IQGAP1 Binds to Yes-associated Protein (YAP) and Modulates Its Transcriptional Activity*

Samar Sayedyahossein, Zhigang Li, Andrew C. Hedman, Chase J. Morgan, and David B. Sacks

From the Department of Laboratory Medicine, National Institutes of Health, Bethesda, Maryland 20892

During development, the Hippo signaling pathway regulates key physiological processes, such as control of organ size, regeneration, and stem cell biology. Yes-associated protein (YAP) is a major transcriptional co-activator of the Hippo pathway. The scaffold protein IQGAP1 interacts with more than 100 binding partners to integrate diverse signaling pathways. In this study, we report that IQGAP1 binds to YAP and modulates its activity. IQGAP1 and YAP co-immunoprecipitated from cells. In vitro analysis with pure proteins demonstrated a direct interaction between IQGAP1 and YAP. Analysis with multiple fragments of each protein showed that the interaction occurs via the IQ domain of IQGAP1 and the TEAD-binding domain of YAP. The interaction between IQGAP1 and YAP has functional effects. Knock-out of endogenous IQGAP1 significantly increased the formation of nuclear YAP-TEAD complexes. Transcription assays were performed with IQGAP1-null mouse embryonic fibroblasts and HEK293 cells with IQGAP1 knockdown by CRISPR/Cas9. Quantification demonstrated that YAP-TEAD-mediated transcription in cells lacking IQGAP1 was significantly greater than in control cells. These data reveal that IQGAP1 binds to YAP and modulates its co-transcriptional function, suggesting that IQGAP1 participates in Hippo signaling.

The Hippo signaling pathway comprises numerous proteins that regulate organ size and shape, regeneration, and stem cell biology (1). The Hippo pathway responds to several stimuli, such as stress, polarity, and adhesion cues and has been implicated in tumorigenesis (2). The primary components of this cascade are a kinase module and a transcriptional module. The transcriptional module consists of yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ), which together drive the activities downstream of the Hippo pathway (3). Importantly, YAP shuttles between the cytoplasm and nucleus. Nevertheless, nuclear translocation of YAP is not sufficient for induction of transcriptional activity because YAP does not contain a DNA-binding region. Nuclear YAP activity is elicited by binding to transcription factors. Among these, TEA domain family members (TEADs) are the major transcription factors driving YAP-mediated gene transcription (4, 5). YAP activity is regulated through phosphorylation-dependent and -independent mechanisms. When Hippo is ON, YAP gets phosphorylated, and its co-transcriptional activity is inhibited. The kinase module of the Hippo pathway, including large tumor suppressor 1 and 2 (LATS1/2) and mammalian STE20-like protein kinase 1 and 2 (MST1/2), inhibits YAP by catalyzing phosphorylation at Ser127 or Ser381 (6). Phosphorylation of YAP at Ser127 induces its interaction with 14-3-3, which leads to retention of YAP in the cytoplasm, thereby inhibiting transcriptional activity. Phosphorylation of YAP at Ser381 (Ser397 in mice) corresponds to residue Ser397 in humans; all reference to phosphorylation at this site will be described as Ser397) leads to its ubiquitination and cytoplasmic degradation. Furthermore, other regulatory proteins, such as NF2 (neurofibromatin 2) and KIBRA (kidney and brain expressed protein), form a complex to activate the Hippo kinase module, thereby inhibiting YAP nuclear activity (7). YAP can also be modulated by direct protein-protein interactions. For example, the angiomit (AMOT) family of proteins binds to YAP and promotes its cytoplasmic retention (8). In contrast, direct binding of YAP to multiple ankyrin repeats single KH domain-containing protein (MASK) in the nucleus potentiates YAP-mediated transcriptional activity (9).

IQGAPs are evolutionary conserved, scaffold proteins with multiple functions. Three IQGAP family members (named IQGAP1, IQGAP2, and IQGAP3) have been identified in mammals (10). The expression of IQGAP2 is restricted to liver (11), and IQGAP3 is reported to be expressed in lung, brain, testis, small intestine, and colon (12). IQGAP1 is ubiquitously expressed and contains five major domains: a calponin homology domain, a WW domain, an IQ domain, a RasGAP-related domain, and a RasGAP-like domain (13). These domains mediate binding to a wide range of partners, thereby forming multi-protein complexes (13). For example, the IQ region of IQGAP1 binds to calmodulin (14), the myosin essential light chain Mt1c (15), the calcium and zinc ion binding protein S100B (16), and EGFR receptor (17). Through these interactions, IQGAP1 is a component of several signaling cascades including G-protein-coupled receptor, transforming growth factor β, and EGF receptor, which regulate processes such as proliferation, differentiation, and transcription (18).
Accumulating evidence suggests that IQGAP1 also participates in nuclear processes. Some nuclear functions of IQGAP1 are elicited via direct interaction with and regulation of transcription factors, such as estrogen receptor (ER) and nuclear factor erythroid-related factor 2 (20). In addition, IQGAP1 binds to catenin and enhances its nuclear accumulation and transcriptional co-activation (21). Because IQGAP1 regulates transcription and is a component of receptor signaling cascades, which integrate with the Hippo pathway, we postulated that IQGAP1 may participate in Hippo signaling. In this study we demonstrate that IQGAP1 and YAP associate directly in vitro and in cells. Moreover, IQGAP1 inhibits formation of YAP-TEAD complexes in the nucleus, thus impairing YAP-mediated transcriptional activity.

Results

IQGAP1 Associates with YAP—To determine whether IQGAP1 binds to YAP, we examined their association using HeLa cells, which express appreciable amounts of both proteins. Cell lysates were incubated with full-length GST-IQGAP1 or GST alone was incubated with equal amounts of protein from HeLa cell lysates. Complexes were isolated, washed, and analyzed by SDS-PAGE. The gel was cut at the 100-kDa region. The lower portion of the gel was transferred to PVDF, and the blot was probed with anti-YAP antibody (upper panel). The top part of the gel was stained with Coomassie Blue (lower panel). B and C, HeLa cells were lysed, and equal amounts of protein were loaded directly onto the gel (Lysate) or immunoprecipitated with either polyclonal anti-IQGAP1 or rabbit IgG antibodies in (B) or monoclonal anti-YAP or mouse IgG antibodies (as a negative control) in (C). Immune complexes were analyzed by SDS-PAGE and transferred to PVDF membrane. Blots were probed with anti-IQGAP1 and anti-YAP antibodies. All data are representative of at least three independent experiments. D, to activate the Hippo pathway, HEK293T cells were cultured in medium containing 10% FBS (+) or starved of serum (−) for 16 h. Equal amounts of protein lysate were resolved by SDS-PAGE and Western blotting. The blots were probed with the indicated antibodies. E, cells, treated as described for D, were lysed, and equal amounts of protein were loaded onto the gel (Lysate) or immunoprecipitated with either polyclonal anti-YAP or rabbit IgG (negative control) antibodies. Immune complexes were analyzed by SDS-PAGE and transferred to PVDF membrane. The blots were probed with anti-IQGAP1 and anti-YAP antibodies. The IQGAP1 bands were quantified with Image Studio 2.0 (LI-COR Biosciences) and corrected for the amount of immunoprecipitated YAP in the corresponding sample. The data are expressed as means ± SEM (error bars) (n = 2) with FBS-treated samples set as 1.

Accumulating evidence suggests that IQGAP1 also participates in nuclear processes. Some nuclear functions of IQGAP1 are elicited via direct interaction with and regulation of transcription factors, such as estrogen receptor α (ERα) and nuclear factor erythroid-related factor 2 (19, 20). In addition, IQGAP1 binds to β-catenin and enhances its nuclear accumulation and transcriptional co-activation (21). Because IQGAP1 regulates transcription and is a component of receptor signaling cascades, several of which integrate with the Hippo pathway, we postulated that IQGAP1 may participate in Hippo signaling. In this study we demonstrate that IQGAP1 and YAP associate directly in vitro and in cells. Moreover, IQGAP1 inhibits formation of YAP-TEAD complexes in the nucleus, thus impairing YAP-mediated transcriptional activity.

IQGAP1 Modulates YAP Activity

To investigate the interaction of IQGAP1 and YAP in a normal cell environment, we conducted immunoprecipitation studies. Endogenous YAP co-immunoprecipitated with endogenous IQGAP1 from HeLa cell lysates (Fig. 1B). In contrast, no YAP was precipitated with GST alone, showing specificity of binding. The Coomassie stain shows the GST-IQGAP1 (Fig. 1A, upper panel).

To assess the effect of Hippo pathway activation on the association between IQGAP1 and YAP, we cultured HEK293T cells at high density and starved them of serum. These conditions induce Hippo activation (22), which was confirmed by phosphorylation and activation of LATS (Fig. 1D). Note that the abundance of YAP decreases considerably because of degradation induced by Hippo activation. The amount of IQGAP1 that co-immunoprecipitated with YAP from cells incubated with

Results

IQGAP1 Associates with YAP—To determine whether IQGAP1 binds to YAP, we examined their association using HeLa cells, which express appreciable amounts of both proteins. Cell lysates were incubated with full-length GST-IQGAP1 or GST as a control. GST-IQGAP1 pulled down endogenous YAP from lysates (Fig. 1A, upper panel). By contrast, no YAP was precipitated with GST alone, showing specificity of binding. The Coomassie stain shows the GST-IQGAP1 (Fig. 1A, upper panel).
and without serum was virtually the same (Fig. 1E). These results demonstrate that Hippo pathway activation does not regulate IQGAP1 binding to YAP.

The IQ Region of IQGAP1 Binds to the TEAD-binding Region of YAP—The data presented in Fig. 1 reveal an association between IQGAP1 and YAP in cell lysates. It is not possible to establish from these data whether the two proteins bind directly or whether their association is indirect via intermediary binding partners. To ascertain whether IQGAP1 and YAP bind directly, we performed analysis with an in vitro transcription and translation (TnT) system and purified recombinant proteins. Selected regions of IQGAP1 (Fig. 2A) were expressed using TnT and labeled with [35S]methionine. The constructs were incubated with GST-YAP and isolated with glutathione-Sepharose beads. After washing, samples were resolved by SDS-PAGE, and gels were dried and processed by autoradiography. The data are representative of three independent experiments.

FIGURE 2. The IQ region of IQGAP1 binds to the TEAD-binding region of YAP. A and C, schematic representation of IQGAP1 (A) and YAP (C) constructs. Full-length (FL) and deletion mutants are depicted. The specific amino acid residues in each construct are indicated. CHD, calponin homology domain; WW, tryptophan containing domain; GRD, RasGAP related domain; RGCT, RasGAP C terminus; TBD, TEAD-binding; TAD, transcription activation domain. B, [35S]methionine-labeled IQGAP1 constructs generated by TnT were incubated with equal amounts of GST-YAP (top panel) or GST alone (middle panel). Samples were run on SDS-PAGE, and gels were dried and analyzed by autoradiography. The bottom panel depicts 5% input of TnT peptides used for pulldown. D, [35S]methionine-labeled YAP constructs generated by TnT were incubated with equal amounts of GST-IQGAP1 (top panel) or GST alone (middle panel). Samples were run on SDS-PAGE, and gels were dried before being analyzed by autoradiography. Input represents 1% of the protein included in the pulldown. The data are representative of three independent experiments.
GST alone (Fig. 2B, middle panel). The expression levels of TNT products were comparable among samples (Fig. 2B, bottom panel). To establish whether the IQ region of IQGAP1 is necessary for YAP binding, we used a construct (termed IQGAP1ΔIQ) that lacks this region. Autoradiography revealed that deletion of the IQ region from IQGAP1 abrogated binding to YAP (Fig. 2B). Collectively, these data demonstrate that the IQ region of IQGAP1 is both necessary and sufficient for YAP binding.

To determine which region of YAP mediates binding to IQGAP1, we generated constructs that contain selected regions of YAP (Fig. 2C) and expressed them using the TNT system. Full-length YAP binds directly to IQGAP1 (Fig. 2D, top panel). Neither the construct containing the WW region of YAP (YAP-M, amino acids 115–341) nor the C-terminal portion of YAP (amino acids 322–454) binds to IQGAP1. Analysis of shorter fragments in the N-terminal region of YAP revealed that amino acids 2–114, but not amino acids 2–85, associate with pure IQGAP1 (Fig. 2D, top panel). No binding was observed to GST alone (Fig. 2D, middle panel), validating specificity. Collectively, these data indicate that IQGAP1 through its IQ domain binds to the TEAD-binding region of YAP.

**Generation of IQGAP1 Knockdown Cells Using CRISPR/Cas9—**

To investigate the effect of IQGAP1 on YAP function, we generated IQGAP1 knock-out HEK293 cells using the CRISPR/Cas9 system. A double nicking strategy was employed to reduce the likelihood of off target gene modification. With this method, paired guide RNAs are used with a mutant Cas9 (D10A mutation) that cleaves single strands. This system generates a specific double-strand break within the IQGAP1 gene, whereas off target single strand nicks will be repaired without mutation. Both sequencing (Fig. 3A) and Western blotting (Fig. 3B) confirmed the loss of IQGAP1 expression. Deletion of IQGAP1 from HEK293 cells did not alter the expression level of IQGAP2 or IQGAP3 (Fig. 3B).

**IQGAP1 Modulates YAP Transcriptional Activity—**

We evaluated YAP function by analyzing YAP-TEAD transcriptional activity. Luciferase-based assays are widely used to study endogenous YAP transcriptional activity in different cell types (23, 24). We transfected HEK293 cells with a synthetic TEAD
IQGAP1 modulates YAP activity. Several experiments showed that the expression level of YAP was not significantly changed by YAP-mediated transcription. Western blotting demonstrated that the abundance of TEAD, LATS1, pYAP-S127, and pYAP-S381 was similar in control and IQGAP1-null cells (Fig. 4A). To verify these findings, we evaluated YAP-TEAD-mediated transcription in a different cell line. Analysis was performed using mouse embryonic fibroblasts (MEFs) generated from embryos of IQGAP1+/− mice and their wild-type littermate controls (25). Analogous to the data in HEK293 cells, YAP-TEAD-mediated transcriptional activity was increased by 3.8 ± 0.7-fold when IQGAP1 was eliminated from MEFs (Fig. 4B). Western blotting demonstrated that the abundance of TEAD, LATS1, pYAP-S127, and pYAP-S381 is not altered in IQGAP1-null MEFs. Collectively, our data using two independent methods of IQGAP1 depletion revealed that IQGAP1 suppresses YAP-mediated transcriptional activity in two different cell types.

To substantiate that the increased YAP-TEAD transcriptional activity is due to the absence of IQGAP1, we performed rescue experiments. Transfecting IQGAP1 knock-out HEK293 cells with full-length IQGAP1 significantly reduced YAP transcriptional activity, although not to levels in control cells (Fig. 4C). By contrast, reconstituting HEK293 cells with IQGAP1ΔIQ, which does not bind YAP (Fig. 2B), did not significantly change YAP-mediated transcription. Western blotting analysis confirmed the expression of the IQGAP1 constructs and showed that the expression level of YAP was not altered (Fig. 4C, lower panels). These data indicate that IQGAP1 attenuates YAP-TEAD transcriptional activity by interacting with YAP.

To extend these findings, we examined the possible effects of IQGAP1 on the ability of YAP to activate the transcription of luciferase from four independent experiments, each performed in duplicate. The data were normalized to IQGAP1+/− cells transfected with empty vector. The data represent the means ± S.E. (error bars) of three independent experiments, each performed in triplicate. *, p < 0.05 compared with control. Western blots show whole cell lysates analyzed by immunoblot probed with the specified antibodies. D, total RNA was extracted from control and IQGAP1-null MEFs. Quantitative RT-PCR (qRT-PCR) was performed to measure AMOTL2 and CTGF hnRNA. The amount of hnRNA was normalized to IQGAP1+/− MEF cells was set as 1. The data represent the means ± S.E. (error bars) of three independent experiments, each performed in triplicate. *, p < 0.01; **, p < 0.001 compared with control cells. E and F, total RNA was extracted from control MEFs transfected with empty vector (V) and IQGAP1-null MEFs transfected with empty vector (V), full-length IQGAP1 (FL), or IQGAP1ΔIQ (ΔIQ). Quantitative RT-PCR was performed to measure AMOTL2 (E) and CTGF (F) hnRNA. The amount of hnRNA was corrected for β-actin hnRNA in the same sample. hnRNA in IQGAP1+/− MEFs transfected with empty vector was set as 1. The data represent the means ± S.E. (error bars) of at least four independent experiments, each performed in triplicate. *, p < 0.05 compared with IQGAP1+/− cells transfected with empty vector. Western blots show whole cell lysates probed with anti-IQGAP1 polyclonal antibodies. Actin was used as a loading control.
two endogenous YAP-responsive genes, namely AMOTL2 and CTGF. Gene expression was measured by quantitative RT-PCR. Analysis revealed that AMOTL2 hnRNA was 1.8 ± 0.1-fold greater in IQGAP1-deficient MEFs than in control cells (Fig. 4D). Similarly, the absence of IQGAP1 increased by 4.1 ± 0.4-fold the amount of CTGF hnRNA.

To confirm that the increased YAP-TEAD transcriptional activity in knock-out cells is due to lack of IQGAP1, we reconstituted IQGAP1-null MEFs. Compared with vector, exogenous expression of full-length IQGAP1 reduced the transcription of AMOTL2 by 43%, almost completely restoring it to levels in control cells (Fig. 4E). This is equivalent to a 95% “rescue” of the increase in transcription caused by the absence of IQGAP1. By contrast, transfection with IQGAP1ΔIQ did not decrease AMOTL2 transcription (Fig. 4E). Transcription of another endogenous YAP-responsive gene, CTGF, was significantly decreased in IQGAP1-null MEFs when transfected with full-length IQGAP1, whereas IQGAP1ΔIQ had essentially no effect (Fig. 4F). Note the reduced magnitude of the effect of the absence of IQGAP1 on gene transcription when cells were transfected with empty vector (Fig. 4, compare E and F with D). This difference is likely because of effects of transfection on the analysis. The level of expression of full-length IQGAP1 and IQGAP1ΔIQ were equivalent and similar to IQGAP1 levels in control MEFs (Fig. 4E, lower panels). These results, which are consistent with our observations using luciferase-based assays, confirm that IQGAP1 modulates YAP transcriptional activity.

YAP-TEAD Nuclear Interaction Is Increased in the Absence of IQGAP1—Binding of YAP to TEAD in the nucleus is essential for YAP-TEAD-mediated transcriptional activity (4). To elucidate the molecular mechanism by which IQGAP1 modulates transcriptional activity of YAP, we examined the effect of IQGAP1 on the formation of YAP-TEAD complexes in the nucleus. The interaction between endogenous YAP and TEAD was analyzed by proximity ligation assay (PLA). In this technique, cells are incubated with specific antibodies. A signal is generated only when the antibodies are in close proximity (nm range). PLA analysis with anti-YAP and anti-TEAD antibodies revealed an interaction between YAP and TEAD in the nucleus of MEFs (Fig. 5A). Quantification demonstrated that the number of stable nuclear YAP-TEAD complexes was 5.9-fold greater in IQGAP1−/− MEFs than in control cells (n = 130) (Fig. 5B). These data suggest that IQGAP1 regulates the formation of YAP-TEAD complexes in the nucleus.

To confirm that the complex formation is modulated by IQGAP1, we reconstituted IQGAP1-null MEFs with GFP-tagged IQGAP1 (Fig. 5C). GFP alone was used as control. Exogenous expression of IQGAP1 significantly attenuated the number of YAP-TEAD complexes in the nucleus of IQGAP1-null cells, with YAP-TEAD complex formation being restored almost to the level seen in control cells (Fig. 5, C and D).

Subcellular Localization of YAP Is Not Altered by IQGAP1—To investigate the subcellular localization of YAP, control and IQGAP1−/− MEFs were separated into cytoplasmic and nuclear fractions. Western blotting revealed that the amounts of YAP in the cytoplasmic fractions of IQGAP1-null and control MEFs were virtually identical (Figs. 6, A and B). Similarly, the abundance of YAP in the nucleus was not altered by the absence of IQGAP1. Minimal IQGAP1 was detected in the nuclear fraction of IQGAP1-expressing cells (Fig. 6A). To further confirm our findings, we immunostained YAP in control and IQGAP1−/− MEFs. The intensity of the YAP signal in the cytoplasm and nucleus in IQGAP1−/− MEFs was comparable to that in control cells (Fig. 6C), suggesting that IQGAP1 does not influence YAP subcellular localization in MEFs.

The possible effect of Hippo activation on the subcellular distribution of IQGAP1 was also examined. MEFs were cultured in serum-free medium, which activated the Hippo pathway, as indicated by LATS phosphorylation and YAP degradation (Fig. 6D). Activation of the Hippo pathway did not alter the subcellular localization of IQGAP1 (Fig. 6E). Collectively, these data suggest that IQGAP1 does not influence the subcellular localization of YAP and that the Hippo pathway does not regulate IQGAP1 localization.

Discussion

IQGAP1 interacts with a wide range of binding partners and forms important signaling complexes, such as scaffolding components of the MAPK pathway or the actin cytoskeleton (26, 27). A growing body of evidence suggests that the association of IQGAP1 with these signaling complexes modulates physiological functions (13). IQGAP1 has been predominantly studied in the cytoplasm, and its roles in the nucleus and in the regulation of transcription are less characterized (18). Here we document that IQGAP1 interacts with the transcriptional co-activator YAP. Further, we demonstrate that IQGAP1 negatively modulates YAP transcriptional activity, establishing a link between IQGAP1 and the Hippo pathway.

An important observation in our study is a direct interaction between YAP and IQGAP1. Binding to YAP is mediated through the N-terminal half of IQGAP1, which contains the IQ region. The strong binding of the isolated IQ region to YAP and loss of YAP association when amino acids 764–863 of IQGAP1 are deleted confirm that the IQ region is both necessary and sufficient for YAP binding. The IQ region of IQGAP1 interacts with several proteins, including calmodulin (28), S100B (16), and EGF receptor (17). The association of many proteins with IQGAP1 is modulated through signaling inputs. For example, Ca2+ regulates the interaction of the IQ domain of IQGAP1 with calmodulin (14), whereas estradiol modulates its association with ERα (19). Thus, it is likely that diverse stimuli affect the association of different proteins with the IQ region of IQGAP1. Therefore, it seems reasonable to postulate that IQGAP1 forms distinct complexes with different proteins, including YAP, depending on extra- and intracellular cues.

Binding to IQGAP1 alters YAP function. When the Hippo pathway is OFF, YAP translocates to the nucleus where it binds TEAD, thereby activating gene transcription (Fig. 7). Activation of the Hippo pathway decreases YAP in the nucleus, attenuating transcription. Our data reveal that hnRNA levels of the endogenous YAP target genes, AMOTL2 and CTGF, were significantly higher in IQGAP1-null MEFs than in IQGAP1+/+ MEFs. Similarly, elimination of IQGAP1 from either HEK293 or MEF cells augmented YAP-TEAD-mediated transcription by 3.8–4.5-fold over control cells. Importantly, re-expression of full-length IQGAP1, but not IQGAP1ΔIQ, in IQGAP1-null cells suppressed YAP-dependent transcription.
HEK293 cells resulted in a significant reduction in YAP-mediated transcription. Reconstitution of IQGAP1-null MEFs with full-length IQGAP1 restored AMOTL2 and CTGF transcription essentially to levels seen in control cells. By contrast, incomplete suppression was observed for YAP-TEAD-mediated transcription measured with the luciferase assay. There are several possible explanations for these differences. The luciferase assay was performed in HEK293 cells, whereas endogenous YAP target gene hnRNA was isolated from MEFs. The differences observed may be due to the nature of the assay or cell types examined. The luciferase assay was conducted 24 h after transfection with IQGAP1 constructs, whereas RT-PCR was performed 48 h after transfection. It is possible that longer induction of IQGAP1 expression is required to restore its full function in the luciferase assay in HEK293 cells. Furthermore, transient transfection of multiple plasmids required in the luciferase-based assay may affect their expression, resulting in an incomplete rescue. Regardless of the explanation, our findings strongly suggest that binding of IQGAP1 to YAP modulates Hippo signaling. Collectively, our data imply that IQGAP1 is required for inhibition of YAP transcriptional activity in the Hippo pathway.

Analogous to depletion of IQGAP1, loss of some Hippo pathway activators increases YAP nuclear activity. For example, liver specific knock-out of MST1/2 kinases results in increased YAP-mediated transcription (29). Further, knock-out of MOB1 from mouse keratinocytes promotes YAP nuclear activity (30). Accumulating evidence implicates IQGAP1 in transcriptional regulation (18). IQGAP1 binds directly to ERα and promotes ERα-mediated gene transcription induced by estradiol (19). Moreover, IQGAP1 binds the transcriptional co-activator, β-catenin (21), an integral component of the canonical Wnt signaling pathway. This interaction augments β-catenin function by promoting its association with importin-β5, which is

FIGURE 5. Increased YAP-TEAD nuclear interaction in the absence of IQGAP1. A, IQGAP1+/+ and IQGAP1−/− MEFs were fixed and stained with both anti-YAP and anti-TEAD antibodies. PLA was performed using Duolink in situ detection reagents as described under “Experimental Procedures.” Red spots indicate positive PLA. DNA is stained by Hoescht (blue). Dotted lines outline the nuclei. Scale bar, 10 μm. B, PLA spots in the nucleus were quantified from confocal images of 130 cells. The number of spots per nucleus in control (black bar) and IQGAP1-null (white bar) MEFs were quantified using IMARIS software. The data are expressed as means ± S.E. (error bars) with the number of spots in control cells set to 1. *, p < 0.001 Student’s t test. C, IQGAP1+/+ MEFs were transfected with GFP-tagged IQGAP1 or GFP alone (control). Samples were processed for PLA as described for A. Red spots indicate positive PLA. DNA is stained by Hoescht (blue). Scale bar, 10 μm. D, PLA spots in the nucleus were quantified from confocal images of 35 cells. The number of spots per nucleus in GFP control and GFP-IQGAP1 expressing MEFs were quantified using IMARIS software. The data are expressed as means ± S.E. with the number of spots in GFP transfected cells set to 1. *, p < 0.001 Student’s t test.
necessary for nuclear localization of β-catenin (31). In contrast to the stimulatory effect of IQGAP1 on ERα and β-catenin, IQGAP1 negatively regulates some transcription factors. As we observed with YAP in the current study, depletion of IQGAP1 enhances transcriptional activation of nuclear factor of activated T-cells (32). In cells lacking IQGAP1, phosphorylation and nuclear translocation of nuclear factor of activated T-cells were increased. These observations reveal a complex role for IQGAP1 in gene regulation, where it positively regulates some transcription factors and negatively regulates transcription of other selected genes.

Investigation of the molecular mechanisms by which IQGAP1 impairs YAP-TEAD-mediated transcriptional activity revealed that IQGAP1 markedly reduced the formation of YAP-TEAD complexes in the nucleus. Because nuclear YAP-TEAD interaction is required for the transcriptional activation (4), a reduction in complex formation will attenuate YAP co-transcriptional function in the nucleus. Importantly, reconstitution of IQGAP1-null MEFs with IQGAP1 reduced YAP-TEAD complex formation close to the level in control cells. There are several possible mechanisms by which IQGAP1 could decrease YAP-TEAD association in the nucleus. These
include: 1) Retention in the cytoplasm. The activating kinase module of Hippo, which includes MST1/2, MOB1, NF2, and SAV, induces LATS1/2-mediated phosphorylation of YAP at Ser127 (Fig. 6). This promotes YAP interaction with 14-3-3, which causes retention of YAP in the cytoplasm (33). IQGAP1 is a scaffold in several signaling cascades that integrate with Hippo signaling. For example, IQGAP1 binds to AKT and modulates its activity (34). AKT phosphorylates YAP at Ser127 and induces its interaction with 14-3-3 in cells stimulated with EGF (35). Therefore, IQGAP1 may regulate phosphorylation-dependent YAP localization in response to specific signals, such as EGF-induced activation of AKT. Direct protein-protein interactions also regulate subcellular localization of YAP in a phosphorylation-independent manner. Several proteins bind to YAP and prevent its translocation to the nucleus. These proteins include Axin (36), AMOT (37), and Expanded (38). We previously observed that IQGAP1 modulates nuclear translocation of β-catenin (21) and nuclear factor of activated T-cells (32). Thus, it is possible that IQGAP1 may reduce YAP levels in the nucleus by binding YAP and sequestering it in the cytoplasm. Although we failed to detect a significant difference in YAP subcellular localization in MEFs with and without IQGAP1, it is possible that our analytic methods do not have adequate sensitivity to detect small changes in YAP nuclear distribution. 2) Increased YAP destruction. Phosphorylation of YAP at Ser381 promotes its ubiquitination, thereby inducing proteasomal degradation (Fig. 7) (39). IQGAP1 has been shown to regulate ubiquitination and degradation of TGF-β receptor II (40). However, the amounts of both total YAP and Ser381 phosphorylated YAP in IQGAP1−/− and IQGAP1+/+ cells were comparable, suggesting that IQGAP1 does not influence YAP degradation. 3) Altered TEAD levels. TEAD is predominantly located in the nucleus, and reduced TEAD levels could decrease nuclear YAP-TEAD complex formation. We observed that elimination of IQGAP1 did not alter the abundance of TEAD, indicating that decreased YAP-TEAD complex formation is not due to a change in TEAD protein levels. 4) Direct competition. Although primarily in the cytoplasm, IQGAP1 has been detected in the nucleus and nuclear envelope (41). We demonstrated that IQGAP1 binds to the TEAD-binding motif of YAP, raising the possibility that IQGAP1 and TEAD compete for YAP binding. Thus, IQGAP1 in the nucleus could bind YAP and inhibit the formation of a YAP-TEAD complex (Fig. 7). The putative mechanisms listed above are not mutually exclusive, and it is possible that more than one operates in the cell.

One recent study has linked YAP to IQGAP1. Adenoviral overexpression of IQGAP1 in mouse liver increased total YAP expression in hepatocytes without significantly altering the level of pYAP (42). Based on the reduction of the ratio of pYAP to total YAP, the authors concluded that IQGAP1 overexpression in the liver activates YAP. By contrast, we did not observe a change in YAP expression or phosphorylation with overexpression of IQGAP1 in HEK293 cells (data not shown). Similarly, elimination of IQGAP1 from HEK293 or MEF cells did not change YAP expression or phosphorylation. However, our data reveal that YAP-TEAD-mediated transcriptional activity was dramatically increased by IQGAP1 knock-out. The differences in findings between the two studies are likely due to differences in the experimental models, techniques used to evaluate YAP function, and cell types examined. In particular, hepatocytes have minimal IQGAP1 but high levels of IQGAP2 (43), which may substantially influence analysis of the role of IQGAP1 in YAP function.

In this paper, we describe a previously unrecognized association of the transcriptional co-activator YAP with IQGAP1. We have shown that IQGAP1 inhibits YAP transcriptional activa-
tion. These observations expand our understanding of both YAP and the roles of IQGAP1 in transcriptional regulation. Our findings establish a new role for IQGAP1 as an activator of the Hippo pathway and negative regulator of YAP function.

Experimental Procedures

Materials—HEK293, HEK293T, and HeLa cells were obtained from American Type Culture Collection. MEF cells were generated from embryos of IQGAP1+/− mice and littermate controls (25). All reagents for tissue culture were from Life Technologies. Protein A-Sepharose and glutathione-Sepharose were purchased from GE Healthcare. PVDF membranes were purchased from Millipore Corp. Anti-GAPDH (ADI-CSA-335-E) was from Enzo Life Sciences. Anti-YAP (catalog nos. 12395 and 14074), anti-pYAP Ser127 (catalog no. 13008), anti-pYAP Ser397 (catalog no. 13619), anti-pLATS1 (catalog no. 8654), and anti-LATS1 (catalog no. 3477) antibodies were purchased from Cell Signaling. Anti-IQGAP1 polyclonal antibodies have been characterized previously (28). To produce anti-IQGAP2 antibody, the region corresponding to amino acids 303–695 of the IQGAP2 gene was amplified using pΩ-IQGAP2 as a template. 5′-CGGGATCCCAAGCTTCGTTGGAACC-3′ was used as the forward primer, and 5′-CGGAAATTCCTACACACTTCCTTCTTTG-3′ was used as the reverse primer. The PCR product was inserted into pGEX2T. GST-IQGAP2 (303–695) was purified. Anti-IQGAP2 antiserum was raised by injecting rabbits with the purified fragment (Cocalico Biologicals, Inc.), and antibody specificity was validated by Western blotting (data not shown). Anti-IQGAP3 antibody (catalog no. SAB1401986-50UG) was purchased from Sigma. Anti-actin antibody (catalog no. 8432) was from Santa Cruz, and anti-TEAD1 antibody (catalog no. 610922) was from BD Biosciences. Hoechst 33342 (catalog no. 561908) was purchased from BD Pharmingen. Blocking buffer and infrared dye-conjugated (IRDye) antibodies, both anti-mouse and anti-rabbit, were obtained from LI-COR Biosciences. All restriction enzymes were from New England Biolabs.

Plasmid Construction and Expression—The construction of Myc-tagged IQGAP1, IQGAP1-N (N-terminal half, comprising amino acids 2–864), IQGAP1-C (C-terminal region, amino acids 865–1657), IQGAP1ΔIQ (amino acids 764–863 deleted), and IQGAP1−717–916 has been described previously (28, 44). To generate YAP constructs, pEGFP YAP (plasmid 17843, Addgene) (35) was digested and inserted into pcDNA3 (Invitrogen). GST-tagged YAP was generated by inserting YAP into pGEX4T-1 (GE Life Sciences) at BamHI and EcoRI sites.

Preparation of Fusion Proteins—GST-IQGAP1 was expressed in Escherichia coli and isolated using glutathione-Sepharose chromatography essentially as described previously (28). GST-YAP was expressed and isolated as described for GST-IQGAP1. The size and purity of the GST proteins were evaluated by SDS-PAGE and Coomassie staining. All proteins were at least 90% pure.

In Vitro Binding Assay—HeLa cells were lysed with 1 ml of buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-100) supplemented with complete protease and phosphatase inhibitors (Thermo Scientific), 1 mM PMSF, and 1 mM EGTA (called Buffer B after the addition of inhibitors). The lysate was precleared with 40 μl of glutathione-Sepharose beads for 1 h and then incubated with 1 μg of GST-IQGAP1 or GST, both on glutathione beads, for 3 h at 4 °C. After washing the beads five times with Buffer A, the samples were resolved by SDS-PAGE. The gel was cut at the 100-kDa region. The bottom part of the gel was transferred to PVDF, blocked with blocking buffer (LI-COR Biosciences) for 1 h at 25 °C, and then probed with anti-YAP monoclonal antibody overnight at 4 °C. The membrane was incubated with IRDye-conjugated anti-mouse antibody for 1 h, and antigen-antibody complexes were detected using the Odyssey imaging system (LI-COR Biosciences). The top portion of the gel was stained with Coomassie Blue.

Transcription and Translation (TnT) Product Production and Binding Analysis—[35S]Methionine-labeled TnT products were synthesized using the TnT quick coupled transcription/translation system (Promega) as described previously (45). Briefly, 1 μg of each plasmid was incubated with 40 μl of TnT Quick Master mix and 20 μCi of [35S]methionine (PerkinElmer Life Sciences) for 90 min at 30 °C. TnT products were confirmed by SDS-PAGE and autoradiography before being used in pulldown assays. To identify the region of IQGAP1 that binds to YAP, TnT products of IQGAP1 were incubated with GST-YAP (GST alone was used as control) for 3 h at 4 °C. Complexes were washed five times with buffer A and separated by SDS-PAGE. The gels were dried, and autoradiography was performed. To identify the region of YAP that binds to IQGAP1, portions of YAP were expressed with TnT. Radiolabeled products were incubated with GST-IQGAP1 and processed as described above.

Cell Culture and Transfection—HEK293 and MEF cells were maintained in DMEM supplemented with 10% FBS. All the experiments were done at ~80% confluence. MEF and HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) or TransIT®-2020 transfection reagent (Mirus Bio) according to the manufacturers’ instructions.

Immunoprecipitation—HeLa cells were plated in 10-cm dishes to reach 80% confluence. The following day, the cells were washed with ice-cold PBS and lysed with 500 μl of Buffer A. Lysates were subjected to two rounds of sonication for 10 s each, and insoluble material was precipitated by centrifugation at 20,000 × g for 10 min at 4 °C. Supernatants were precleared with glutathione-Sepharose beads for 1 h. Equal amounts of protein lysate were incubated with protein A-Sepharose beads and inserted into pcDNA3-myc at BamHI and XbaI sites or cut with BamHI and EcoRI and inserted into pGEX2T (GE Life Sciences) at BamHI and EcoRI sites.
and anti-IQGAP1 polyclonal antibodies or anti-Yap monoclonal antibody for 3 h at 4 °C. Rabbit IgG and mouse IgG were used as controls for polyclonal and monoclonal antibody immunoprecipitations, respectively. Samples were washed five times with Buffer A, resolved by SDS-PAGE, and processed by Western blotting.

Generation of a Cell Line with Stable IQGAP1 Knockdown by CRISPR/Cas9—To generate IQGAP1 knock-out cells, we used the CRISPR/Cas9 strategy. HEK293 cells were transfected with plasmids expressing a Cas9 D10A nickase mutant and a pair of sgRNA-IQGAP1 N1 and N2 that target Exon1 of the IQGAP1 gene at the following sequences: N1, AACCTCTGGTCCGGGACA and N2, GGGCCGGCCGACTA. The sequences were identified using the CRISPR Design tool (Feng Zhang laboratory at MIT 2015). These sequences and their reverse complements were annealed and ligated into the pSPCas9n-2A-BB-Puro plasmid (Addgene catalog no. 62987 from the laboratory of Feng Zhang) following the described method (46). HEK293 cells were transfected using Lipofectamine 2000, and clones were selected 48 h later using puromycin. Individual clones were plated, and IQGAP1 deletion was confirmed by sequencing genomic DNA, which had been extracted with Quick-Extract buffer (Epicenter catalog no. QE09060), using primer CCGGCAAGAAGGGAGATGACC. Loss of IQGAP1 protein expression was confirmed by Western blotting. HEK293 cells transfected with pSpCas9n(BB)-2A-Puro empty vector were selected with puromycin and used as a control.

Transcriptional Activity—Equal numbers (0.3 × 10^6) of cells were cultured in 12-well plates for 24 h at 80% confluence in serum-containing medium. Under these conditions, the Hippo pathway is minimally activated. The cells were transfected with 2.5 μg of plasmid containing a YAP/TAZ-responsive synthetic promoter driving the luciferase expression (8xGTTIC-luciferase, Addgene catalog no. 34615) and 2.5 μg of Renilla luciferase-polymerase III (Addgene catalog no. 37380) using TransIT®-2020 transfection reagent (Mirus Bio LLC) for MEFs and Lipofectamine 2000 (Invitrogen) for HEK293 cells. After 24 h, cells were lysed using lysis buffer supplied in the kit. Luciferase and Renilla (control) signals were measured using a Dual-Luciferase reporter assay kit (Promega catalog no. E1910) according to the manufacturer’s instructions. Luminescence was measured using a Tecan Infinite M200 microplate reader (PerkinElmer Life Sciences). The data are expressed as ratios of luciferase to the Renilla signal. For rescue experiments, HEK293 cells were transfected with 2.5 μg of myc-IQGAP1 or myc-IQGAP1ΔIQ using TransIT®-2020 transfection reagent according to the manufacturer’s instructions. After 6 h, the cells were washed with PBS and replenished with fresh medium. After an additional 24 h, cells were transfected with TEAD reporter luciferase plasmid and Renilla luciferase-polymerase III for 24 h, and the Dual-Luciferase reporter assay was conducted as described above.

Quantitative RT-PCR—To measure AMOTL2 and CTGF hnRNA, MEFS were cultured for 24 h. Then total RNA was isolated from the cells using an RNA isolation kit (Qiagen). 1 μg of RNA was reverse transcribed to cDNA using a high capacity cDNA reverse transcriptase kit (Applied Biosystems) according to the manufacturer’s instructions. RT-PCR was performed on a StepOnePlus Real Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and 200 nm forward and reverse primers. The primers used were: AMOTL2-2020 transfection reagent (Mirus Bio LLC) for MEFs and 2020 transfection reagent according to the manufacturer’s instructions. Luciferase and CTGF expression was performed as described for AMOTL2, except the primers were: CTGF, 5′-CAAGGACCGGACACAGCAG-3′ (forward) and 5′-AGCACGGCGCTCACCCTCTG-3′ (reverse). For rescue experiments, MEFs at 50% confluence were transfected with 2.5 μg of empty vector, full-length IQGAP1, or IQGAP1ΔIQ constructs using TransIT®-2020 transfection reagent according to the manufacturer’s instructions. The following day, the transfection was repeated. 48 h after the first transfection, total RNA was isolated from the cells, and quantitative RT-PCR was performed as described above.

Proximity Ligation Assay—MEFs were plated in 24-well dishes on coverslips at 80% confluence. Under these conditions, the Hippo pathway is minimally activated. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton-PBS, and blocked overnight in 10% FBS. Anti-YAP (1/100 dilution) and anti-TEAD1 (1/100 dilution) antibodies were incubated with the cells for 1 h. For the PLA, donkey anti-mouse PLUS and donkey anti-rabbit MINUS secondary antibodies from Duolink were used, and PLA analysis was performed using Duolink in situ detection reagents Red (DUO92008 SIGMA) following the manufacturer’s protocol. DNA was stained using Hoechst 33342, and coverslips were mounted. The cells were examined using a confocal microscope (LSM880; Carl Zeiss Microscopy). Fluorescence images were collected using a 63× objective lens (N.A. 1.4). Extended field of view tile images (2 × 2 tile matrix) were acquired with 5% image overlap, and with 0.7-μm X-Y pixel size and an optical slice thickness of 2.0 μm. The tiled images were aligned and stitched together using the stitching algorithm in the Zeiss Zen Blue (v. 2.0) software and saved as single large images. The number of PLA spots in each cell nucleus was counted using ImarisCell module of Imaris (v. 8.1.2) image analysis software (Bitplane USA). 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. The number of individual PLA objects per nucleus was counted. The same image processing and analysis parameters were used for all of the images in the data set. For rescue experiments, IQGAP1-null MEFS were transfected with GFP-tagged full-length IQGAP1 (47) or GFP as a control. After 48 h cells were processed for PLA.

Miscellaneous Methods—Statistical analysis was performed by Student’s t test or analysis of variance as described in individual experiments using Prism 6 (GraphPad). Subcellular fractionation was conducted using NE-PER™ nuclear and cyto-
plasmic extraction kit (ThermoFisher, catalog no. 78833), following the manufacturer's instruction. Western blotting images were quantified with Image Studio 2.0 (LI-COR Biosciences) according to the manufacturer's instructions. Protein concentrations were determined using Bio-Rad protein assay dye reagent (500-0006).

Author Contributions—S. S., Z. L. and D. B. S. designed the study. S. S. and Z. L. conducted experiments. S. S., Z. L., A. C. H. and C. J. M. generated the IQGAP1 knock-out and control HEK293 cell lines. S. S. and Z. L. conducted experiments. S. S., Z. L., A. C. H., and C. J. M. generated the IQGAP1 knock-out and control HEK293 cell lines.

Acknowledgments—We thank Jessica Smith for conducting initial experiments on this project and Michael Krahuk (Experimental Immunology Branch, National Institutes of Health) for expert assistance with confocal microscopy.

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