cells were exposed to dithiobis(succinimidyl propionate) (DSP) that reacts with primary amine groups and crosslinks bound proteins. Cells were then lysed, and endogenous YAP was immunoprecipitated with anti-YAP monoclonal antibody. Immunoglobulin G (IgG) was used as a negative control. Analysis of the immunoprecipitated samples by SDS-PAGE and Western blotting revealed that endogenous calmodulin coimmunoprecipitates with YAP (Fig. 1A).

In order to ascertain if calmodulin also binds to other components of the Hippo pathway, we investigated whether it interacts with LATS1, the kinase that inhibits YAP. We immunoprecipitated endogenous LATS1 from HeLa cells. Western blotting demonstrated that calmodulin coimmunoprecipitated with LATS1 (Fig. 1B). YAP was also present in the immunoprecipitates. The absence of signal from the samples precipitated with IgG validates the specificity of the interactions. Together, our data imply that YAP and LATS1 interact with calmodulin in cells. Furthermore, coimmunoprecipitation of both calmodulin and YAP with LATS1 suggests that these proteins could form a ternary complex in cells.

To validate these observations, we reciprocally immunoprecipitated calmodulin from HeLa cell lysates and probed the Western blot for YAP and LATS1. Both YAP and LATS1 coimmunoprecipitate with calmodulin (Fig. 1C). Again, the absence of signal from the samples precipitated with IgG validates the specificity of the interactions. These data confirm
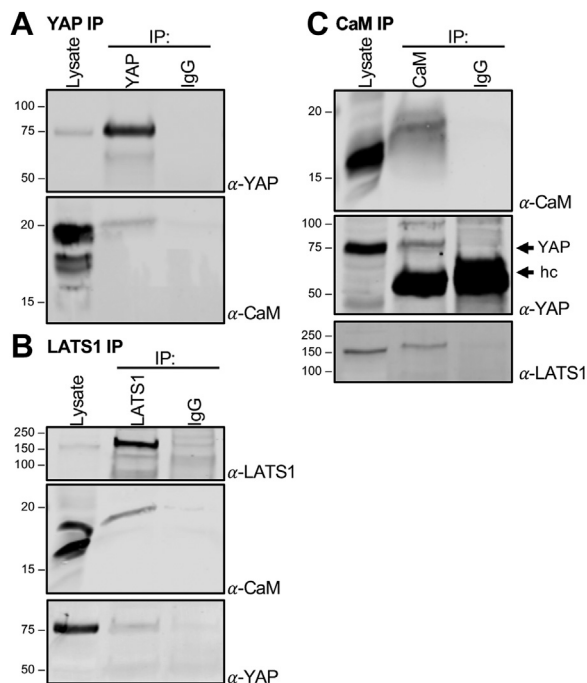

Figure 1. Calmodulin (CaM) interacts with YAP and LATS1 in cells. A, HeLa cells were cultured, incubated with DSP, and lysed. Equal amounts of protein from cell lysates were subjected to immunoprecipitation with anti-YAP monoclonal antibody. Control precipitation was carried out with rabbit IgG. Samples were resolved by SDS-PAGE followed by Western blotting and probed with anti-YAP and anti-CaM antibodies. Unfractionated cell lysate was processed in parallel. B, immunoprecipitation was carried out from HeLa cell lysates as described for (A), except anti-LATS1 monoclonal antibody was used. Western blots of the samples were probed with anti-LATS1, anti-CaM, and anti-YAP antibodies. C, cell lysates were immunoprecipitated with anti-CaM monoclonal antibody or mouse IgG. Western blots were probed with anti-CaM, anti-YAP, and anti-LATS1 antibodies. The *arrowheads* indicate positions of migration of YAP and the heavy chains (hc) of the antibodies. The three panels are from the same Western blotting membrane. All data shown in this figure are representative of three independent experiments. The positions of migration of molecular weight markers are indicated on the *left*. DSP, dithiobis(succinimidyl propionate); IgG, immunoglobulin G; LATS1, large tumor suppressor 1; YAP, Yes-associated protein.

that endogenous YAP and LATS1 interact with endogenous calmodulin in cells and support the premise that the three proteins could form a complex in HeLa cells.

Evaluation of the effect of Ca^{2+} on the binding of calmodulin to YAP and LATS1

To further characterize the binding of calmodulin to YAP and LATS1, we evaluated whether Ca^{2+} regulates their interaction. To do so, human embryonic kidney 293 (HEK293) cells were lysed in buffer containing 1 mM of either $CaCl_2$ or of the Ca^{2+}-chelator EGTA. Equal amounts of protein lysates were incubated with calmodulin-Sepharose, and samples were resolved by Western blotting. The amount of YAP bound to calmodulin was not significantly altered by the presence of Ca^{2+} or EGTA (Fig. 2, A and B). This result confirms that YAP binds to calmodulin, regardless of the presence/absence of Ca^{2+}, in cell lysates.

Similar analysis was performed to examine the possible effect of Ca^{2+} on the association of LATS1 with calmodulin. In
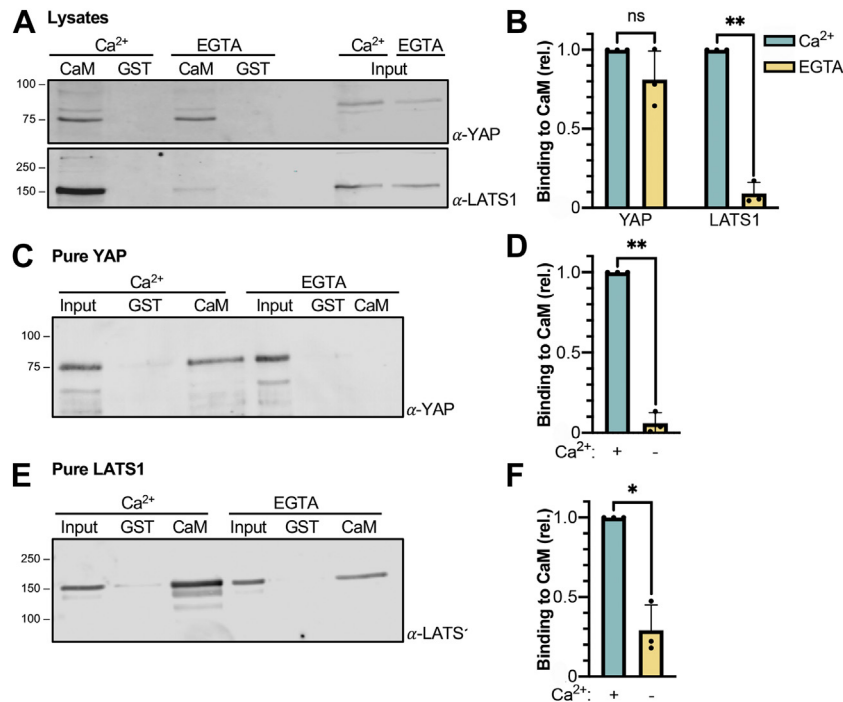
Figure 2. YAP and LATS1 bind directly to calmodulin (CaM) in a Ca^{2+}-regulated manner. *A*, HEK293 cells were lysed in buffer containing 1 mM CaCl$_2$ or 1 mM EGTA. The cell lysate (1 mg) was incubated with CaM-Sepharose (CaM) or control GST-Sepharose (GST) beads. After centrifugation and washing, bound proteins were eluted in Laemmli sample buffer. Cell lysates not subjected to pull-down were processed in parallel. Samples were resolved by SDS-PAGE and Western blotting and then probed with anti-YAP and anti-LATS1 antibodies. Blots are representative of three independent experiments. *B*, the YAP and LATS1 bands were quantified using Image Studio 2.0 (LI-COR Biosciences). Samples containing Ca^{2+} were set as 1. Data represent the mean ± SD of three independent experiments. *C* and *E*, 2 μg of purified human recombinant YAP (*C*) or LATS1 (*E*) were incubated with CaM-Sepharose or GST-Sepharose (control) in the presence of 1 mM CaCl$_2$ or 1 mM EGTA. Beads were pelleted by centrifugation, washed, and attached proteins were eluted in Laemmli sample buffer. Input is 100 ng purified YAP (*C*) or LATS1 (*E*) not subjected to pull-down. Samples were resolved by SDS-PAGE followed by Western blotting and probed with anti-YAP (*C*) or anti-LATS1 (*E*) antibodies. All blots shown are representative of three independent experiments. *D* and *F*, the YAP and LATS1 bands observed after each pull-down with CaM-Sepharose were quantified using Image Studio 2.0, with samples containing Ca^{2+} set as 1. Data represent the mean ± SD of three independent experiments. All statistical analyses performed in this figure are one-sample *t* tests (**p* ≤ 0.05; ***p* ≤ 0.01; ns). GST, glutathione-*S*-transferase; HEK293, human embryonic kidney 293 cell line; LATS1, large tumor suppressor 1; ns, not significant; YAP, Yes-associated protein.

contrast to YAP, the amount of LATS1 bound to calmodulin in the presence of Ca^{2+} is 11-fold greater than when Ca^{2+} is chelated with EGTA (Fig. 2, *A* and *B*). These data imply that the interaction of calmodulin with endogenous LATS1 is regulated by Ca^{2+}. No YAP or LATS1 was detected in samples incubated with glutathione-*S*-transferase (GST)–Sepharose (Fig. 2*A*), demonstrating the specificity of binding of YAP and LATS1 to calmodulin.

YAP and LATS1 bind directly to calmodulin

To determine whether YAP binds directly to calmodulin, we used pure proteins. Purified human recombinant YAP was incubated with calmodulin-Sepharose in the presence of either CaCl$_2$ or EGTA. Western blotting reveals that pure YAP binds directly to pure calmodulin in the presence of Ca^{2+} (Fig. 2*C*). Importantly, chelation of Ca^{2+} by EGTA almost completely abolishes the calmodulin–YAP interaction (Fig. 2, *C* and *D*). This shows that YAP binds directly to calmodulin in a Ca^{2+}-dependent manner. This further suggests that the interaction between YAP and calmodulin that was observed in the absence of Ca^{2+} in cell lysates is indirect and occurs in a complex with (an)other protein(s).

Analogous binding assays were carried out with purified human recombinant LATS1. Similar to YAP, our data show

that LATS1 binds directly to Ca^{2+}/calmodulin (Fig. 2*E*). The binding of LATS1 to calmodulin is significantly reduced (by 70.9 ± 12.9%, mean ± SD), but not eliminated, by removal of Ca^{2+} with EGTA (Fig. 2, *E* and *F*). These data demonstrate that pure LATS1 binds directly to pure calmodulin in a Ca^{2+}-regulated manner, similar to what was observed in cell lysates. The absence of YAP and LATS1 from the pull-downs with GST-Sepharose validates the specificity of their binding to calmodulin. Taken together, our data demonstrate direct binding of YAP and LATS1 to Ca^{2+}/calmodulin.

Formation of a Ca^{2+}-dependent YAP–LATS1–calmodulin ternary complex *in vitro*

Coimmunoprecipitation of both YAP and calmodulin with LATS1, as well as YAP and LATS1 with calmodulin, suggests that the three proteins form a ternary complex in cells. To assess whether YAP, LATS1, and calmodulin can form a ternary complex *in vitro*, we simultaneously incubated purified YAP and LATS1 with calmodulin-Sepharose. Both YAP and LATS1 bind to calmodulin in the presence of Ca^{2+} (Fig. 3*A*). No binding was detected when Ca^{2+} was chelated with EGTA. These data support the concept that YAP, LATS1, and calmodulin form a Ca^{2+}-dependent ternary complex *in vitro*. To further test this hypothesis, we incubated pure YAP,
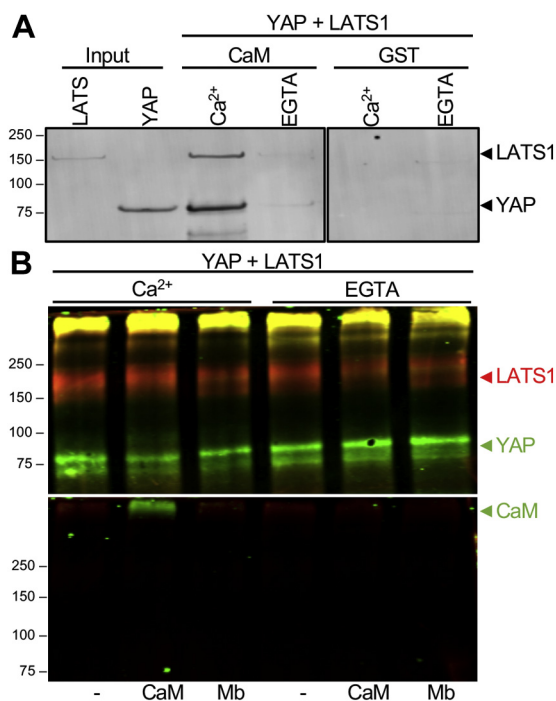
Figure 3. Pure YAP, LATS1, and calmodulin (CaM) form a Ca²⁺-dependent ternary complex *in vitro*. *A*, 2 μg of purified YAP and LATS1 were simultaneously incubated with CaM-Sepharose (CaM) or GST-Sepharose (GST, control) in the presence of 1 mM CaCl₂ or 1 mM EGTA. Beads were pelleted by centrifugation, washed, and the attached proteins were eluted in Laemmli sample buffer. Input is 100 ng purified YAP or LATS1 not subjected to pull-down. Blots were probed with anti-YAP and anti-LATS1 antibodies. Both panels are from the same PVDF membrane and have the same exposure. An irrelevant lane has been removed. *B*, 600 ng of purified YAP and LATS1 were incubated with no added proteins (–) or 600 ng pure CaM or myoglobin (Mb) in the presence of 1 mM CaCl₂ or 1 mM EGTA. After crosslinking with DTSSP, samples were analyzed by SDS-PAGE and Western blotting. The blot was first probed for YAP and LATS1 (*green* and *red bands*, respectively, *upper panel*). *Yellow bands* indicate comigration of YAP and LATS1. After stripping, the membrane was probed for CaM (*green band*, *lower panel*). DTSSP, dithiobis(sulfosuccinimidyl propionate); GST, glutathione-*S*-transferase; LATS1, large tumor suppressor 1; PVDF, polyvinylidene difluoride; YAP, Yes-associated protein.

LATS1, and calmodulin, and then crosslinked the proteins with 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP). Samples were analyzed by Western blotting. LATS1 and YAP were detected at their expected size (Fig. 3*B*, *upper panel*, *red* and *green bands*, respectively). In addition, we observed bands that migrated at higher molecular mass; these comprise both YAP and LATS1 (Fig. 3, *B*, *upper panel*, *yellow bands*). These bands were not influenced by the presence or the absence of Ca²⁺. These data indicate the formation of Ca²⁺-independent YAP–LATS1 complexes. Importantly, probing the same membrane for calmodulin reveals that it is in the high molecular mass complex with YAP and LATS1, only when Ca²⁺ is present (Fig. 3*B*, *lower panel*). Together, these data demonstrate that pure YAP, LATS1, and calmodulin form a Ca²⁺-dependent complex *in vitro*.

Calmodulin promotes phosphorylation of YAP when Hippo is activated

We next studied whether the binding of calmodulin to YAP and LATS1 affects Hippo signaling. Initially, we investigated

whether calmodulin modulates phosphorylation of YAP on Ser[127], which occurs when Hippo is activated (8).

Serum starvation is known to activate the Hippo signaling network (23, 24). To validate this observation under our assay conditions, we quantified phosphorylated YAP (pYAP) as an indicator of Hippo activation. HeLa cells were grown in complete (10% fetal bovine serum [FBS]) or serum-free medium. Equal amounts of protein lysates from these cells were analyzed by Western blotting and probed for pYAP (Ser[127]) and total YAP. Starved cells display 2.1-fold more YAP phosphorylation than fed cells (Fig. 4*A*), confirming Hippo activation. To determine whether calmodulin influences Hippo activation by serum starvation, we used the highly selective cell-permeable calmodulin antagonist CGS9343B (25). Starved HeLa cells were incubated with 40 μM CGS9343B. Dimethyl sulfoxide (DMSO) was the vehicle control. Western blotting of cell lysates reveals that YAP phosphorylation is significantly reduced by 74% by CGS9343B (Fig. 4*A*). The amount of total YAP was not significantly altered (Fig. 4*A*), validating that the decreased pYAP signal reflects diminished protein phosphorylation rather than protein degradation. Collectively, these data indicate that inhibition of calmodulin impairs the phosphorylation of YAP induced when Hippo is activated by serum starvation.

In order to determine whether the effect of calmodulin is restricted to Hippo activation by serum starvation, we examined its potential role using a different signal to activate Hippo. Cells grown at low confluency have minimal activation of Hippo, whereas increased cell–cell contact at higher density activates signaling (7, 26). We initially validated these observations in our assay system by comparing the abundance of pYAP in HEK293 cells grown at low or high confluency (Fig. 4*B*). Consistent with published data (7, 26), we observed that cells at high confluency had 1.5-fold more YAP phosphorylation than those at low confluency (Fig. 4*C*). Inhibition of calmodulin with CGS9343B reduced by 12% the amount of pYAP in cells at high confluency (Fig. 4*D*), albeit without statistical significance. Taken together, our data demonstrate that calmodulin function is required for maximal YAP phosphorylation in cells when the Hippo kinase cascade is activated by cell starvation and, to a lesser extent, by cell–cell contact.

Calmodulin inhibits expression of Hippo target genes when the pathway is active

When Hippo is active, LATS1/2-mediated phosphorylation of YAP on Ser[127] hinders its translocation to the nucleus, hence inhibiting expression of Hippo target genes (7). Here, we assessed the level of expression of two endogenous Hippo target genes, connective tissue growth factor (*CTGF*) and cysteine-rich angiogenic inducer 61 (*CYR61*), in fed and serum-starved cells. Quantitative real-time PCR analysis revealed, as anticipated, that the expression level of *CTGF* and *CYR61* is significantly lower in starved cells than in cells cultured with FBS (Fig. 5*A*). This result confirms Hippo activation by serum starvation. We used this assay to determine whether calmodulin antagonism modulates Hippo gene
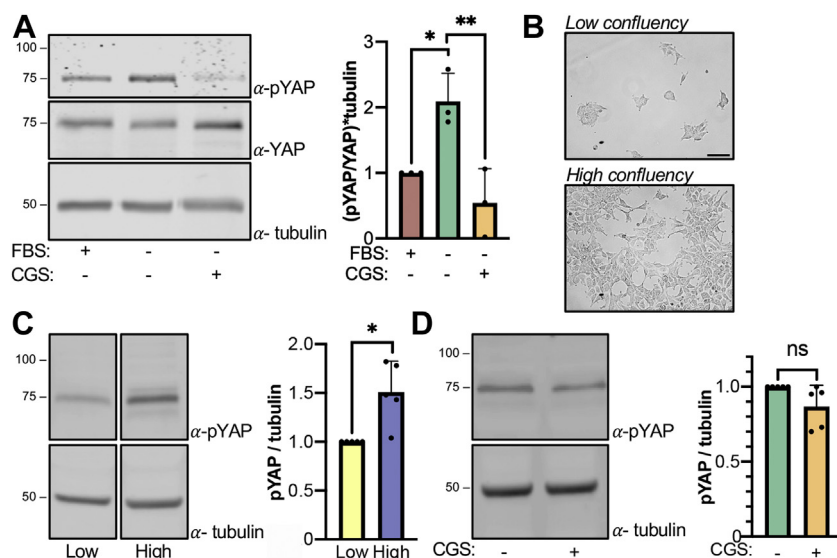
Figure 4. Calmodulin modulates phosphorylation of YAP when Hippo is activated. *A*, HeLa cells cultured in complete (10% FBS, +) or serum-free (−) medium were incubated with 40 µM CGS9343B (CGS, +) or vehicle (DMSO, −). After 16 h, cells were lysed. Equal amounts of protein lysate were resolved by SDS-PAGE, followed by Western blotting. Blots were probed with anti-phosphoYAP (Ser^{127}) (pYAP), anti-YAP, and anti-tubulin (loading control) antibodies. The pYAP, YAP, and tubulin bands were quantified using Image Studio 2.0 (LI-COR Biosciences) for calculation of the pYAP/YAP ratio normalized to the amount of tubulin ([pYAP/YAP] * tubulin). Fed cells were set as 1. Data represent the means ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA ($F_{2,6} = 12.64$; $p ≤ 0.01$) with Tukey's post hoc test (*$p ≤ 0.05$; **$p ≤ 0.01$). *B*, HEK293 cells were plated in 6 cm dishes at low ($1.25 × 10^5$ cells) or high ($5 × 10^5$ cells) density. Once adherent, cells were imaged in transmitted light. The scale bar represents 100 µm. *C*, HEK293 cells grown at low or high confluency were lysed. Equal amounts of protein lysate were resolved by SDS-PAGE and Western blotting and probed with anti-pYAP and anti-tubulin antibodies with further quantification of the pYAP/tubulin ratio. All blot panels are from the same membrane. Data represent the means ± SD of five independent experiments, with cells cultured at low confluency set as 1. *D*, HEK293 cells plated at high density were incubated with 40 µM CGS9343B (CGS, +) or vehicle (−). After 16 h, cells were processed as described for (*C*), with quantification of the pYAP/tubulin ratio. Data show the means ± SD of five independent experiments, with vehicle-treated cells set as 1. Statistical analyses for (*C*) and (*D*) were performed with one-sample *t* tests (*$p ≤ 0.05$; ns). All Western blots shown in this figure are representative of three to five independent experiments. DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HEK293, human embryonic kidney 293 cell line; ns, not significant; YAP, Yes-associated protein.

expression. HeLa cells cultured in serum-free medium were incubated with 40 µM CGS9343B or DMSO. Quantitative real-time PCR analysis shows that CGS9343B increases expression of *CTGF* and *CYR61* by 2.6-fold and 4.0-fold, respectively (Fig. 5*B*). This result suggests that inhibiting calmodulin promotes YAP-mediated transcription when Hippo is activated by serum starvation.

We also examined the effect of cell confluency on *CTGF* and *CYR61* expression. Cells at high confluency have a significantly lower expression of *CTGF* and *CYR61* than cells at low density (Fig. 5*C*), as expected. We investigated whether calmodulin antagonism influences YAP-mediated gene expression induced by cell–cell contact. CGS9343B significantly increases expression of both *CTGF* and *CYR61* in cells at high density (Fig. 5*D*). Collectively, these data reveal that calmodulin impairs expression of at least two endogenous Hippo target genes when the pathway is activated by either cell–cell contact or cell starvation.

Inhibiting calmodulin increases YAP–TEAD nuclear interaction

When Hippo is inactive, YAP binding to TEAD transcription factors in the nucleus triggers expression of target genes. By contrast, Hippo activation prevents YAP from translocating to the nucleus and interacting with TEAD (10). To gain insight into the molecular mechanism by which calmodulin modulates expression of Hippo target genes, we measured the interaction of YAP with TEAD. We assessed the formation of YAP–TEAD complexes in the nucleus by proximity ligation assay (PLA). In this assay, fixed permeabilized cells are incubated with antibodies specific to YAP and TEAD1. A fluorescence signal is generated only when the two antibodies are in close proximity (nanometer range). Consistent with the observation that serum starvation activates Hippo (Fig. 4*A*), starved cells have 60% fewer YAP–TEAD complexes than cells cultured in FBS (Fig. 6, *A*–*C*). Importantly, starved cells incubated with CGS9343B have 2.2-fold more nuclear YAP–TEAD complexes than DMSO-treated cells (Fig. 6, *A*–*C*). We verified by Western blotting that the amount of TEAD is not significantly altered by serum starvation and/or calmodulin antagonism (Fig. 6*D*). Taken together, these data demonstrate that calmodulin impairs YAP–TEAD nuclear interactions when Hippo is activated by serum starvation.

Ca²⁺/calmodulin stimulates LATS1 kinase activity

Calmodulin modulates the activity of numerous kinases in response to changes in intracellular free Ca²⁺ concentration (27). To gain insight into the molecular mechanism by which calmodulin modulates Hippo signaling, we evaluated whether calmodulin directly influences LATS1 kinase. We used purified active LATS1 kinase (Active Motif). Cellular activation of LATS1 requires its phosphorylation at Ser^{909} and Thr^{1079} (28). To validate that the purified kinase was active, we evaluated its phosphorylation at Ser^{909} and Thr^{1079}. Analysis of purified LATS1 by Western blotting with specific antibodies revealed that the LATS1 is phosphorylated at both Ser^{909} and Thr^{1079} (Fig. 7*A*).
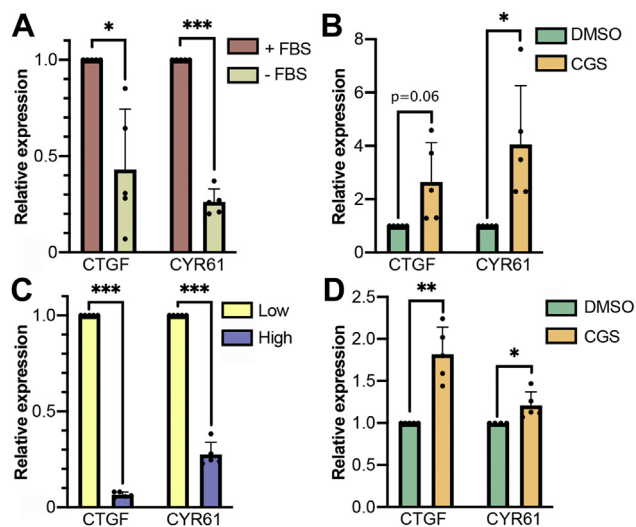
Figure 5. Calmodulin inhibits expression of Hippo target genes when the pathway is active. *A*, HeLa cells were incubated for 16 h in complete (+FBS) or serum-free (−FBS) culture medium. Total RNA was extracted, and expression of *CTGF* and *CYR61* was measured by quantitative RT–PCR (qRT–PCR). The level of expression of these two genes was normalized to that of *GAPDH* in the same sample. Expression of *CTGF* and *CYR61* in fed cells was set as 1. *B*, HeLa cells cultured in serum-free medium were incubated for 16 h with 40 μM CGS9343B (CGS) or an equivalent volume of DMSO (vehicle). RNA extraction and qRT–PCR were carried out. Expression of *CTGF* and *CYR61* in cells treated with vehicle was set as 1. *C*, HEK293 cells were plated at low or high density, as described under the Experimental procedures section. RNA extraction and qRT–PCR were performed. Expression of *CTGF* and *CYR61* in cells at low confluency was set as 1. *D*, cells plated at high density were incubated for 16 h with 40 μM CGS or vehicle DMSO. RNA extraction and qRT–PCR were carried out. Expression of *CTGF* and *CYR61* in cells treated with vehicle was set as 1. For all panels, data represent means ± SD of five independent experiments, each performed in triplicate. Statistical analyses were performed with one-sample *t* tests (*$*p \le 0.05$; **$p \le 0.01$; and ***$p \le 0.001$). *CTGF*, connective tissue growth factor; *CYR61*, cysteine-rich angiogenic inducer 61; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HEK293, human embryonic kidney 293 cell line.

The activity of purified active LATS1 was measured *in vitro* by homogeneous time-resolved fluorescence (HTRF) using the KinEASE STK S1 kit. The assay quantifies phosphorylation of a peptide substrate by LATS1 using FRET between a donor antibody that recognizes phosphorylated sites on the peptide and an acceptor molecule that targets the peptide substrate. The effect of purified calmodulin on LATS1 activity was examined in the presence or the absence of Ca^{2+}. Calmodulin significantly stimulates LATS1 kinase activity in a dose-dependent manner in the presence of Ca^{2+} (Fig. 7B). In contrast, calmodulin does not increase LATS1 kinase activity in the absence of Ca^{2+}. Importantly, addition of pure myoglobin instead of calmodulin to the reaction mixture does not stimulate LATS1 kinase (Fig. 7B). The lack of effect of myoglobin, which has similar molecular weight to calmodulin, validates the specificity of Ca^{2+}/calmodulin. In negative control reactions carried out either without LATS1 or without ATP, basal HTRF signal was low and not increased by Ca^{2+}/calmodulin (Fig. 7B). Collectively, our data reveal that Ca^{2+}/calmodulin directly stimulates active LATS1 kinase.

Discussion

The Hippo pathway, which has a fundamental role in controlling cell growth and proliferation, is dysregulated in many

human pathologies including cancer (29). A comprehensive understanding of the regulatory mechanisms of Hippo signaling is therefore required to develop new therapeutic strategies. Here, we describe a previously unidentified association of the Ca^{2+} sensor calmodulin with the two Hippo proteins YAP and LATS1 and unravel the consequences of these interactions on Hippo activation.

In this study, we demonstrate direct binding of YAP and LATS1 to calmodulin by *in vitro* analyses with pure proteins. Ca^{2+} modulates the interaction of calmodulin with numerous binding partners. Although Ca^{2+}-free calmodulin binds to some proteins, particularly those with IQ motifs (30), Ca^{2+}/calmodulin binds a substantially larger number of proteins (31). When Ca^{2+} binds to the four EF-hands of calmodulin, the protein undergoes a conformational change exposing hydrophobic surfaces for Ca^{2+}-dependent interactions (31). We observed here that the binding of pure YAP and LATS1 to calmodulin is regulated by Ca^{2+}. Chelation of Ca^{2+} with EGTA abrogates the interaction of calmodulin with pure YAP and significantly decreases its binding to pure LATS1. These findings establish both YAP and LATS1 as newly identified calmodulin interactors.

We examined the association of YAP and LATS1 with calmodulin in a normal cellular milieu. Calmodulin-Sepharose pull-downs from cell lysates demonstrate that both endogenous YAP and LATS1 bind to calmodulin. Coimmunoprecipitation analyses further reveal that endogenous calmodulin binds to endogenous YAP and LATS1 in cultured cells. Analogous to pure LATS1, cellular LATS1 binds to calmodulin predominantly in its Ca^{2+}-bound form. In contrast, endogenous YAP associates with both Ca^{2+}/calmodulin and apocalmodulin. Since pure YAP did not bind apocalmodulin *in vitro*, the association of cellular YAP with apocalmodulin most likely is indirect, through an intermediary molecule. The large number of known apocalmodulin interactors (30) precludes us from speculating as to the identity of the intermediary molecule(s).

Importantly, our data with pure proteins reveal that YAP, LATS1, and calmodulin form a Ca^{2+}-dependent complex *in vitro*. Coimmunoprecipitation of both calmodulin and YAP with LATS1, and of both YAP and LATS1 with calmodulin, suggests that a ternary YAP–LATS1–calmodulin complex may be present in cells. It is possible that other Hippo-related proteins, like mammalian Ste20-like kinases 1 and 2 or Mps1-binder kinase activator 1, may also be in the complex, or facilitate the formation of that complex.

To evaluate whether the interaction of calmodulin with YAP and LATS1 influences Hippo signaling in cells, we inhibited calmodulin function using the highly specific cell-permeable calmodulin antagonist CGS9343B. This calmodulin inhibitor, which has been widely used by our group and others (19, 21, 32, 33), was demonstrated to be specific for calmodulin at concentrations of up to 1 mM (25). To minimize the possibility of off-target effects, all assays were carried out at a CGS9343B concentration of 40 μM, which is only 4% of the concentration up to which it has been documented to be specific. Using this approach, we observed that calmodulin antagonism in cells
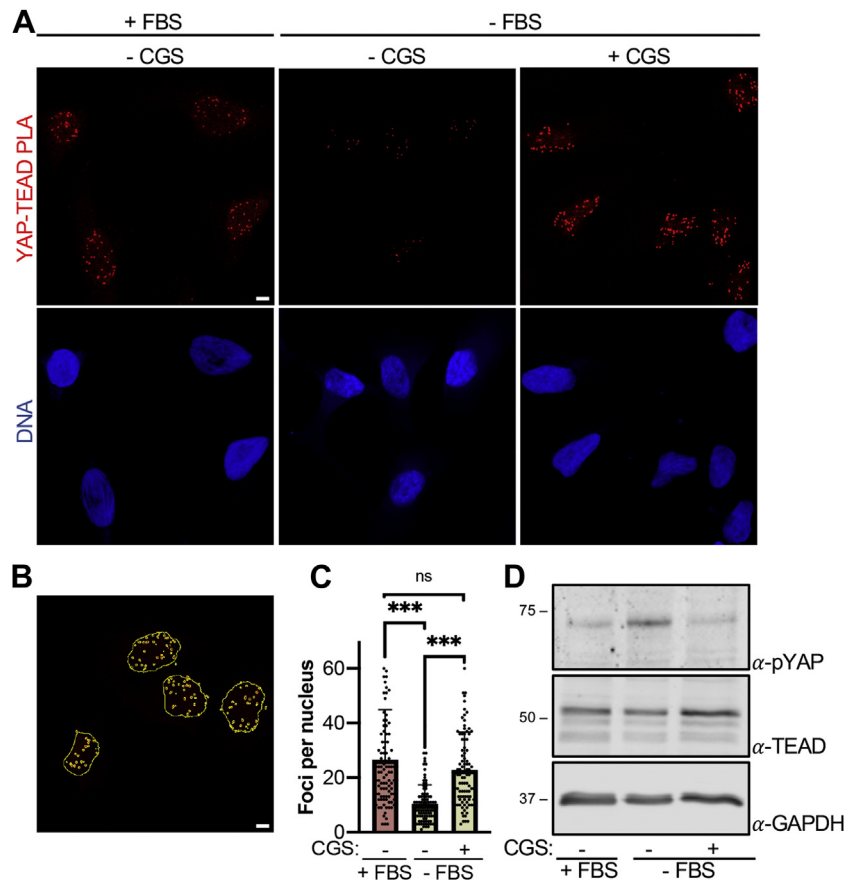
Figure 6. Inhibiting calmodulin increases YAP–TEAD nuclear interaction. *A*, HeLa cells were grown for 16 h in complete (+FBS) or serum-free (−FBS) medium. Cells were then incubated for 16 h with CGS9343B (+CGS) or vehicle (−CGS). Cells were fixed, permeabilized, and incubated with both anti-YAP and anti-TEAD1 antibodies. PLA was carried out using the Duolink *in situ* PLA probes and detection reagents. Cell images were acquired with a confocal microscope (Zeiss LSM880). DNA was stained by Hoechst (*blue*). *Red spots* indicate positive PLA. The scale bar represents 10 μm. *B*, PLA foci in the nucleus were quantified from confocal images by delimiting the area that corresponds to the nucleus (*outer yellow line*) and then defining the PLA foci (*red dots*) within the nucleus. Analysis was carried out using ImageJ. The scale bar represents 10 μm. *C*, PLA foci in the nucleus were quantified in 100 cells for each condition. Data are expressed as means ± SD. Statistical analyses were performed using one-way ANOVA ($F_{2,297}$ = 17.60; p ≤ 0.0001) with Tukey's post hoc test (***p ≤ 0.001; ns). *D*, HeLa cells were cultured in complete or serum-free medium and treated with CGS9343B or vehicle as described for (*A*). After 16 h, cells were lysed. Equal amounts of protein lysate were resolved by SDS-PAGE followed by Western blotting. Blots were probed with anti-pYAP (Ser[127]), anti-TEAD1, and anti-GAPDH (loading control) antibodies. Samples analyzed are from one of the three replicates of the experiment shown for Figure 4*A*, with the top anti-pYAP blot being one of the three quantitatively analyzed in that figure. FBS, fetal bovine serum; ns, not significant; PLA, proximity ligation assay; pYAP, phosphorylated YAP; TEAD, transcriptional enhanced associate domain; YAP, Yes-associated protein.

decreases phosphorylation of YAP on Ser[127], one of the sites catalyzed by LATS1 (8). The reduced phosphorylation occurs regardless of whether Hippo is activated by cell–cell contact or serum starvation. These findings demonstrate that calmodulin function is required for maximal activation of the Hippo kinase cascade.

To better understand how calmodulin modulates activation of the Hippo kinase cascade, we investigated its potential influence on LATS1 kinase activity *in vitro*. Importantly, we observed that Ca^{2+}/calmodulin directly stimulates the kinase activity of pure LATS1 in a dose-dependent manner. In contrast, apocalmodulin fails to stimulate LATS1. The latter observation is consistent with our finding that Ca^{2+} chelation by EGTA significantly decreases LATS1 binding to calmodulin. Interestingly, the Ca^{2+}-binding protein S100B stimulates nuclear Dbf2-related protein kinase (NDR)1 and NDR2, two members of the NDR/LATS1 kinase family, in a Ca^{2+}-dependent manner (34, 35). Binding of Ca^{2+}/S100B induces NDR1 autophosphorylation, leading to its activation (36, 37). In

contrast, we observed that calmodulin stimulates the activity of activated LATS1. Since the identification in 1970 that Ca^{2+}/calmodulin activates phosphodiesterase type 1 (38), numerous other enzymes, particularly kinases, have been shown to be stimulated by Ca^{2+}/calmodulin. *Via* this mechanism, calmodulin participates in diverse cellular functions, ranging from cell proliferation and cyclic nucleotide metabolism to cytoskeletal rearrangements (39). Our results identify for the first time that active LATS1 kinase is stimulated by calmodulin in a Ca^{2+}-dependent manner.

We also demonstrate that calmodulin antagonism alters YAP function. Inhibiting calmodulin in cells with CGS9343B increases expression of the endogenous Hippo target genes *CTGF* and *CYR61*. Because of the lack of a DNA-binding domain, YAP regulates gene expression by binding to transcription factors of the TEAD family in the nucleus (10). To gain insight into the mechanism by which calmodulin inhibition influences Hippo target genes, we evaluated by PLA the association of YAP with TEAD. We demonstrated that

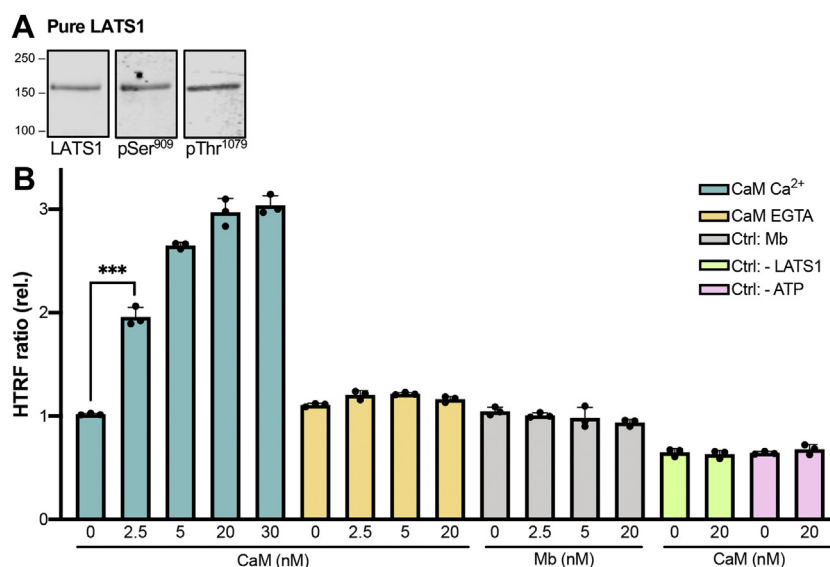**Figure 7. Ca^{2+}/calmodulin (CaM) stimulates LATS1 kinase activity.** *A*, purified LATS1 was analyzed by SDS-PAGE and Western blotting. Blots were probed with anti-LATS1, anti-pLATS1(pSer^{909}), and anti-pLATS1(pThr^{1079}) antibodies. *B*, the activity of LATS1 was measured as described under the Experimental procedures section using the KinEASE STK S1 kit. About 1 μM STK S1 substrate was incubated with 25 nM purified LATS1 and CaM at the concentrations indicated in the presence of Ca^{2+} (*blue bars*) or EGTA (*orange bars*). LATS1 kinase activity was quantified by the HTRF ratio. Control (ctrl) reactions were carried out in the presence of Ca^{2+} with myoglobin (Mb, *gray bars*). Negative controls contained Ca^{2+} and CaM, but either LATS1 (−LATS1, *green bars*) or ATP (−ATP, *pink bars*) was omitted. Data represent the means of three independent experiments ± SD. The HTRF ratio measured without CaM in the presence of Ca^{2+} was set as 1. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test (***$p \leq 0.001$). HTRF, homogeneous time-resolved fluorescence; LATS1, large tumor suppressor 1.

CGS9343B significantly increases the number of YAP–TEAD complexes in the nucleus. Collectively, our results strongly suggest that calmodulin antagonism stimulates expression of Hippo target genes by promoting YAP–TEAD association. These findings imply that calmodulin function inhibits YAP cotranscriptional activity by altering the interaction of YAP with TEAD in the nucleus.

Several possible mechanisms could explain how calmodulin impairs the nuclear interaction of YAP with TEAD. (1) Increased retention of YAP in the cytosol. When activated, the Hippo kinase cascade induces LATS1/2-mediated phosphorylation of YAP on Ser^{127}. This promotes interaction of YAP with 14-3-3, which causes retention of YAP in the cytoplasm (7). By directly activating LATS1, calmodulin probably increases phosphorylation of YAP on Ser^{127} and, consequently, prevents its nuclear translocation. Another mechanism through which calmodulin could promote YAP phosphorylation is by scaffolding YAP and LATS1. Indeed, calmodulin is structurally organized into two globular lobes linked by a flexible tether (40), and each lobe can simultaneously bind different target sequences. For example, calmodulin can interact with two distinct regions of the same polypeptide chain to yield novel structural features (41). Moreover, calmodulin has been suggested to mediate the assembly of heterodimeric structures, as indicated by the calmodulin-dependent complex formation between calcineurin and the nuclear receptor interacting protein (42). Since our coimmunoprecipitation data suggest that calmodulin might form a complex with YAP and LATS1 in cells, it is feasible that calmodulin scaffolds YAP and LATS1. By placing the two proteins in close proximity, calmodulin would increase

LATS1-mediated phosphorylation of YAP on Ser^{127}, resulting in its cytoplasmic retention. The subcellular distribution of YAP is also regulated by direct protein–protein interactions, independently of phosphorylation. For example, axin (43), angiomotin (44), and expanded (45) bind directly to YAP and prevent its translocation to the nucleus. It is noteworthy that calmodulin regulates the subcellular distribution of several binding partners. Among others, calmodulin sequesters the transcription factor c-rel in the cytoplasm (46). Similarly, calmodulin could increase retention of YAP in the cytoplasm by binding to it. (2) Increased protein degradation. Calmodulin has been shown to influence the stability of some of its interactors, notably the estrogen receptor (47). Therefore, calmodulin could potentially inhibit YAP by enhancing its degradation. However, we observed that CGS9343B does not significantly change total YAP concentration, implying that calmodulin does not influence YAP degradation. Alternatively, calmodulin could induce degradation of TEAD, impairing the formation of nuclear YAP–TEAD complexes. We excluded this possibility by showing that calmodulin antagonism does not alter the abundance of TEAD. (3) TEAD–calmodulin competition. Calmodulin in the nucleus could directly compete with TEAD for YAP binding, reducing the formation of YAP–TEAD complexes. These mechanisms are not mutually exclusive, and more than one could operate in the cell.

Recent evidence indicates that changes in intracellular Ca^{2+} levels modulate Hippo signaling (22, 48, 49). Intriguingly, some studies show that Ca^{2+} signaling activates Hippo, whereas others report an inhibitory effect of Ca^{2+} (22). These observations indicate that the Hippo–Ca^{2+} interconnection is complex and likely determined by spatial and temporal factors.

The Ca^{2+}-binding protein S100A1 associates with LATS1 in hepatocellular carcinoma cells (50). Knockdown of S100A1 stimulates the Hippo kinase cascade (50), which is opposite to our observation that calmodulin antagonism decreases Hippo activation. Our study, by demonstrating a functional interaction of YAP and LATS1 with calmodulin, uncovers a further mechanism by which Ca^{2+} can influence the Hippo pathway.

YAP is considered to be an oncogene because of its ability to induce expression of genes involved in cell migration and proliferation (10). Consistently, YAP hyperactivation and/or overexpression has been observed in many human cancers (29). Chemical YAP inhibitors are currently under clinical investigation for chemotherapy (51). Similarly, chemical calmodulin antagonists are also being considered as potential chemotherapeutic agents. Most studies, carried out in cell models of oncogenesis, report that calmodulin inhibitors prevent tumorigenesis (52). For example, calmodulin antagonism inhibits migration and invasiveness of non–small cell lung cancer cells (53) and impairs growth of human breast cancer cell lines (54–56). In contrast, we observed here that CGS9343B increases expression of Hippo target genes involved in cell migration and proliferation, implying that inhibition of calmodulin could promote YAP-induced tumorigenesis. Taken together with previously published work from others, our data suggest that, by regulating the activity of numerous targets, calmodulin likely influences tumorigenesis in a complex multimodal manner. Moreover, our results emphasize the importance of fully understanding the effects of calmodulin antagonists as putative chemotherapeutic agents before undertaking clinical trials because antagonizing calmodulin in patients could promote cell proliferation and invasion by dysregulating Hippo.

In conclusion, our data provide insight into the functional consequences of calmodulin binding to the Hippo proteins YAP and LATS1. While signaling pathways were initially perceived to be linear processes independent from one another, it is now clear that crosstalk and interconnections between signaling networks can occur at multiple levels. Our study reveals a previously unidentified intersection between the Ca^{2+}/calmodulin and Hippo signaling pathways. Fundamentally, these observations expand our understanding of the regulatory mechanisms of the Hippo cascade. Moreover, since Hippo is dysregulated in numerous human pathologies including cancer (29), our findings open new perspectives to develop innovative therapeutic approaches.

Experimental procedures

Materials

Dulbecco's modified Eagle's medium (DMEM), FBS, DMSO, and Halt protease and phosphatase inhibitor cocktail were purchased from Thermo Fisher Scientific. Glutathione-Sepharose, calmodulin-Sepharose, protein A-Sepharose, and protein G-Sepharose beads were from GE Healthcare. CGS9343B was from Tocris. Immobilon-FL polyvinylidene difluoride membrane and ATP were purchased from Sigma–Aldrich. DSP and DTSSP were from Pierce. Normal rabbit and

mouse IgG were from Santa Cruz Biotechnology. ProLong glass antifade mountant was from Invitrogen. Hoechst 33342 was purchased from BD Biosciences. Blocking buffer and IR dye–conjugated secondary antibodies were purchased from LI-COR Biosciences. Table 1 lists the antibodies and dilutions used in this study.

Cell lines and culture conditions

HEK293 and HeLa cells (purchased from American Type Culture Collection) were grown at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS. Cells were seeded in 6 cm dishes at low and high densities with 1.25×10^5 and 5×10^5 cells, respectively, and harvested after 16 h of culture. For serum starvation, cells were cultured for 16 h in FBS-free DMEM. Where indicated, cells were incubated with the calmodulin antagonist CGS9343B (40 μM) or an equivalent volume of DMSO as vehicle for 16 h.

Immunoprecipitations

Cells were washed with ice-cold PBS and exposed to 1 mM DSP for 30 min at 22 °C. The reaction was stopped by the addition of Tris–HCl, pH 7.4, to a final concentration of 50 mM. Cells were washed again and lysed *via* sonication in 1 ml buffer A (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% Triton X-100, and 1 mM EGTA) supplemented with protease and phosphatase inhibitors. Insoluble fractions were pelleted by centrifugation at 15,000*g* for 10 min at 4 °C. Cell lysates were precleared with glutathione-Sepharose beads for 1 h at 4 °C, then incubated with anti-calmodulin, anti-LATS1, or anti-YAP antibodies for 16 h. Samples processed in parallel with IgG served as negative controls. Immune complexes were isolated using protein G (for calmodulin immunoprecipitations) or protein A (for LATS1 and YAP immunoprecipitations) beads. Beads were washed four times with buffer A and resuspended in Laemmli sample buffer. Samples were heated at 95 °C for 5 min and analyzed by SDS-PAGE, followed by Western blotting. The anti-calmodulin antibody pulls down only a fraction of calmodulin from the lysate under our

Table 1
Antibodies used in this study

Protein detected	Source—catalog no. (lot no./reference)	Dilution
Calmodulin	Monoclonal antibody (57)	1:1000 (WB) 1:100 (IP)
LATS1	Cell Signaling—3477S (7)	1:1000 (WB) 1:100 (IP)
pLATS1(Ser^{909})	Cell Signaling—9157S (2)	1:1000 (WB)
pLATS1(Thr^{1079})	Cell Signaling—8654S (7)	1:1000 (WB)
YAP	Cell Signaling—12395S (3) Abcam—ab52771 (GR3266441-6) Cell Signaling—14074S (4)	1:1000 (WB) 1:70 (IP) 1:100 (PLA)
pYAP(Ser^{127})	Cell Signaling—13008S (5)	1:1000 (WB)
TEAD1	BD Biosciences—610922 (8314623)	1:1000 (WB) 1:100 (PLA)
Tubulin	Sigma—T5201 (079M4869V)	1:1000 (WB)
GAPDH	Cell Signaling—2118S (5)	1:1000 (WB)

Abbreviations: IP, immunoprecipitation; PLA, proximity ligation assay; WB, Western blotting.

immunoprecipitation conditions. Note that calmodulin is commonly detected as a doublet on Western blots, where the lower and upper bands correspond to Ca^{2+}/calmodulin and apocalmodulin, respectively.

Binding assays

Binding of purified proteins to calmodulin was assessed using calmodulin-Sepharose beads. We precleared 2 µg of purified recombinant human LATS1 (active motif; catalog no.: 81209) and/or YAP (Origene; catalog no.: TP325864) with glutathione-Sepharose for 1 h at 4 °C. The proteins were then incubated with calmodulin-Sepharose for 3 h at 4 °C in buffer B (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, and 1% Triton X-100) supplemented with 1 mM $CaCl_2$ or 1 mM EGTA as indicated in the figure legends. Control pull-downs with GST-Sepharose were carried out in parallel. After pelleting by centrifugation, beads were washed four times with buffer B containing 1 mM Ca^{2+} or 1 mM EGTA and resuspended in Laemmli sample buffer. Samples were heated at 95 °C for 5 min and analyzed by SDS-PAGE, followed by Western blotting.

For the calmodulin pull-downs from cell lysates, HEK293 cells were lysed in 1 ml buffer B supplemented with protease and phosphatase inhibitors in the presence of 1 mM $CaCl_2$ or 1 mM EGTA as indicated in the figure legends. Samples were precleared with glutathione-Sepharose for 1 h at 4 °C, followed by incubation with calmodulin-Sepharose or GST-Sepharose (control) for 3 h at 4 °C. The beads were processed as described previously. The samples were analyzed by SDS-PAGE and Western blotting.

YAP, LATS1, and calmodulin crosslinking in vitro

Purified YAP, LATS1, calmodulin (Sigma–Aldrich; catalog no.: 208694), and/or myoglobin (Origene; catalog no.: TP312352) (600 ng each) were incubated in 20 µl PBS for 15 min at 22 °C in the presence of 1 mM $CaCl_2$ or 1 mM EGTA and then 2.5 mM DTSSP was added to crosslink the proteins. After 30 min, 50 mM Tris–HCl (pH 7.4) was added for 15 min at 22 °C to stop the reaction. Samples were resuspended in nonreducing Laemmli sample buffer and analyzed by SDS-PAGE, followed by Western blotting. Blots were probed first with anti-YAP and anti-LATS1 antibodies. After stripping, the blots were probed with anti-calmodulin monoclonal antibody.

Quantitative RT–PCR

To quantify the expression of *CTGF* and *CYR61*, total RNA was isolated from cells using the PureLink RNA Mini kit (Invitrogen) according to the manufacturer's instructions. Reverse transcription and quantitative PCR were performed with the Power SYBR Green RNA-to-C_T 1-Step kit (Applied Biosystems) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Each reaction was carried out with 100 ng RNA. The primers used (100 nM per reaction) are listed in Table 2. Reverse transcription was carried out at 48 °C for 30 min, followed by PCR enzyme activation at 95 °C for 10 min and 40 amplification cycles (30 s at 95 °C and 1 min at

Table 2
Quantitative RT–PCR primers used in this study

Gene	Primer sequence
CTGF	Forward: 5′-GCCACAAGCTGTCCAGTCTAATCG-3′
	Reverse: 5′-TCGATTCTCCAGCCATCAAGAGAC-3′
CYR61	Forward: 5′-GAGTGGGTCTGTGACGAGGAT-3′
	Reverse: 5′-GGTTGTATAGGATGCGAGGCT-3′
GAPDH	Forward: 5′-ATGGGGAAGGTGAAGGTCG-3′
	Reverse: 5′-GGGGTCATTGATGGCAACAATA-3′

60 °C). All samples were assayed in technical triplicates, with the use of *GAPDH* as a housekeeping gene. The results were analyzed using the ΔΔCT method.

PLA

HeLa cells were seeded on coverslips in 24-well plates. When 20% confluency was reached, cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. After blocking in 10% FBS for 16 h, coverslips were incubated for 1 h at 37 °C with rabbit anti-YAP and mouse anti-TEAD1 antibodies. Donkey anti-mouse PLUS and anti-rabbit MINUS oligonucleotide-labeled secondary antibodies (PLA probes; Sigma–Aldrich) were then added to the cells for 1 h at 37 °C. DNA ligation and amplification were carried out using the Duolink *In Situ* Detection Reagents Red (Sigma–Aldrich) following the manufacturer's protocol. DNA was stained with Hoechst 33342. Coverslips were mounted using slide mounting medium, and the cells were examined by confocal microscopy (LSM880; Carl Zeiss Microscopy). Fluorescence images were collected with a 63× objective lens (numerical aperture of 14). The number of PLA spots in each cell nucleus was counted using Fiji/ImageJ (National Institutes of Health). Briefly, individual nuclei were segmented based on the Hoechst staining, and the PLA spots were segmented as individual objects based on the red fluorescence intensity above background. A total of 100 cells were quantified for each condition.

In vitro LATS1 kinase assay

LATS1 kinase activity was evaluated *in vitro* using the HTRF KinEASE STK S1 kit (Cisbio Bioassays) according to the manufacturer's instructions. Briefly, 25 nM of human recombinant LATS1 was incubated with 1 µM of the STK S1 substrate in the kinase buffer provided in the kit (supplemented with 5 mM $MgCl_2$ and 1 mM DTT) with 1 mM $CaCl_2$ or 1 mM EGTA and various concentrations (2.5, 5, 20, and 30 nM) of calmodulin or myoglobin. The reaction was initiated by the addition of 100 µM ATP. Control reactions omitted either LATS1 or ATP. After incubation at 22 °C for 1 h, 62.5 nM streptavidin-XL665 and 5 µl STK antibody cryptate were added to the reaction mixture. Samples were incubated for 1 h at 22 °C, and the fluorescence emission at 665 and 620 nm after excitation at 330 nm was measured with a microplate reader (BioTek Synergy 4). The HTRF ratio was calculated as the fluorescence emission at 665 nm over that measured at 620 nm. An increase in this ratio reflects greater kinase activity.

Statistical analyses

All statistical analyses were carried out using Prism 9 (GraphPad Software, Inc). Comparisons to data normalized to one were carried out with one-sample *t* tests. Other datasets were analyzed with ANOVA. Bands of interest on Western blots were quantified with Image Studio 2.0 (LI-COR Biosciences) according to the manufacturer's instructions. Where indicated, the signal intensity of the band of interest was normalized to that of the loading control band (tubulin or GAPDH).

Data availability

This study includes no data deposited in external repositories.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: *CTGF*, connective tissue growth factor; *CYR61*, cysteine-rich angiogenic inducer 61; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DSP, dithiobis(succinimidyl propionate); DTSSP, 3,3'-dithiobis(sulfosuccinimidyl propionate); FBS, fetal bovine serum; GST, glutathione-*S*-transferase; HEK293, human embryonic kidney 293 cell line; HTRF, homogeneous time-resolved fluorescence; IgG, immunoglobulin G; LATS1, large tumor suppressor 1; LATS2, large tumor suppressor 2; NDR, nuclear Dbf2-related protein kinase; PLA, proximity ligation assay; pYAP, phosphorylated YAP; TAZ, transcriptional coactivator with PDZ-binding motif; TEAD, transcriptional enhanced associate domain; YAP, Yes-associated protein.

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