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The Hippo pathway plays an important role in regulating tissue homeostasis, and its effectors, the transcriptional co-activators Yes-associated protein (YAP) and WW domain–containing transcription regulator 1 (WWTR1 or TAZ), are responsible for mediating the vast majority of its physiological functions. Although YAP and TAZ are thought to be largely redundant and similarly regulated by Hippo signaling, they have developmental, structural, and physiological differences that suggest they may differ in their regulation and downstream functions. To better understand the functions of YAP and TAZ in the Hippo pathway, using CRISPR/Cas9, we generated YAP KO, TAZ KO, and YAP/TAZ KO cell lines in HEK293A cells. We evaluated them in response to many environmental conditions and stimuli and used RNA-Seq to compare their transcriptional profiles. We found that YAP inactivation has a greater effect on cellular physiology (namely, cell spreading, volume, granularity, glucose uptake, proliferation, and migration) than TAZ inactivation. However, functional redundancy between YAP and TAZ was also observed. In summary, our findings confirm that the Hippo pathway effectors YAP and TAZ are master regulators for multiple cellular processes but also reveal that YAP has a stronger influence than TAZ.

The Hippo pathway is an important regulator of tissue homeostasis and plays a critical role in development and regeneration. The core kinase cascade of the Hippo pathway consists of MST1/2 (mammalian STE20-like 1/2), MAP4Ks (mitogen-activated protein kinase kinase kinase kinases), and LATS1/2 (large tumor suppressor 1/2). In response to a wide range of signals, MST1/2 and the mitogen-activated protein kinase kinase kinases phosphorylate and activate the LATS[3] kinases. When the core kinase cascade is activated, LATS1/2 phosphorylates and inactivates the downstream effectors of the Hippo pathway, the transcriptional co-activators YAP (Yes-associated protein 1) and TAZ (WW domain–containing transcription regulator 1). Because YAP and TAZ do not have their own DNA binding motifs, when dephosphorylated they translocate to the nucleus and interact with a host of transcription factors, primarily TEAD1–4 (TEA domain family members 1–4), to induce expression of genes promoting cell growth (1). YAP and TAZ are not only inhibited by LATS1/2, but they are also involved in a negative feedback loop to regulate Hippo pathway kinase activity (2). YAP and TAZ are, if not the only, the most important downstream effectors of LATS1/2 mediating the physiological functions of the Hippo pathway.

The Hippo pathway is highly conserved, with YAP/Yorkie (the ortholog of YAP) first appearing in single-cell eukaryotes (3). However, during evolution, TAZ only appeared much later in vertebrates (4). Although YAP and TAZ are generally thought to be functionally redundant, there are structural and physiological clues that suggest they may have additional, non-overlapping roles.

Structurally, although YAP and TAZ share high protein sequence similarity (60%), there are significant distinctions as well (Fig. 1*A* and Fig. S1) (5–7). First, although both contain WW domains that mediate protein–protein interactions, including interactions with LATS1/2 and AMOT, YAP contains two tandem WW domains, whereas TAZ contains only one. Additionally, YAP contains an SH3-binding motif and an N-terminal proline-rich region believed to be involved in mRNA processing, both of which are absent from TAZ. Moreover, GSK3β has been shown to directly phosphorylate TAZ to create a second, additional phosphodegron not present in YAP that contributes to TAZ's protein stability being much more dynamically regulated in response to phosphorylation than that of YAP (8). Finally, although all residues necessary for YAP–TAZ interaction with TEAD1–4 are conserved, there are also differences within the TEAD binding domain. The TEAD bind-

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This article contains Figs. S1–S6.

The results from the RNA-sequencing can be accessed through the Gene Expression Omnibus (GEO) Database under accession number GSE111583.

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[3] The abbreviations used are: LATS, large tumor suppressor; YAP, Yes-associated protein; KO, knockout; HEK, human embryonic kidney; LPA, lysophosphatidic acid; CTGF, connective tissue growth factor; RNA-seq, RNA sequencing; HA, hemagglutinin; AMOT, angiomotin; qPCR, quantitative PCR; PET, polyethylene terephthalate.

ing domain of YAP features an extended P*XX*OP loop (where O is a hydrophobic residue) not found in TAZ (9, 10). In addition, a recent report found that TAZ-TEAD can form a heterotetramer complex that may affect DNA target selectivity and induce stronger expression of certain target genes (11). Together, these structural differences suggest that there may be differences in how YAP and TAZ are regulated and how they interact with TEAD1–4 to induce gene expression.

There are physiological differences between YAP and TAZ as well. YAP knockout mice are embryonic lethal at embryonic day 8.5 because of severe developmental defects (12). Conversely, TAZ knockout is only partially lethal, with one-fifth of the mice being viable, although they develop renal cysts and lung emphysema (13–15). Thus, YAP and TAZ are not completely redundant because TAZ is unable to compensate for the loss of YAP. What is not clear, however, is whether this is due to differences in tissue distribution and expression or actual regulatory or transcriptional differences between the two genes.

Therefore, there are several open questions in Hippo biology: what are the differences in the transcriptional profiles of YAP and TAZ, and what are the downstream physiological implications of these differences? To this end, we used CRISPR/Cas9 to create YAP or TAZ single knockout and LATS1/2 and YAP/TAZ double knockout cell lines and performed a wide array of assays and comparisons to delineate any differences between YAP and TAZ and to better characterize the consequences of dysregulated Hippo pathway signaling.

Results

Comparison of YAP and TAZ in TEAD interaction and target gene expression

We used CRISPR/Cas9 to create LATS1/2 knockout (KO), YAP KO, TAZ KO, and YAP/TAZ KO cell lines in HEK293A cells (16). In addition to sequencing, we also performed siRNA and rescue experiments to ensure that our knockouts were specific (Fig. S2, *A* and *B*). The first question we addressed was how the loss of YAP or TAZ affects activation and regulation of the upstream Hippo pathway kinase cascade. Serum is one of the most potent activators of YAP/TAZ; lysophosphatidic acid (LPA) present in serum activates G protein–coupled receptors to inactivate the Hippo pathway, resulting in dephosphorylated, nuclear, and transcriptionally active YAP and TAZ. In the presence of serum, YAP and TAZ are dephosphorylated and nuclear in all cell lines (Fig. 1, *B* and *C*). Following starvation, YAP and TAZ are phosphorylated in all cell lines except the LATS1/2 KO cells; this is expected because LATS1/2 are the primary kinases for YAP/TAZ in response to starvation, so following their deletion, the cell is unable to compensate to inactivate YAP/TAZ. TAZ protein levels were significantly elevated in LATS1/2 KO cells, supporting the notion that TAZ is destabilized by LATS-dependent phosphorylation. However, expression and activation of the upstream components LATS1/2 and MOB1A/B appear to be unaffected by loss of YAP, TAZ, or both YAP/TAZ.

Moreover, the same trends are evident when we look at downstream transcriptional activity. The YAP/TAZ target genes connective tissue growth factor (CTGF) and cysteine-rich angiogenic inducer 61 (CYR61) are both induced, whereas leucine-rich repeat containing G protein–coupled receptor 5 (LGR5) is repressed by active YAP and TAZ. Following serum stimulation, CTGF and CYR61 expression was induced in all cell lines except the YAP/TAZ KO cell line (Fig. 1, *D* and *E*), indicating that CTGF and CYR61 induction is YAP/TAZ-dependent. Deleting YAP had a more dramatic effect on CTGF and, to a lesser extent, on CYR61 expression than deletion of TAZ, as induction in the YAP KO cell line was significantly reduced relative to the WT and TAZ KO cell lines. LGR5 expression was increased in all YAP KO, TAZ KO, and YAP/TAZ KO cell lines compared with the WT and LATS1/2 KO cell lines and, consistently, was strongly repressed in LATS1/2 KO cells (Fig. 1*F*). These data suggest that both YAP and TAZ are involved in repression of LGR5 so that deleting either YAP or TAZ is sufficient to cause increased LGR5 expression, whereas YAP is more important than TAZ in the induction of CTGF and CYR61.

The most noticeable structural difference between YAP and TAZ is the WW domains, of which YAP has two but TAZ only has one (Fig. 1*A*). We questioned whether these structural differences may yield some insight into potential differences in how YAP and TAZ are regulated or how they interact with TEAD to induce transcription. We deleted each of the WW domains in YAP and assayed the effect on YAP activity by comparing CTGF induction after expressing the YAP mutants in YAP/TAZ KO cells. We found that single deletion of a WW domain had little effect, whereas deletion of both WW domains partially compromised CTGF induction (Fig. S3, *A–C*). Phosphorylation is the major mechanism for YAP/TAZ regulation. We created point mutations for each of the conserved serine residues, except those in the C-terminal transactivation domain, to determine which potential phosphorylation sites are most important for YAP transcriptional activity (Fig. S3, *A–C*). Our goal was that, if we identified serines critical for YAP transcriptional activity, then we could also determine whether they are critical for TAZ transcriptional activity, which may yield some insight into how YAP/TAZ are differentially regulated. However, none of these mutations had a significant effect on downstream target gene (CTGF) expression relative to WT YAP, with the exception of S94A (which prevents YAP–TEAD binding) and S127A (which prevents YAP–14-3-3 binding), both of which are already well-established and conserved in TAZ (Fig. S3*C*). These observations support Ser-94 and Ser-127 as key regulatory phosphorylation sites and highlight the importance of interaction with the TEAD transcription factors and cytoplasmic localization by 14-3-3 binding in controlling YAP function.

TEADs are the main transcription factors and nuclear binding partners of YAP/TAZ. When TEADs are not in the nucleus, even unphosphorylated YAP cannot be localized in the nucleus (17). Any physiological or transcriptional differences between YAP and TAZ could be due to differences in their binding affinities or interactions with TEADs. Thus, we examined the interaction between TEADs and YAP and TAZ by co-immunoprecipitation. Our data showed that YAP or TAZ displayed similar interactions with TEAD1, TEAD2, and TEAD4 (Fig. S3*D*). We

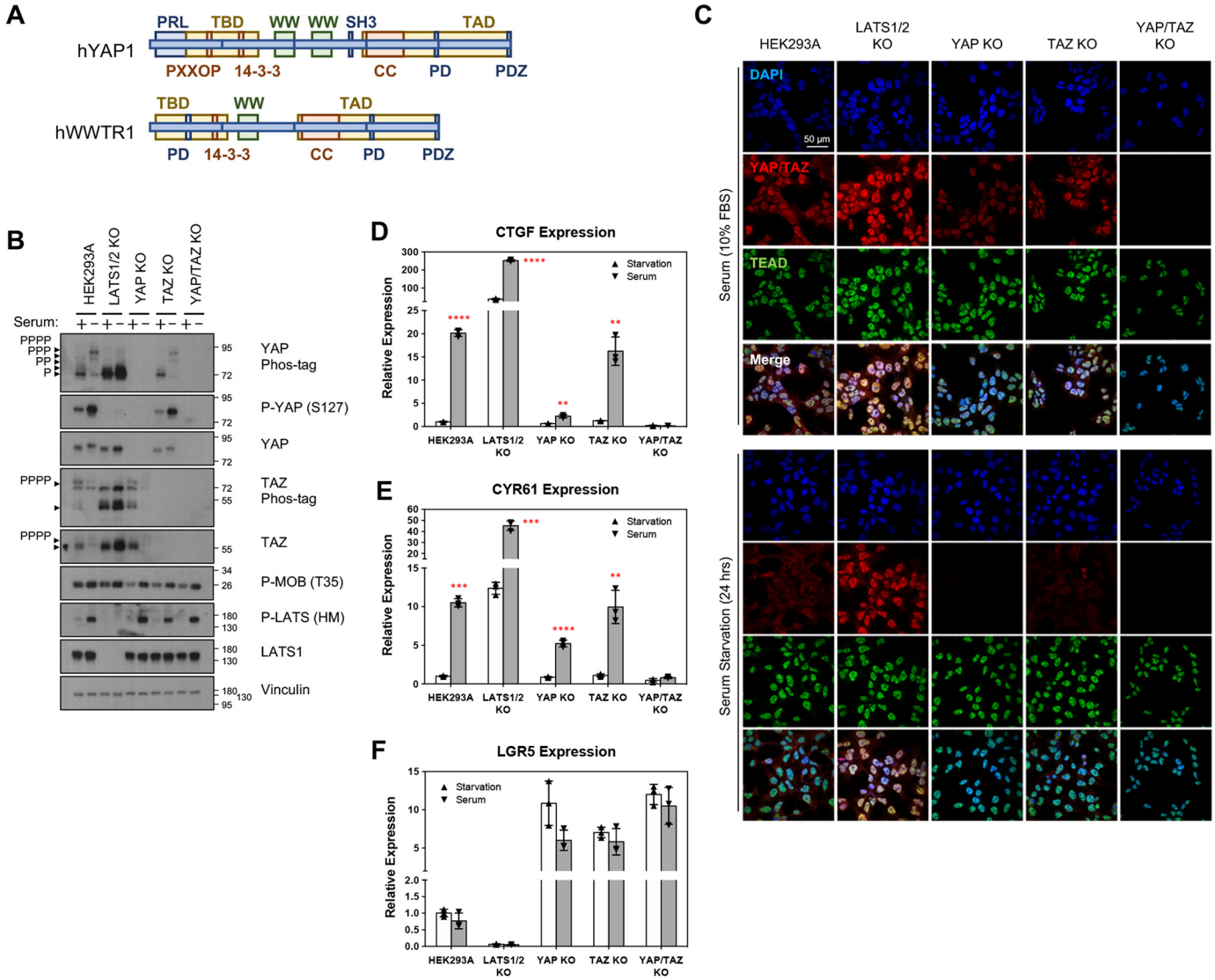

Figure 1. Comparison of YAP and TAZ knockout cells. *A*, schematic of the structural differences between YAP and TAZ. The domains are labeled proline-rich region (*PRL*), TEAD binding domain (*TBD*), WW domain (*WW*), SH3 binding domain (*SH3*), transcriptional activation domain (*TAD*), P*XX*OP site (*PXXOP*), 14-3-3 binding site (*14-3-3*), coiled-coil region (*CC*), PDZ binding domain (*PDZ*), and phosphodegron (*PD*). *B*, Western blots showing loss of LATS1/2, YAP, and/or TAZ. Cells were serum-starved overnight and either harvested or subsequently stimulated with serum for 60 min. Total cell lysates were used for Western blotting with the indicated antibodies. *C*, immunofluorescence showing YAP/TAZ (*red*), TEAD1–4 (*green*), and 4′,6-diamidino-2-phenylindole (*DAPI*, a DNA stain, *blue*) localization following either overnight serum starvation or overnight serum starvation followed by 60 min of serum stimulation. *D–F*, qPCR of CTGF, CYR61, and LGR5 following either overnight serum starvation (*white columns*) or overnight serum starvation followed by 60 min of serum stimulation (*gray columns*). Data are represented as ± S.D. **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

did not include TEAD3 because it is lowly expressed in HEK293A cells (Fig. S3*E*). This indicates that the differential effect of YAP and TAZ on gene expression is not likely to be due to a difference in TEAD binding because YAP and TAZ interacted similarly with each of the TEADs. Interestingly, the protein levels of TEAD1, TEAD2, and TEAD4 were decreased in YAP KO, TAZ KO, and YAP/TAZ KO cells (Fig. S3*F*), suggesting that YAP/TAZ and proper TEAD expression are linked, although it may not be a direct regulation. Nevertheless, this supports an interdependent relationship between YAP/TAZ and TEADs.

Effect of YAP and TAZ on cell growth and size

First, we observed that dysregulation of the Hippo pathway affected cell spreading (Fig. 2, *A* and *B*). When plated on a dish, LATS1/2 KO cells showed increased cell spreading, whereas YAP KO and YAP/TAZ KO cells were significantly smaller. TAZ KO cells showed no difference from WT cells. This was also observed when cells were plated on poly-lysine (Fig. 2*C*).

Because cell spreading is only one measure of cell size, we also used FACS to compare cell volume and granularity. Consistent with what we observed with cell spreading, LATS1/2 KO cells exhibited a significant increase in volume and granularity relative to WT cells, whereas YAP KO and YAP/TAZ KO cells showed significant decreases in both volume and granularity (Fig. 2, *D–F*). Consistent with what we observed transcriptionally (Fig. 1, *D* and *E*), deleting YAP had a greater impact on cell size than deleting TAZ.

Although it is not clear what might account for the changes in granularity, one potential explanation may be differences in

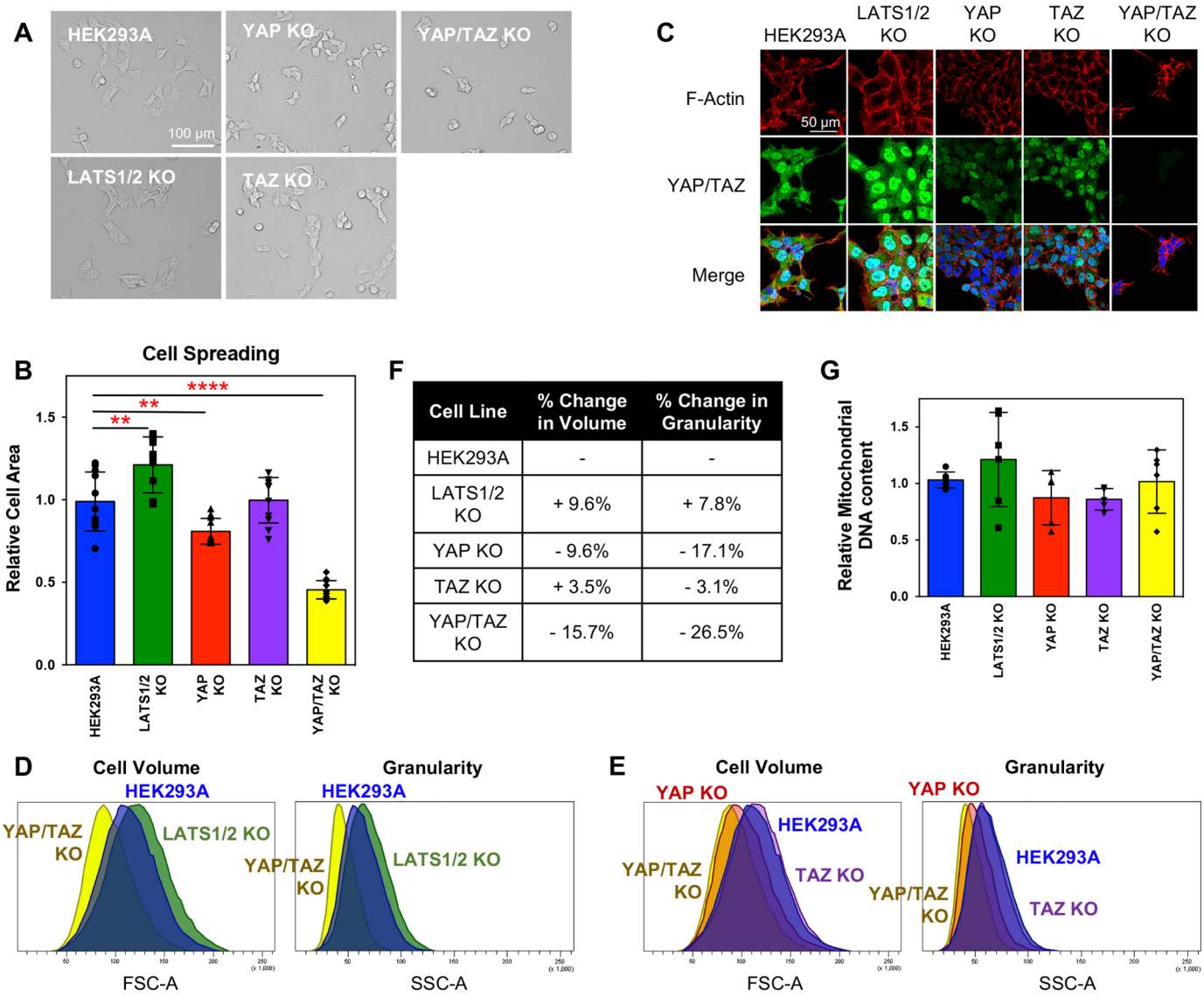

Figure 2. Loss of YAP results in decreased cell spreading, volume, and granularity. *A*, bright-field images showing cell spreading on a dish. *B*, quantification of *A*. Data are represented as ± S.D. *C*, immunofluorescence showing F-actin (*red*), YAP/TAZ (*green*), or 4′,6-diamidino-2-phenylindole (*DAPI*, a DNA stain, *blue*). *D* and *E*, FACS data showing differences in cell volume (forward scatter (*FSC*)) and granularity (side scatter (*SSC*)) for double knockout cells (*D*) and single knockout cells (*E*). *F*, quantification of *D* and *E*. *G*, PCR quantification of the relative mitochondrial DNA content for each cell line. Data are represented as ± S.D. **, $p \leq 0.01$; ***, $p \leq 0.001$.

mitochondrial number; if LATS1/2 KO cells have increased mitochondria, that might also explain their increased growth capacity. However, no differences in the relative mitochondrial content of the different cell lines were observed (Fig. 2*G* and Fig. S4*A*).

Effect of YAP and TAZ on cell physiology

To compare the physiological consequences of dysregulated YAP and TAZ, we first looked at glucose uptake and metabolism. Cells were plated and given fresh medium for 6 h, after which the remaining glucose levels in the culture media were measured. LATS1/2 KO cells took up glucose at a faster rate and had lower remaining glucose levels in the culture medium than WT cells, whereas YAP KO and YAP/TAZ KO cells' glucose uptake was reduced (Fig. 3*A*). Interestingly, TAZ KO cells were not significantly different from WT cells.

Next, we compared rates of cell proliferation. As expected, LATS1/2 KO cells with constitutively active YAP and TAZ proliferated at a rate slightly faster than WT cells (Fig. 3*B*). The modest effect of LATS1/2 KO on cell growth is likely because, under normal growth conditions (low cell density and the presence of abundant serum and glucose), LATS activity is repressed. TAZ KO cells clustered closely with WT cells, whereas YAP KO and YAP/TAZ KO cells showed a dramatically decreased rate of proliferation. When we performed a cell cycle analysis of these cells under complete growth conditions, there were no significant differences between any of the cell lines (Fig. 3*C*), indicating that YAP KO and YAP/TAZ KO cells proliferate slower overall but are not specifically impaired at any stage of the cell cycle.

The migratory capacity of each cell line was also tested in a trans-well migration assay utilizing an 8-μm PET pore mem-

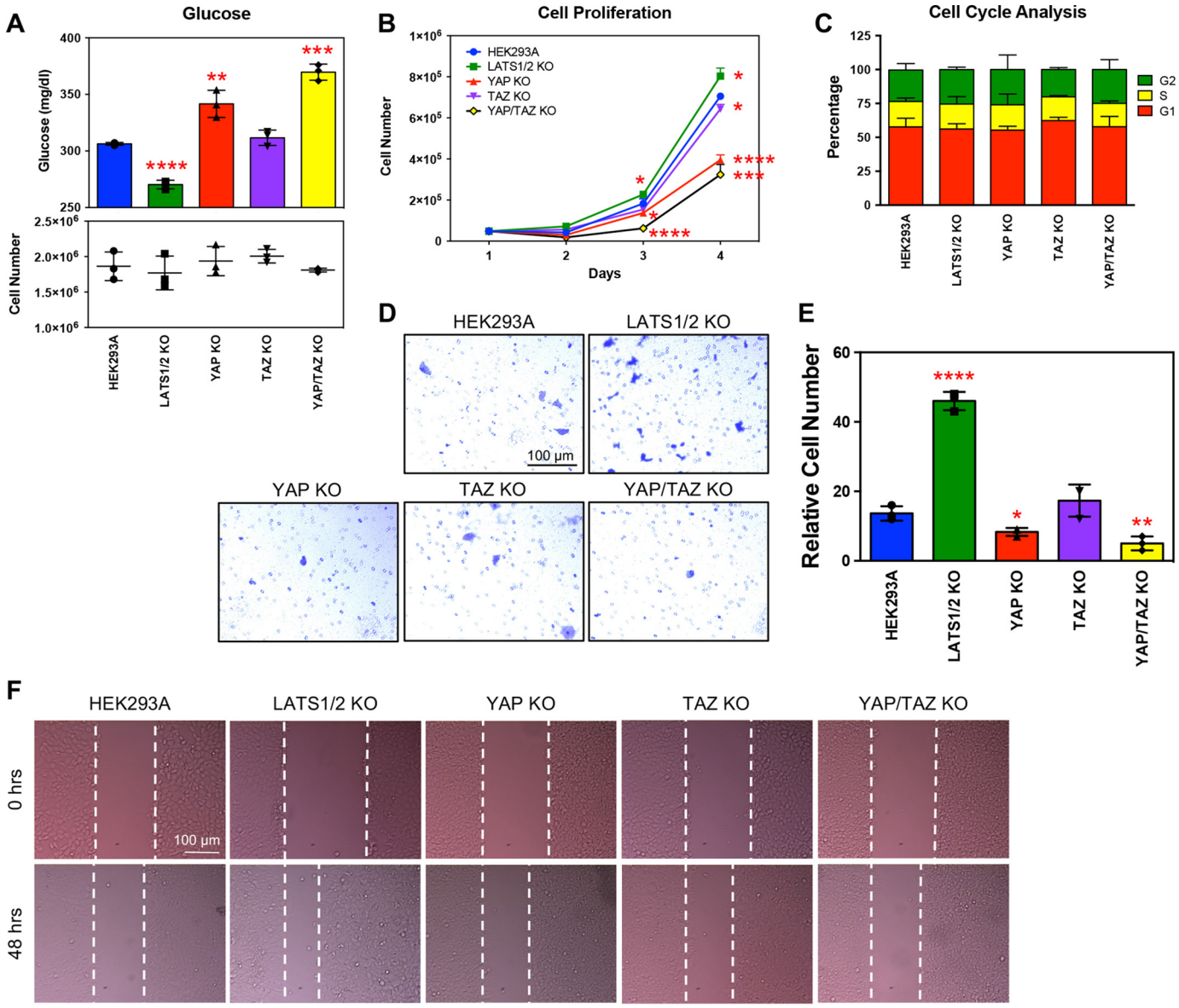

Figure 3. Loss of YAP results in decreased glucose uptake, proliferation, and migration. *A*, glucose levels remaining in the culture medium following 6 h of incubation. Data are represented as ± S.D. *B*, cell proliferation curves for each of the cell lines under normal growth conditions. Data are represented as ± S.D. *C*, cell cycle analysis of each of the cell lines under normal growth conditions. Data are represented as ± S.D. *D*, bright-field images showing migration through an 8-μm PET pore membrane after 8 h. *E*, quantification of *D*. Data are represented as ± S.D. *F*, bright-field images showing migration following a scratch assay. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

brane. 8 h after plating, LATS1/2 KO cells showed significantly increased migratory capacity relative to WT cells, whereas YAP KO and YAP/TAZ KO cells both showed decreased migratory capacity (Fig. 3, *D* and *E*).

Finally, we tested the migratory potential of each cell line using a scratch assay. Although the LATS1/2 KO cells showed increased migration over 48 h, there were no differences between the WT, YAP KO, TAZ KO, and YAP/TAZ KO cell lines (Fig. 3*F* and S4*B*). To ensure that the wound closure we observed is due to migration and not cell proliferation, cells were maintained in serum-free medium. Under prolonged starvation conditions, YAP and TAZ are both phosphorylated and inactivated, which may explain why the WT, YAP KO, TAZ KO, and YAP/TAZ KO cells behaved similarly.

Effect of YAP and TAZ on cell signaling

We next compared whether there were any kinetic differences between the regulation of YAP and TAZ. It is possible that regulation of one is primarily responsible for inducing immediate response genes, whereas regulation of the other is responsible for inducing a second set of slower-responding genes. However, when we performed a time course of serum stimulation on each of these cell lines, YAP and TAZ activation appeared to be unaffected in the knockout cell lines, and both showed dephosphorylation beginning around 30 min following stimulation (Fig. S4*C*). Moreover, this was consistent when we compared YAP and TAZ nuclear localization (Fig. 4*A*). However, one difference we did note was that, following 60 min of serum stimulation, YAP became completely dephosphorylated

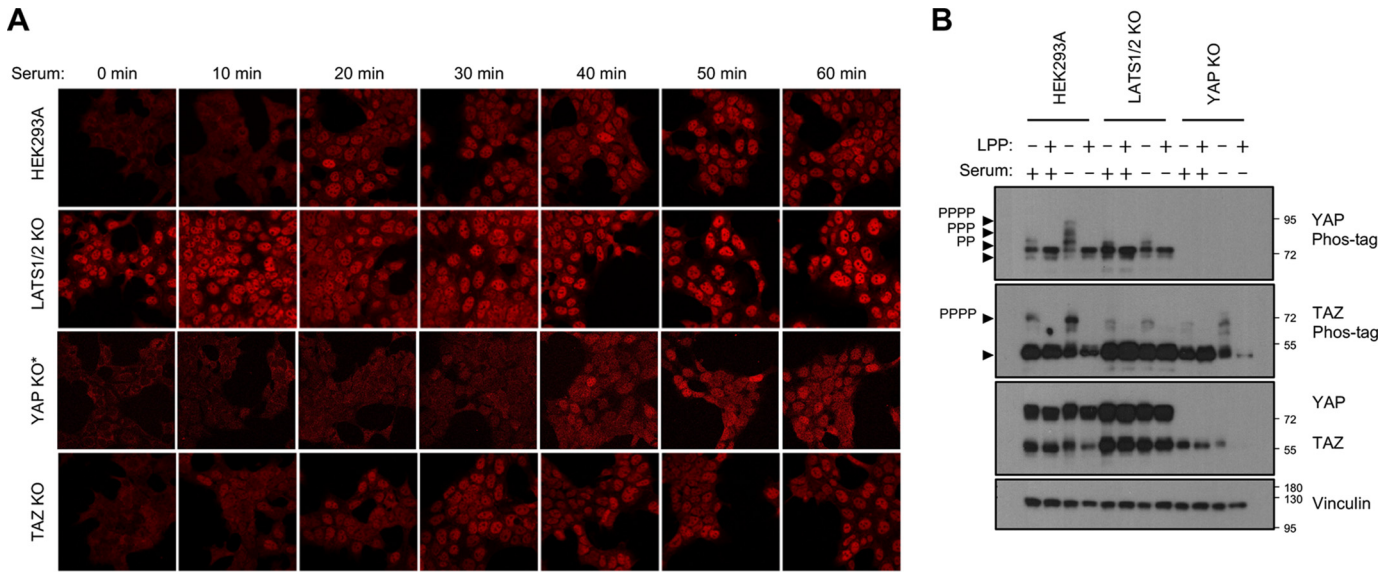

Figure 4. Comparing the kinetics of YAP and TAZ activation. *A*, immunofluorescence showing a time course of YAP/TAZ (*red*) localization in response to serum stimulation following overnight serum starvation. *B*, Western blots showing YAP and TAZ phosphorylation in response to serum starvation and λ phosphatase (*LPP*) treatment.

(Fig. S4*C*). Conversely, under the same conditions, TAZ was only partially dephosphorylated based on Phos-tag gel. Even in LATS1/2 KO cells, when YAP was completely dephosphorylated, almost 50% of TAZ remained highly phosphorylated. We have yet to identify any conditions under which TAZ is completely dephosphorylated. To confirm whether this upper band was due to phosphorylation or some other post-translational modification, we treated the lysates with λ phosphatase (Fig. 4*B*). Following treatment, the upper band of TAZ disappeared, demonstrating that TAZ is not completely dephosphorylated in response to serum stimulation. This raises the possibility that there are additional LATS1/2-independent kinases that specifically phosphorylate TAZ but not YAP. However, under stimulation conditions, TAZ is largely nuclear, suggesting that this LATS1/2-independent phosphorylation does not affect TAZ protein localization. Thus, it is unclear whether this remaining phosphorylation has any effect on TAZ co-transcriptional activity, although it appears that it is not a compensatory mechanism because, even following loss of YAP, TAZ does not become "more fully" activated in response.

We next investigated whether the physiological differences we observed between YAP and TAZ could be due to differences in protein expression. Although mRNA expression of TAZ is slightly higher than that of YAP by RNA-seq, this does not always translate into corresponding differences in protein expression (Fig. 5*A*). Because TAZ has two phosphodegrons, its protein stability is much more dynamically regulated than that of YAP, which only has one phosphodegron. For instance, following 6 h of serum starvation, TAZ protein levels decreased much more dramatically than that of YAP (Fig. 5*B*). To compare the relative protein levels of endogenous YAP and TAZ, we first needed to determine the relative sensitivity of the YAP/TAZ antibody that recognizes both YAP and TAZ. To calibrate the YAP/TAZ antibody, we transfected HA-YAP and HA-TAZ and compared the expression levels as detected with the HA antibody, which should be equally sensitive to both HA-YAP and HA-TAZ. The HA-YAP and HA-TAZ in the same samples were then also detected with the YAP/TAZ antibody (Fig. 5*C*). Based on the above analyses, our data indicate that the YAP/TAZ antibody detects both YAP and TAZ with similar sensitivity. Thus, according to the Western blot signals of endogenous YAP and TAZ detected by the YAP/TAZ antibody, we concluded that the endogenous YAP protein levels are more than twice that of TAZ in HEK293A cells under normal growth conditions. Therefore, even though TAZ has higher mRNA expression relative to YAP, its lower protein stability may contribute to the lower protein levels. The higher YAP protein levels may explain why deleting YAP has a greater effect on cell size and physiology than deleting TAZ.

To confirm that the transcriptional differences we observed in CTGF induction are primarily due to changes in protein expression, we transfected varying amounts of HA-YAP and HA-TAZ in YAP/TAZ KO cells along with a CTGF-luciferase reporter (Fig. 5, *D* and *E*). Induction of the luciferase reporter was equivalent between HA-YAP and HA-TAZ when they were equally expressed, suggesting that the biggest difference of endogenous YAP and TAZ in their ability to induce CTGF and potentially other downstream target genes is due to their protein expression.

In addition, we also wanted to determine whether regulation of YAP/TAZ phosphorylation and this YAP/TAZ-dependent mechanism of CTGF induction were unique to HEK293A cells or more broadly applicable. We also generated knockouts in HeLa and MCF7 epithelial cells (Fig. 5*F* and Fig. S4*D*). In HeLa cells, knockout of YAP/TAZ was sufficient to completely ablate CTGF expression. Furthermore, in MCF7 cells, inactivation of LATS1/2 was sufficient to result in dephosphorylated YAP and increased CTGF expression, even under starvation conditions, demonstrating that these mechanisms are more broadly conserved.

Finally, we also tested the possibility that YAP/TAZ may play an important role in response to different environmental cues

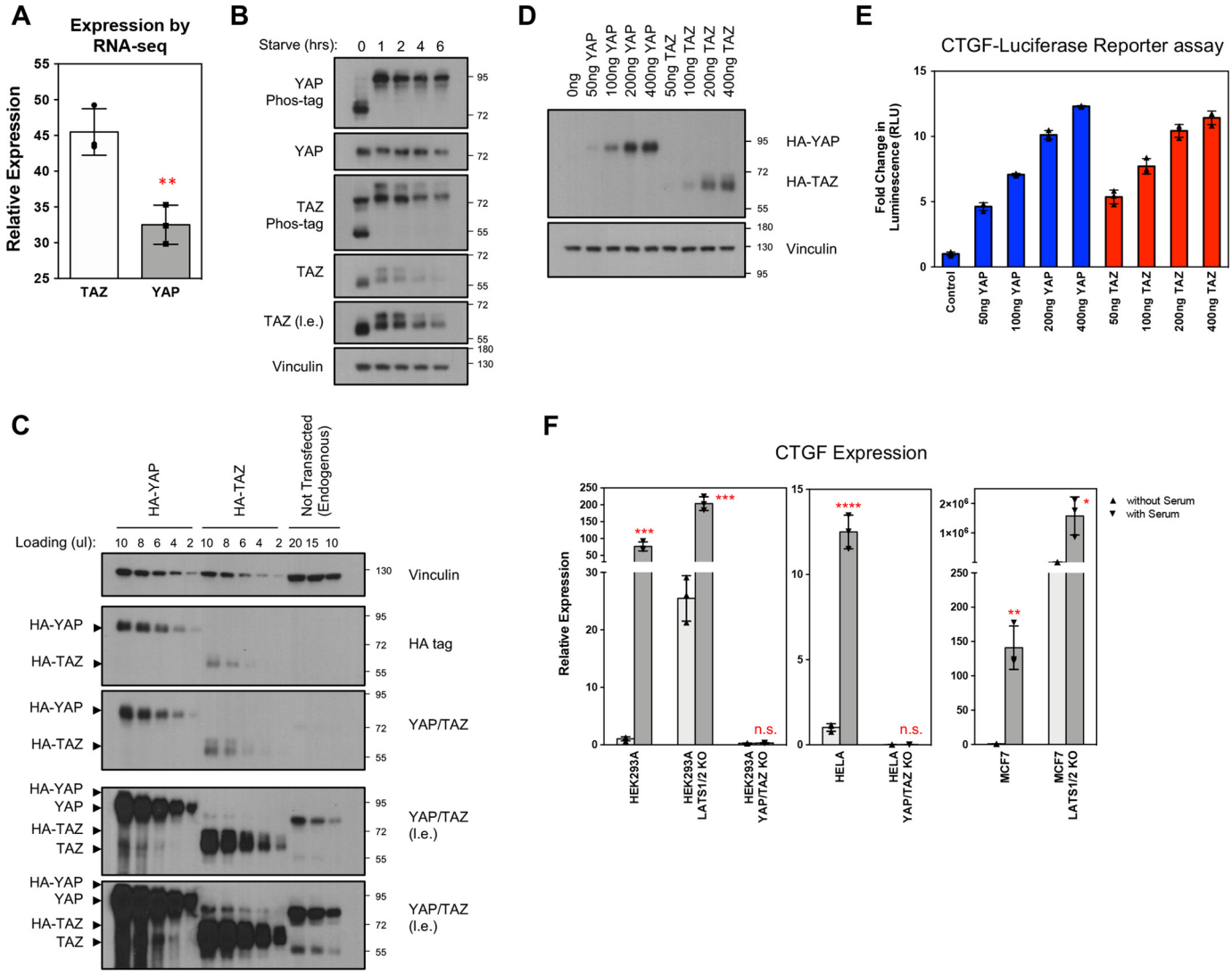

Figure 5. Comparing YAP and TAZ protein expression. *A*, mRNA expression levels of TAZ and YAP as detected by RNA-seq. Data are represented as ± S.D. *B*, Western blots showing changes in YAP and TAZ phosphorylation and protein levels following serum starvation for the indicated times. (*l.e.* indicates longer exposure.) *C*, Western blots comparing detection of HA-YAP and HA-TAZ using either an HA antibody or the YAP/TAZ antibody and detection of endogenous YAP and TAZ using the YAP/TAZ antibody. *D*, Western blots showing HA-YAP and HA-TAZ expression following transfection along with a CTGF-luciferase reporter in YAP/TAZ KO cells. *E*, luciferase activity following transfection of a CTGF-luciferase reporter with varying amounts of either HA-YAP or HA-TAZ in YAP/TAZ KO cells. Data are represented as mean ± S.D. *F*, qPCR of CTGF expression in various cell lines as indicated following either overnight starvation (*white columns*) or overnight starvation followed by serum stimulation for 90 min (*gray columns*). Data are represented as mean ± S.D. ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

or cellular stresses. To this end, we first compared YAP and TAZ phosphorylation in response to a variety of stresses, including glucose starvation, actin disruption by Latrunculin B, activation of protein kinase A (PKA) by Forskolin/3-isobutyl-1-methylxanthine, and exposure to cerivastatin (Fig. S5*A*). We observed similar regulation of YAP and TAZ phosphorylation in the different cell lines in response to the various treatments. Next, we compared WT, LATS1/2 KO, and YAP/TAZ KO cells in response to long-term exposure to several types of stress, including exposure to lipopolysaccharides, toxin (Streptolysan O), dobutamine (a β1 receptor agonist), rapamycin (inhibits mTOR), hypoxia, serum starvation, osmotic stress, endoplasmic reticulum stress, phosphatase inhibitors, cerivastatin (a 3-hydroxy-3-methylglutaryl–CoA reductase inhibitor), apoptosis, DNA damage, AMP-activated protein kinase (AMPK) inhibitors, and glucose starvation (Fig. S5*B*). However, we did not see any significant differences in long-term survival between any of the cell lines under these conditions, indicating that YAP/TAZ do not play an important role in response to these specific stresses.

Effect of YAP and TAZ on transcription

Because serum activates many other pathways in addition to YAP and TAZ, we focused on LPA, which is the most potent YAP/TAZ activator in serum (18). Using a low concentration of LPA, we performed a time course in each of the cell lines to determine whether there were differences in their transcriptional responses to LPA stimulation (Fig. 6*A*). Similar to what we saw following serum stimulation (Fig. 1, *D–F*), deleting YAP had a greater effect on CTGF induction than deletion of TAZ. To further delineate whether there are other differences in the transcriptional programs of YAP and TAZ, we performed

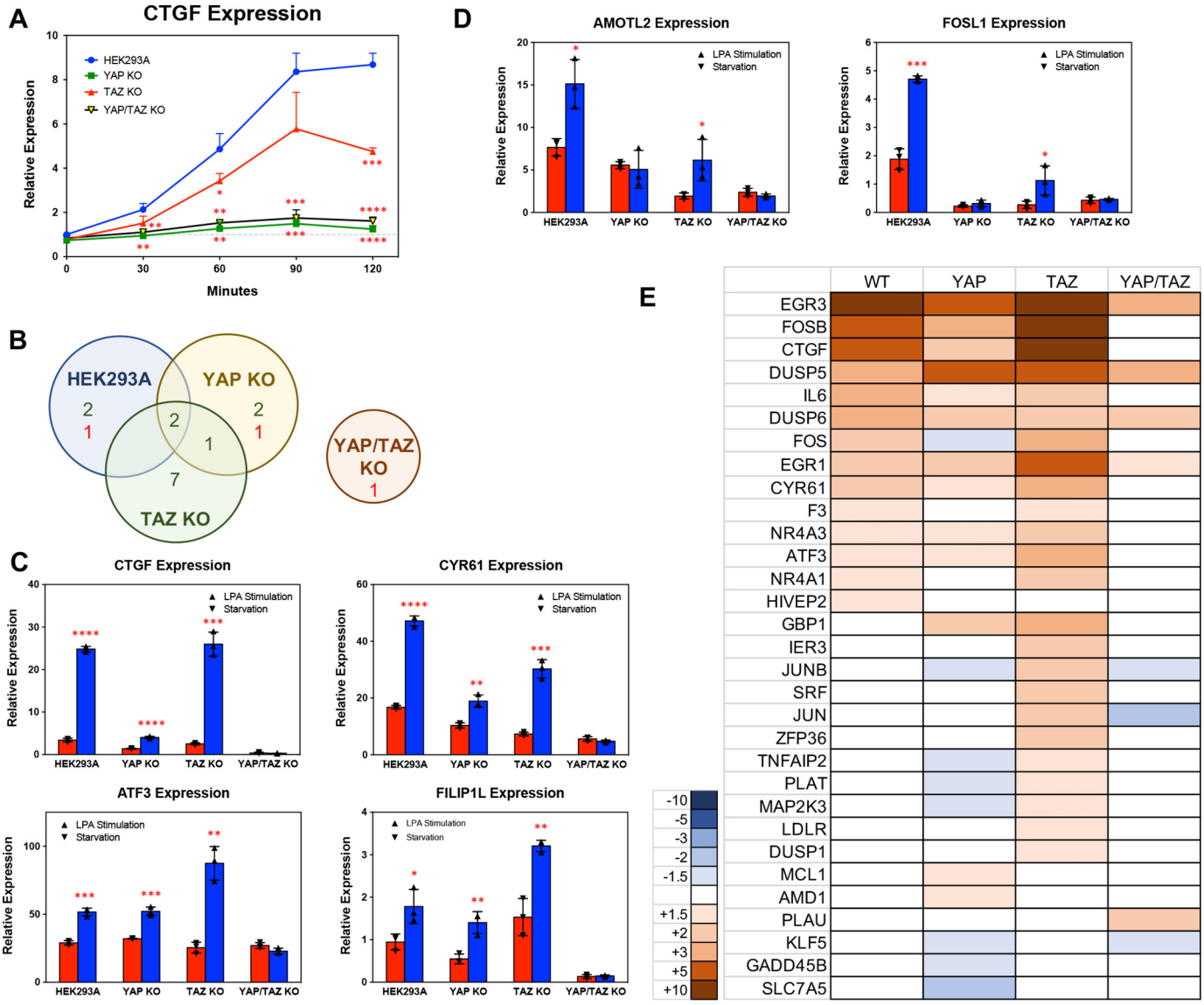

Figure 6. Using RNA-seq to examine transcriptional differences between YAP and TAZ. *A*, qPCR time course of CTGF expression following overnight serum starvation and treatment with LPA for the indicated times in WT, YAP KO, TAZ KO, and YAP/TAZ KO cells. Data are represented as ± S.D. *B*, schematic representing genes that were statistically significant and showed more than a 2-fold change following LPA stimulation. Genes in *green* represent a 2-fold induction, and genes in *red* represent a 2-fold decrease in expression following stimulation. *C*, relative expression from RNA-seq of genes that are YAP/TAZ-dependent following serum starvation (*red columns*) or LPA stimulation (*blue columns*). Data are represented as ± S.D. *D*, relative expression from RNA-seq of genes that are YAP-dependent following serum starvation (*red columns*) or LPA stimulation (*blue columns*). Data are represented as ± S.D. *E*, heatmap summarizing induction of genes in the WT, YAP KO, TAZ KO, and YAP/TAZ KO cell lines in response to LPA stimulation by RNA-seq. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

RNA-seq for the WT, YAP KO, TAZ KO, and YAP/TAZ KO cell lines under starvation and LPA stimulation conditions (100 nM, 90 min). In doing so, our goal was two-fold: first, we wanted to identify *bona fide* YAP/TAZ target genes; second, we wanted to identify any differences between the transcriptional programs of YAP and TAZ.

Surprisingly, there were very few genes that were statistically significant and showed greater than a 2-fold change in expression following stimulation (Fig. 6*B*). This is probably because the LPA stimulus was intentionally weak to identify YAP/TAZ-dependent responses while hopefully minimizing any secondary responses. The two genes that showed the biggest induction in the WT, YAP KO, and TAZ KO cell lines were CTGF and CYR61. Additionally, they showed no induction in YAP/TAZ KO cells, confirming that induction of CTGF and CYR61 expression depends on both YAP and TAZ.

To summarize some of the genes that showed at least a 1.5-fold induction, there were several genes that showed induction in the WT, YAP KO, and TAZ KO cell lines but not the YAP/TAZ KO cell line (Fig. 6*C*). These genes included CTGF, CYR61, activating transcription factor 3 (ATF3), and filamin A–interacting protein 1–like (FILIP1L). Induction of all of these in response to LPA stimulation depended on both YAP and TAZ; both YAP and TAZ were involved because they compensated for the loss of the other, as LPA-induced expression was seen in single knockouts but not YAP/TAZ KO cells.

There were also genes such as angiomotin-like 2 (AMOTL2) and Fos-like antigen 1 (FOSL1) that were induced in the WT

and TAZ KO cell lines but not in the YAP KO or YAP/TAZ KO cell lines (Fig. 6*D*). Induction of these genes was YAP-dependent. AMOTL2 is part of the AMOT (angiomotin) family of proteins, which induces LATS2 phosphorylation and YAP cytoplasmic sequestration. The fact that AMOTL2 is induced in WT and TAZ KO cells following LPA stimulation could indicate a potential YAP-dependent feedback mechanism.

Among the genes that did show at least a 1.5-fold induction in WT cells following LPA stimulation, we found that a number of them were immediate-early response genes, and several of them were compromised in the YAP KO and YAP/TAZ KO cell lines (Fig. 6*E*) (19). Indeed, a gene ontology (GO) pathway analysis of these genes revealed that many of them are transcription factors or growth factors, supporting an important role for YAP in mediating induction of immediate-early response genes (Fig. S6*A*). Overall, deleting YAP had a greater effect on downstream transcription than deleting TAZ. However, before LPA stimulation, cells were starved overnight, and based on what we observed previously (Fig. 5*B*), TAZ protein levels were probably significantly reduced at this point because of degradation; this might explain why there does not appear to be much difference between starved WT cells and TAZ KO cells.

One important caveat to note is that baseline expression for many genes may already be significantly altered following deletion of either YAP or TAZ. To compare how deleting YAP or TAZ affects the baseline transcriptional states of the cells, we compared the YAP KO, TAZ KO, and YAP/TAZ KO cell lines relative to WT cells under baseline starvation conditions (Fig. S6*B*). In total, the YAP KO cell line had 294 differentially expressed genes, the TAZ KO cell line had 202, and the YAP/TAZ KO cell line had 324 compared with WT cells. This suggests that deleting YAP or TAZ alone is sufficient to cause widespread changes in the transcriptional landscapes of the cells. Of the 294 differentially expressed genes in YAP KO cells, 81% of them were similarly differentially expressed in YAP/TAZ KO cells. For TAZ KO cells, this percentage drops to 49%. This suggests that deleting YAP has a greater effect on the transcriptional landscape of the cell than deleting TAZ. Thus, changes in induction between the cell lines may also be due to differences in their basal transcriptional levels.

Discussion

The Hippo pathway plays an important role in regulating cell growth, proliferation, and tissue homeostasis. Thus, it is not surprising that dysregulation of the Hippo pathway results in significant cellular changes and has been implicated in many human diseases, particularly cancer (20). To better characterize the cellular effects of dysregulated Hippo signaling, we analyzed the physiological consequences of inactivating the key effectors of the Hippo pathway, YAP and TAZ.

By comparing LATS1/2 KO cells, in which YAP/TAZ are constitutively active, and YAP/TAZ KO cells, we were able to clearly delineate the consequences of dysregulated YAP/TAZ signaling. First, it is clear that YAP/TAZ are master regulators for a variety of cellular processes, including cell spreading, controlling cell volume, glucose uptake and metabolism, cell proliferation, migration, and downstream gene expression, and that dysregulation of YAP/TAZ alone can have significant consequences for the cell. Especially given that several of these phenotypes, particularly control of cell proliferation, cell size, and migration, are correlated with cancer stem cell–like properties and metastasis, these findings reaffirm the oncogenic potential of YAP/TAZ and their attractiveness as therapeutic targets. When comparing the differences between cell lines, YAP KO cells clustered more closely with YAP/TAZ KO cells with regards to many of these phenotypes; thus, targeting YAP may be more efficacious than targeting TAZ. Although TAZ does have some contribution, as attested to by the differences between YAP KO and YAP/TAZ KO cells, it appears that loss of TAZ is largely compensated for by the presence of YAP. Biochemically, the nuclear translocation and TEAD binding of YAP and TAZ are similar. However, YAP protein levels are significantly higher than that of TAZ in HEK293 cells; therefore, we posit that the difference in YAP and TAZ protein levels may contribute to their functional differences in these cells. Interestingly, we found that there remains significant LATS1/2-independent phosphorylation of TAZ. Although it remains unclear under which conditions TAZ may become completely dephosphorylated or whether this phosphorylation plays any role in regulating TAZ protein stability or transcriptional activity, this raises the possibility that TAZ may have some context-specific or even cell type– dependent activity or that there may still be some mechanisms by which regulation of YAP and TAZ diverge. Further work is warranted.

Furthermore, even though it is clear that inactivating YAP in HEK293A cells has a greater effect than inactivating TAZ, it is also evident that YAP and TAZ are not completely functionally redundant. There is evidence for this from RNA-seq with genes such as AMOTL2 and FOSL1, whose induction is YAP-dependent but not TAZ-dependent. This again raises the possibility that, although there is certainly significant overlap, YAP and TAZ may induce slightly different transcriptional profiles. This may be dependent on the type and severity of the stimuli. Therefore, it will be interesting to perform additional comparisons across other types of stimuli and cell types to better understand what the differences might be and obtaining a more complete understanding of the significance of why TAZ only appears in vertebrates and what evolutionary role it might play.

Finally, as previously reported, YAP, TAZ, and TEAD1–4 are already nuclear and transcriptionally active in LATS1/2 KO cells, even under starvation conditions. However, serum stimulation was still able to induce CTGF and CYR61 expression in these cells (Fig. 1, *D* and *E*). This additional induction could be due to activation of other transcription factors, such as AP-1, which has been reported to synergize with TEAD1–4 in promoting gene expression (21, 22). This highlights the observation that regulation of TEAD1–4 or other co-transcription factors, such as AP-1, has a major role in amplifying the downstream target gene expression of active YAP/TAZ. Nevertheless, this induction is completely dependent on YAP/TAZ because no such induction is seen in YAP/TAZ KO cells.

In conclusion, we have analyzed the cellular and transcriptional consequences of inactivating YAP and TAZ. One note of caution is that our studies were conducted primarily in HEK293A cells, and it is entirely possible that YAP and TAZ may have tissue-specific roles during development and regen-

eration because of differences in signaling or distribution. Other potential differences between YAP and TAZ may involve tissue-specific or cell type–specific transcription factor binding partners or other mechanisms that regulate YAP/TAZ protein stability and expression. This may explain some of the differences we see between the YAP and TAZ knockout mouse models. However, because YAP knockout mice are embryonic lethal, and several other YAP conditional knockout models are also lethal, it complicates comparing the consequences of inactivating YAP and TAZ *in vivo*. Additionally, many *in vivo* studies only focus on YAP or TAZ but do not compare the two. For example, although lung-specific conditional knockout models have been generated for many Hippo pathway components, lung-specific TAZ knockout mice have not been studied (23). Our goal was to more comprehensively characterize some of the physiological differences between YAP and TAZ *in vitro* in an attempt to gain greater insight into this topic. Although more work remains, this systematic functional analysis of YAP and TAZ provides a useful resource in the quest to better understand the commonalities and differences between YAP and TAZ and the downstream effects of dysregulated Hippo signaling.

Experimental procedures

Generating cell lines

pSpCas9(BB)-2A-Puro (PX459, Addgene plasmid 48139) was a gift from Dr. Feng Zhang (24). Gene-specific single guide RNAs were designed and cloned as described previously (16). HEK293A cells were transfected using PolyJet transfection reagent, selected with puromycin for 3 days, and single-cell sorted by FACS into 96-well plates. Single clones were expanded and screened by Western blotting and confirmed by sequencing.

Cell culture

HEK293A cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated at 1.5×10^5 cells/well into 6-well plates and serum-starved overnight before serum or LPA (100 nM, 90 min) stimulation.

Western blotting

Western blots were performed as described previously (16). 7.5% Phos-tag gels were used to compare YAP and TAZ phosphorylation.

FACS

Cells were fixed in cold 70% ethanol overnight. Cells were then washed twice with PBS, treated with 10 μg/ml RNase at 37 °C for 30 min, and stained with 50 μg/ml propidium iodide.

Glucose measurements

Cells were plated at 8×10^5 cells/well into 6-well plates. When the cells adhered to the dish, the culture medium was replaced with 1 ml of fresh Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin for 6 h. Glucose levels were measured using the FreeStyle Precision Neo glucose monitoring system.

Migration assay

For the migration assay, we used Falcon cell culture inserts (transparent PET membrane, 24-well, 8.0-μm pore size). The bottom of each insert was coated with poly-lysine for 1 h at 37 °C, after which the poly-lysine was removed and the insert allowed to air-dry at room temperature for 1 h. 5×10^4 cells were plated in the insert in serum-free culture medium, whereas the bottom of the well was filled with complete culture medium. Cells were allowed to migrate for 8 h, after which they were fixed and stained with 0.5% crystal violet in methanol.

Scratch assay

6-well plates were coated with poly-lysine for 1 h at room temperature before being washed three times with PBS. Cells were then plated at 1.5×10^6 cells/well into 6-well plates. When the cells adhered to the dish, the culture medium was replaced with serum-free medium, and the scratch was made using a plastic pipette tip.

Immunofluorescence

Immunofluorescence was performed as described previously (16).

λ Phosphatase treatment

Cells were lysed in mild lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40, and protease inhibitor mixture). Then 10 μl of NEBuffer, 10 μl of McCl, and 2 μl of λ phosphatase was added and incubated at 37 °C for 1 h. The reaction was terminated by adding 4× loading buffer (200 mM Tris (pH 6.8), 8% SDS, 0.1% bromphenol blue, 40% glycerol, and 20% 2-mercaptoethanol). Samples were then boiled for 5 min and loaded directly onto the gel for Western blot analysis.

RNA-seq

Total RNA was extracted and used to prepare libraries using Illumina TruSeq Stranded mRNA Library Prep Kit Set A (Illumina, RS-122-2101) or Set B (Illumina, RS-122-2102). The libraries were sequenced using Illumina HiSeq 4000 (single-end 50 bp).

Sequenced reads were aligned to the hg19 reference genome using STAR (25). Only uniquely mapped reads were kept for further analysis. The number of reads for each gene were counted using htseq-count (26) based on the Gencode human annotation release 24. Differentially expressed genes were identified using DESeq2 (27). Specifically, genes with adjusted $p <$ 0.1 were considered differentially expressed. Biological triplicates were used.

Luciferase assay

The luciferase reporter assay was performed as described previously (28). Briefly, cells were transfected with a pGL3-CTGF-Luc reporter. Luciferase activity was assayed using the Neolite Reporter Gene Assay System (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Statistical analysis

Where indicated, experiments were repeated at least three times, and statistical analysis was performed using unpaired t

tests; ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

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