Insulin suppresses transcriptional activity of yes-associated protein in insulin target cells

Samar Sayedyahossein¹, Andrew C. Hedman, and David B. Sacks*
Department of Laboratory Medicine, National Institutes of Health, Bethesda, MD 20892

ABSTRACT Yes-associated protein (YAP), the main transcriptional coactivator of the Hippo pathway, integrates multiple inputs from different signaling cascades. Evidence implicates YAP in the control of cellular nutrient and energy status, but the underlying mechanisms are not fully elucidated. Here we show that insulin modulates YAP transcriptional activity in classic insulin target cells, namely HepG2 and C2C12. Insulin increases YAP phosphorylation and significantly decreases YAP abundance in HepG2 cell nuclei. Proximity ligation assay analysis revealed a marked reduction in the interaction of YAP with TEA domain (TEAD) transcription factors in the nuclei of insulin-exposed cells. Consistent with these findings, insulin impaired both YAP/TEAD-mediated transcription and transcription of YAP target genes in HepG2 and C2C12 cells. Serum starvation abrogated the effect of insulin on YAP phosphorylation and YAP transcription. Both the expression of two gluconeogenesis genes, G6PC and PCK1, and the inhibitory effect of insulin on these genes were attenuated in YAP-deficient HepG2 cells. Our results identify insulin as a previously undescribed suppressor of YAP activity in insulin target cells and provide insight into cross-talk between the insulin and Hippo pathways.

INTRODUCTION
Insulin is a critical regulator of energy homeostasis and metabolism. Dysregulation of the insulin signaling pathway is associated with several diseases, especially type 2 diabetes (Poloz and Stambolic, 2015). Insulin promotes glucose uptake and glycogen synthesis, inhibits glycogenolysis, and modulates transcription of selected genes involved in metabolism (Boucher et al., 2014). Binding of insulin to the insulin receptor (IR) induces autophosphorylation of tyrosine residues in the receptor (De Meyts, 2000), which initiates signaling pathways. These pathways include the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades. Although insulin can elicit effects in diverse tissues, skeletal muscle, fat, and liver are known as “classic” insulin target tissues in which insulin regulates the metabolism of glucose and fat. PI3K is responsible for most of the metabolic effects of insulin (White, 2003), while MAPK predominantly mediates mitogenic cellular responses (Avruch, 2007).

Yes-associated protein (YAP) is a major transcriptional coactivator of the Hippo signaling pathway that integrates signals from multiple receptors to determine cell behavior, organ size, and tissue homeostasis (Zhao et al., 2010a; Liu-Chittenden et al., 2014). When Hippo is on, YAP and its closely related parologue TAZ become phosphorylated. Although YAP/TAZ are retained in the cytoplasm, which reduces their nuclear cotranscriptional activity. 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 activity by inducing its phosphorylation at serine127 (Ser-127; Liu-Chittenden et al., 2014). In the nucleus, YAP interacts with several transcription factors, particularly members of the TEAD domain (TEAD) family, to drive gene transcription (Zhao et al., 2008; Liu-Chittenden et al., 2012). Changes in nuclear localization of YAP through phosphorylation-independent and phosphorylation-dependent mechanisms are

© 2020 Sayedyahossein et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution-Noncommercial-Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-ns/3.0).
Moreover, the published effects of insulin on YAP are not consistent; effects of insulin on YAP activity have not been characterized in detail. To consider when evaluating YAP function.

Several growth factor receptors, including those for transforming growth factor β (Pefani et al., 2016) and epidermal growth factor (EGF) (Ürutasun et al., 2011), modulate YAP activity. However, the effects of insulin on YAP activity have not been characterized in detail. Moreover, the published effects of insulin on YAP are not consistent among studies. Insulin has been reported to have no effect on YAP phosphorylation in some mammalian cells (HeLa and HEK293; Zhao et al., 2007; Yu et al., 2012). In contrast, in human pancreatic ductal adenocarcinoma cells, insulin decreased YAP phosphorylation and promoted both nuclear localization and expression of genes regulated by YAP (Hao et al., 2017). Importantly, no published reports have evaluated whether insulin influences YAP function in classic mammalian insulin target cells.

In this study, we examined the effects of insulin on YAP in HepG2 liver cells and C2C12 skeletal muscle cells. We show that insulin increases YAP phosphorylation and decreases the amount of YAP in the nucleus. Importantly, insulin alters YAP function; insulin significantly down-regulates transcription of YAP target genes, an effect that was abrogated by PI3K inhibition. Our results identify insulin as a suppressor of YAP activity in classic insulin target cells, establishing cross-talk between the insulin and Hippo signaling cascades.

RESULTS

Insulin modulates subcellular localization of yes-associated protein

Investigation of intracellular insulin signaling is usually conducted in cells starved of serum. However, it has been shown in several cell lines that serum starvation induces YAP phosphorylation and ubiquitination, which results in inhibition of YAP transcriptional activity (Yu et al., 2012; Adler et al., 2013). Therefore, we used cells cultured in medium containing 10% charcoal-stripped fetal bovine serum (chFBS). ChFBS has undetectable concentrations of insulin and insulin-like growth factor-1 (IGF-1; see Materials and Methods), as well as low concentrations of steroid and peptide hormones, but contains nutrients (Cao et al., 2009), which prevent the suppressive effects of starvation on YAP function.

To ascertain whether insulin activates IR and promotes signaling under our assay conditions, we examined tyrosine phosphorylation of IR. Cells were incubated with insulin for 5 min either in serum-free medium or in medium containing 1% chFBS. The IR was immunoprecipitated with anti-IR antibodies and phosphorylation was examined with anti-phosphotyrosine antibody. We observed that insulin stimulates IR tyrosine phosphorylation in HepG2 cells in either serum-free or chFBS-containing medium (Figure 1A). Quantification of tyrosine phosphorylation (corrected for the total amount of IR immunoprecipitated) revealed that IR tyrosine phosphorylation was slightly, but not significantly, greater in serum-starved HepG2 cells (Figure 1, A and B). Similar data were obtained in C2C12 cells; insulin significantly augmented IR phosphorylation in cells cultured in either medium (Figure 1, C and D). These results indicate that our conditions using chFBS medium are suitable for assessing the effects of insulin on YAP.

To determine whether insulin signaling influences YAP, we initially examined phosphorylation of YAP at Ser127. C2C12 cells were

![FIGURE 1: Insulin activates IR in chFBS-containing medium.](Image 36x456 to 379x735)

**C2C12**

**pTyr**

**Lysate**

**IR**

**Insulin:**

Starve

chFBS

Starve

chFBS

**IP**

**C2C12**

**pTyr**

**Lysate**

**IR**

**Insulin:**

Starve

chFBS

Starve

chFBS

**IP**

**RESULTS**

Insulin modulates subcellular localization of yes-associated protein

Investigation of intracellular insulin signaling is usually conducted in cells starved of serum. However, it has been shown in several cell lines that serum starvation induces YAP phosphorylation and ubiquitination, which results in inhibition of YAP transcriptional activity (Yu et al., 2012; Adler et al., 2013). Therefore, we used cells cultured in medium containing 10% charcoal-stripped fetal bovine serum (chFBS). ChFBS has undetectable concentrations of insulin and insulin-like growth factor-1 (IGF-1; see Materials and Methods), as well as low concentrations of steroid and peptide hormones, but contains nutrients (Cao et al., 2009), which prevent the suppressive effects of starvation on YAP function.

To ascertain whether insulin activates IR and promotes signaling under our assay conditions, we examined tyrosine phosphorylation of IR. Cells were incubated with insulin for 5 min either in serum-free medium or in medium containing 1% chFBS. The IR was immunoprecipitated with anti-IR antibodies and phosphorylation was examined with anti-phosphotyrosine antibody. We observed that insulin stimulates IR tyrosine phosphorylation in HepG2 cells in either serum-free or chFBS-containing medium (Figure 1A). Quantification of tyrosine phosphorylation (corrected for the total amount of IR immunoprecipitated) revealed that IR tyrosine phosphorylation was slightly, but not significantly, greater in serum-starved HepG2 cells (Figure 1, A and B). Similar data were obtained in C2C12 cells; insulin significantly augmented IR tyrosine phosphorylation in cells cultured in either medium (Figure 1, C and D). These results indicate that our conditions using chFBS medium are suitable for assessing the effects of insulin on YAP.

To determine whether insulin signaling influences YAP, we initially examined phosphorylation of YAP at Ser127. C2C12 cells were

![FIGURE 1: Insulin activates IR in chFBS-containing medium.](Image 36x456 to 379x735)

**C2C12**

**pTyr**

**Lysate**

**IR**

**Insulin:**

Starve

chFBS

Starve

chFBS

**IP**

**RESULTS**

Insulin modulates subcellular localization of yes-associated protein

Investigation of intracellular insulin signaling is usually conducted in cells starved of serum. However, it has been shown in several cell lines that serum starvation induces YAP phosphorylation and ubiquitination, which results in inhibition of YAP transcriptional activity (Yu et al., 2012; Adler et al., 2013). Therefore, we used cells cultured in medium containing 10% charcoal-stripped fetal bovine serum (chFBS). ChFBS has undetectable concentrations of insulin and insulin-like growth factor-1 (IGF-1; see Materials and Methods), as well as low concentrations of steroid and peptide hormones, but contains nutrients (Cao et al., 2009), which prevent the suppressive effects of starvation on YAP function.

To ascertain whether insulin activates IR and promotes signaling under our assay conditions, we examined tyrosine phosphorylation of IR. Cells were incubated with insulin for 5 min either in serum-free medium or in medium containing 1% chFBS. The IR was immunoprecipitated with anti-IR antibodies and phosphorylation was examined with anti-phosphotyrosine antibody. We observed that insulin stimulates IR tyrosine phosphorylation in HepG2 cells in either serum-free or chFBS-containing medium (Figure 1A). Quantification of tyrosine phosphorylation (corrected for the total amount of IR immunoprecipitated) revealed that IR tyrosine phosphorylation was slightly, but not significantly, greater in serum-starved HepG2 cells (Figure 1, A and B). Similar data were obtained in C2C12 cells; insulin significantly augmented IR tyrosine phosphorylation in cells cultured in either medium (Figure 1, C and D). These results indicate that our conditions using chFBS medium are suitable for assessing the effects of insulin on YAP.

To determine whether insulin signaling influences YAP, we initially examined phosphorylation of YAP at Ser127. C2C12 cells were
incubated overnight in serum-free or chFBS-containing medium and then stimulated with 100 nM insulin for 5 min. Cell lysates were examined by SDS–PAGE and Western blotting with an anti-phospho-YAP Ser\(^{127}\) antibody. Insulin significantly enhanced YAP Ser\(^{127}\) phosphorylation twofold in chFBS medium (Figure 2, A and B). Serum starvation increased basal YAP phosphorylation in C2C12 cells (Figure 2A), consistent with data obtained with other cell lines (Yu et al., 2012; Adler et al., 2013). Insulin slightly, but not significantly, enhanced YAP phosphorylation in cells starved of serum (Figure 2, A and B). These results demonstrate that insulin promotes YAP phosphorylation in insulin target cells, but with significant phosphorylation observed only when nutrients are available.

Retention of YAP in the cytoplasm through a phosphorylation-independent mechanism prevents nuclear localization of YAP, thus suppressing its transcriptional activity (Zhao et al., 2011; Li et al., 2014). Phosphorylation of YAP at Ser\(^{127}\) promotes its retention in the cytoplasm and also inhibits YAP nuclear localization (Zhao et al., 2007). To investigate the possible effects of insulin on YAP localization, we performed subcellular fractionation. HepG2 cells were incubated with or without insulin and cell extracts were separated into cytoplasmic and nuclear fractions. Analysis by Western blotting revealed that insulin significantly decreased the abundance of YAP in the nucleus by 42% (Figure 3, A and B), with a concomitant increase in the ratio of YAP phosphorylated on Ser\(^{127}\) in total YAP in the cytoplasmic fraction (Figure 3, A and C, left panel). PhosphoYAP is present predominantly in the cytoplasm, with minimal amounts detected in the nucleus (Figure 3A). Insulin also slightly increased the total amount of YAP in the cytoplasm, although this did not reach statistical significance (Figure 3C).

Binding of YAP to TEAD in the nucleus is essential for YAP/TEAD-mediated transcriptional activity (Zhao et al., 2008). We evaluated the effect of insulin on the formation of nuclear YAP/TEAD complexes in two insulin target cell lines, namely HepG2 and C2C12. The interaction between endogenous YAP and TEAD was analyzed by proximity ligation assay (PLA), in which a signal is generated only when the antibodies against proteins of interest are in close proximity (nm range). PLA analysis revealed an interaction between YAP and TEAD in the nuclei of unstimulated HepG2 cells (Figure 3D). Quantification of PLA spots demonstrated that incubation with insulin for 2 h reduced the number of stable nuclear YAP/TEAD complexes by 85% (Figure 3, D and E). This effect persisted for at least 8 h. In C2C12 cells, insulin slightly reduced the association of YAP/TEAD by 2 h, and this interaction was significantly impaired by 73% after 8 h. Collectively, these data reveal that insulin attenuates nuclear localization of YAP and decreases the formation of stable YAP/TEAD complexes in the nucleus.

Next, we studied the impact of insulin signaling on YAP/TAZ phosphorylation over time by stimulating cultured C2C12 cells with insulin for 30 and 60 min. Western blot analysis revealed a significant increase in the phosphorylation of both YAP (1.7-fold) and TAZ (3.4-fold) at 30 min (Figure 4). At 60 min, insulin-stimulated YAP phosphorylation has ceased, but TAZ phosphorylation remains increased, albeit not significantly. These data suggest that insulin has a greater and more prolonged effect on TAZ phosphorylation than on YAP in C2C12 cells. We also evaluated the possible effect of insulin-like growth factor (IGF-1) and EGF on YAP/TAZ phosphorylation in C2C12 cells. IGF-1 had no significant effect on YAP phosphorylation (Figure 4). In contrast, IGF-1 significantly increased TAZ phosphorylation 4.2- and 2.4-fold at 30 and 60 min, respectively. EGF did not significantly alter phosphorylation of either YAP or TAZ (Figure 4). Our data suggest that insulin increases YAP/TAZ phosphorylation in C2C12 cells.

**Insulin impairs yes-associated protein transcriptional activity**

The effect of insulin on YAP function was evaluated by analyzing YAP transcriptional activity. Two complementary approaches were used. In the first, cells were transfected with a synthetic TEAD promoter that drives luciferase expression (Sayedyahossein et al., 2016). The luciferase signal was measured and corrected for a Renilla control. Addition of insulin to serum-starved C2C12 cells slightly reduced YAP activity, but this change was not statistically significant (Figure 5A). When cells were cultured in chFBS, insulin significantly suppressed YAP function. Note that basal YAP activity in serum-starved C2C12 cells was 53% lower than in cells cultured in chFBS (Figure 5A). We observed that insulin also significantly reduced YAP activity by 20% in HepG2 cells cultured in chFBS (Figure 5B), a magnitude similar to that seen in C2C12 cells.

In the second approach, YAP activity was assessed by measuring the transcription of endogenous YAP-responsive genes. Expression of ACTA2, AMOTL2, and CTGF was measured by quantitative reverse transcriptase-PCR (RT-PCR). Insulin reduced ACTA2 hRNA by 70% in HepG2 cells cultured in chFBS after 24 h (Figure 5C). Similarly, AMOTL2 and CTGF hRNA were decreased by 64% and 54%, respectively, when cells were incubated with insulin. Note that the suppressive effect of insulin on YAP activity begins after ~2 h of insulin stimulation and persists for at least 24 h (Figure 5D).

To evaluate whether the inhibition is specific to insulin, we examined whether EGF alters YAP transcriptional activity in HepG2 cells, which are targets for EGF (Yang et al., 1996). In contrast to insulin, EGF had no significant effect on YAP-mediated transcription of the three endogenous target genes (Figure 5C). Collectively, these data reveal that insulin impairs YAP transcriptional activity in at least two insulin target cell lines cultured in serum.
FIGURE 3: Insulin modulates YAP subcellular localization. (A) HepG2 cells, cultured in chFBS medium, were treated without (–) or with (+) 100 nM insulin for 2 h. Cell lysates were separated into cytoplasmic (Cyt) and nuclear (Nuc) fractions as described under Materials and Methods. Equal amounts of cytoplasmic and nuclear proteins were resolved by SDS–PAGE and Western blotting was performed with the indicated antibodies. Heat shock protein 90 (HSP90) and histone H3 (H3) were used as controls for cytoplasmic and nuclear fractions, respectively. (B, C) The phospho-YAP Ser\(^{127}\) (pYAP), YAP, HSP90, and H3 bands were quantified with Image Studio 2.0 and corrected for the total amount of YAP, HSP90, or H3 in the same sample. Data are expressed as means ± SEM (error bars) with unstimulated (–) samples set to 1. *p < 0.05 Student’s t test. (D) HepG2 (left panels) or C2C12 (right panels) cells were incubated with 100 nM insulin for 0, 2, or 8 h and then fixed and stained with both anti-YAP and anti-TEAD antibodies. Control panels show cells incubated with Duolink secondary antibodies alone. PLA was performed using Duolink in situ detection reagents as described under Materials and Methods. DNA is stained by Hoescht (blue). Scale bar, 10 μm. (E) The numbers of PLA spots in the nucleus were quantified using IMARIS software from confocal images of HepG2 (n ≥ 117) and C2C12 (n ≥ 45) cells. Data are expressed as means ± SEM (error bars) with the number of spots in control unstimulated cells set to 1. *p < 0.001, **p < 0.0001, ANOVA.
Inhibition of PI3K blocks the effect of insulin on yes-associated protein transcriptional activity

To gain insight into the signaling pathway by which insulin modulates YAP function, we used LY294002, a specific and potent inhibitor of PI3K (Vlahos et al., 1994; Joyal et al., 1997). HepG2 cells were incubated with LY294002 and YAP transcriptional activity was evaluated. LY294002 had no significant effect on basal YAP-mediated transcription of ACTA, AMOTL2, or CTGF (Figure 6A). In contrast, inhibition of PI3K abrogated the ability of insulin to reduce transcription of all three YAP-responsive genes examined. Similar data were obtained when YAP activity was measured by TEAD–luciferase reporter assays. LY294002 abolished the suppressive effect of insulin on YAP activity without influencing basal YAP activity (Figure 6B). These results suggest that insulin-induced suppression of YAP function is mediated by the PI3K signaling cascade.

Knockdown of yes-associated protein alters expression of gluconeogenesis genes

In the liver, insulin controls multiple metabolic processes, including inhibition of gluconeogenesis. To investigate whether YAP has a function in insulin action, we examined the effect of altering intracellular YAP levels on transcription of insulin-regulated genes. The expression of two gluconeogenesis genes, glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PCK1), was evaluated. Analysis by quantitative RT-PCR revealed that insulin induced down-regulation of G6PC and PCK1 mRNA in control HepG2 cells by 81% and 54%, respectively (Figure 7, A and B). Knockdown of YAP by siRNA reduces basal G6PC and PCK1 expression. In HepG2 cells with reduced amounts of YAP, the ability of insulin to decrease gluconeogenesis genes was impaired. Insulin further decreased expression of G6PC by 64% in YAP knockdown cells (Figure 7A), but did not significantly decrease PCK1 expression in YAP-deficient cells (Figure 7B). The inhibitory effect of insulin in cells with YAP knockdown may be due to residual YAP expression in these cells. Western blot shows that siRNA reduced, but did not completely eliminate YAP from HepG2 cells (Figure 7C).

DISCUSSION

YAP is a critical regulator of organ size and tissue homeostasis and its nuclear localization is essential for its transcriptional functions (Zhao et al., 2010a). Several studies have focused on signaling pathways that regulate YAP function, including growth factors (Urtasun et al., 2011; Pefani et al., 2016), cell density (Zhao et al., 2007), and metabolic pathways and nutrient-sensing receptors (Yu et al., 2012; DeRan et al., 2014; Mo et al., 2015; Wang et al., 2015). Additionally, changes in the cellular microenvironment, such as mechanical cues, extracellular matrix stiffness, and cell confluence (Dupont et al., 2011), modulate YAP function. While YAP has been shown to regulate both IGF-1 signaling (Xin et al., 2011) and phosphorylation of insulin receptor substrate (IRS; Wang et al., 2016), as well as influencing proliferation of the insulin-producing β-cells of the pancreas (Yuan et al., 2016), the regulation of YAP function by insulin signaling is poorly characterized.

Nutrient deprivation promotes Ser127 phosphorylation and ubiquitination of YAP (Adler et al., 2013) and also inhibits YAP function (Miller et al., 2012; Yu et al., 2012; Mo et al., 2015). Thus, nutrient status is a critical consideration in investigating YAP activity. The majority of published studies that have evaluated the effect of growth factors on YAP have been performed in cells starved of serum. To avoid inhibition of YAP activity by serum deprivation, the current study was performed using cells incubated in medium containing 10% chFBS, which contains adequate nutrients, but extremely low concentrations of insulin and IGF-1. We verified that under our assay conditions insulin is able to activate IR signaling in two insulin target cell lines, HepG2 and C2C12.

Consistent with the published literature (Yu et al., 2012; Adler et al., 2013), we observed that basal YAP phosphorylation was increased in serum-starved cells. Moreover, there was not a significant increase in phosphoYAP in response to insulin in serum-starved cells. In contrast, when cells were cultured in chFBS, insulin induced phosphorylation of YAP at Ser127, reducing the nuclear localization of YAP. We cannot exclude the possibility that insulin may also regulate YAP subcellular localization by a phosphorylation-independent mechanism. Moreover, insulin may modulate YAP by inducing
Insulin suppresses YAP-mediated gene transcription. (A) C2C12 cells were transfected with TEAD reporter luciferase plasmid and Renilla luciferase–Pol III constructs. After 24 h, cells were cultured in serum-free (starve) or chFBS-containing medium and incubated without (−) or with (+) 100 nM insulin for an additional 24 h. Equal numbers of cells were lysed and processed using a dual luciferase assay. The graphs depict the luciferase signal corrected for Renilla. Values were normalized to untreated, serum-free cells. Data are expressed as means ± SEM (error bars), n = 4, for starved and n = 2 for chFBS. *p ≤ 0.01, **p ≤ 0.0001, n.s., not significant by ANOVA. (B) HepG2 cells, cultured in chFBS medium, were transfected as described for panel A for C2C12 cells. Luciferase activity was measured as described for C2C12 cells. Data are expressed as means ± SEM (error bars), n = 9. **p ≤ 0.01, ***p ≤ 0.0001, n.s., not significant by ANOVA. (C) HepG2 cells, cultured in chFBS medium, were transfected as described for panel A for C2C12 cells. Luciferase activity was measured as described for C2C12 cells. Data are expressed as means ± SEM (error bars), n = 9. **p ≤ 0.01, ***p ≤ 0.0001, n.s., not significant by ANOVA. (D) HepG2 cells, cultured in chFBS-containing medium, were incubated for 24 h with vehicle (control), 100 nM insulin, or 100 ng/ml EGF. Total RNA was extracted and quantitative RT-PCR was performed to measure ACTA2, AMOTL2, and CTGF hnRNA. The amount of hnRNA was corrected for β-actin hnRNA in the same sample. hnRNA in vehicle-treated cells was set as 1. Data are expressed as means ± SEM (error bars) of at least four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.0001, ANOVA; n.s., not significant. (E) HepG2 cells, cultured in chFBS-containing medium, were incubated for 24 h with vehicle (control), 100 nM insulin, or 100 ng/ml EGF. Total RNA was extracted and quantitative RT-PCR was performed to measure ACTA2, AMOTL2, and CTGF hnRNA as described for panel C. Data are expressed as means ± SEM (error bars) of four to seven independent experiments. *p < 0.005, **p < 0.0001, ANOVA.

FIGURE 5: Insulin suppresses YAP-mediated gene transcription. (A) C2C12 cells were transfected with TEAD reporter luciferase plasmid and Renilla luciferase–Pol III constructs. After 24 h, cells were cultured in serum-free (starve) or chFBS-containing medium and incubated without (−) or with (+) 100 nM insulin for an additional 24 h. Equal numbers of cells were lysed and processed using a dual luciferase assay. The graphs depict the luciferase signal corrected for Renilla. Values were normalized to untreated, serum-free cells. Data are expressed as means ± SEM (error bars), n = 4, for starved and n = 2 for chFBS. *p ≤ 0.01, **p ≤ 0.0001, n.s., not significant by ANOVA. (B) HepG2 cells, cultured in chFBS medium, were transfected as described for panel A for C2C12 cells. Luciferase activity was measured as described for C2C12 cells. Data are expressed as means ± SEM (error bars), n = 9. **p ≤ 0.01, ***p ≤ 0.0001, n.s., not significant by ANOVA. (C) HepG2 cells, cultured in chFBS medium, were transfected as described for panel A for C2C12 cells. Luciferase activity was measured as described for C2C12 cells. Data are expressed as means ± SEM (error bars), n = 9. **p ≤ 0.01, ***p ≤ 0.0001, n.s., not significant by ANOVA. (D) HepG2 cells, cultured in chFBS-containing medium, were incubated for 24 h with vehicle (control), 100 nM insulin, or 100 ng/ml EGF. Total RNA was extracted and quantitative RT-PCR was performed to measure ACTA2, AMOTL2, and CTGF hnRNA. The amount of hnRNA was corrected for β-actin hnRNA in the same sample. hnRNA in vehicle-treated cells was set as 1. Data are expressed as means ± SEM (error bars) of at least four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.0001, ANOVA; n.s., not significant. (E) HepG2 cells, cultured in chFBS-containing medium, were incubated for 24 h with vehicle (control), 100 nM insulin, or 100 ng/ml EGF. Total RNA was extracted and quantitative RT-PCR was performed to measure ACTA2, AMOTL2, and CTGF hnRNA as described for panel C. Data are expressed as means ± SEM (error bars) of four to seven independent experiments. *p < 0.005, **p < 0.0001, ANOVA.

phosphorylation of YAP at additional residues. For example, phosphorylation of YAP on Ser197, Ser24, and Ser311 has been reported to regulate YAP function (Zhao et al., 2010c; Mo et al., 2015; Wang et al., 2015).

In agreement with the impaired YAP nuclear localization, insulin decreased nuclear YAP/TEAD complex formation. The predominant transcription factors that mediate the biological functions of YAP are members of the TEAD family (Ota and Sasaki, 2008; Zhao et al., 2008). To evaluate the outcome of insulin-induced suppression of the YAP/TEAD interaction, we analyzed YAP-mediated gene transcription using two complementary approaches, a widely employed luciferase-based assay (Tan et al., 2015; Zanconato et al., 2015) and RT-PCR. Both assays revealed that insulin consistently suppressed endogenous YAP target gene expression when cells were cultured in chFBS. In contrast, when cells were starved of serum, YAP transcriptional activity was significantly lower and not responsive to insulin. These data emphasize the importance of assay conditions, particularly nutrient status, in investigating the effects of extracellular stimuli on YAP function.

Few published studies have looked at the effect of insulin on the Hippo pathway, and none was conducted in insulin target cells. The first examination failed to detect any effect of insulin on pYAP Ser127 in HeLa cells (Zhao et al., 2007), despite using supra-physiological insulin concentrations (800 nM). The possible effect of insulin on YAP function was not evaluated in that study. The same group also looked at pYAP Ser127 in HEK293 cells treated with 200 nM insulin and again did not observe an effect of insulin on YAP phosphorylation (Yu et al., 2012). Others investigated the role of Yki, the Drosophila homologue of YAP, in cell proliferation (Strassburger et al., 2012). Drosophila expresses a single insulin-like receptor (INR), which is similar to the IR and IGF-1 receptors in mammals. The investigators observed that the insulin/IGF signaling pathway promoted Yki dephosphorylation in Drosophila S2R+ cells, and this effect was inhibited by the PI3K inhibitor wortmannin (Strassburger et al., 2012). The authors used a very high concentration (1.7 μM) of insulin, although other reports have demonstrated that 200–500 nM insulin is sufficient to promote INR tyrosine phosphorylation and AKT activation in Drosophila cells (Fernandez et al., 1995; Sanchez-Alvarez et al., 2015). Moreover, Strassburger et al. (2012) did not examine the effect of insulin on either YAP phosphorylation or YAP function in mammalian...
cells. Insulin was recently observed to promote dephosphorylation, nuclear localization, and activity of YAP in serum-free, confluent pancreatic ductal adenocarcinoma cells (Hao et al., 2017). The experimental conditions in that study promote increased YAP phosphorylation from both cell confluence and serum starvation, inhibiting basal YAP activity (Dupont et al., 2011; Adler et al., 2013). In our study, we did not use serum starvation to avoid interference from the high basal level of YAP phosphorylation and impaired YAP transcriptional activity. Differences among cell types are also likely to influence regulation of YAP substantially. Our study is the first to evaluate the effect of insulin signaling on YAP in “classic” mammalian insulin target cells, namely skeletal muscle and liver. IR predominates in these classic insulin-responsive tissues, whereas in many other cell types insulin signals through the more abundant IGF-1 receptor (Siddle, 2012). Both HepG2 and C2C12 cells are very widely used to study insulin signaling. Nevertheless, it should be borne in mind that these are transformed cell lines and their signaling pathways could be dysregulated.

Numerous stimuli, including insulin, growth factors (e.g., EGF), G-protein coupled receptors, and integrins, activate PI3K signaling. Prior publications have examined the influence of PI3K on YAP, but the data are conflicting. For example, some studies observed that PI3K inhibitors promote YAP phosphorylation (Strassburger et al., 2012; Li et al., 2013), whereas other studies demonstrate that inhibition of PI3K or AKT decreases YAP phosphorylation and function (Choi et al., 2015; Chen and Harris, 2016). PI3K has a critical role in the biological effects of insulin. Liver-specific deletion of p110β, the catalytic subunit of PI3K, lowers glucose tolerance and insulin sensitivity in mice (Jia et al., 2008). In HepG2 cells, the PI3K-specific inhibitor LY294002 does not change YAP mRNA levels (Urtasun et al., 2011) or basal YAP target gene expression (Figure 5). Nevertheless, we observed that LY294002 abrogated the inhibitory effect of insulin on YAP transcriptional activity, implicating the PI3K pathway in the suppressive effect of insulin on YAP activity.

Insulin controls several aspects of glucose metabolism in the liver, including inhibition of gluconeogenesis (Hatting et al., 2018). One of the mechanisms by which this occurs is modulation of the transcription of genes involved in gluconeogenic control, including G6PC and PCK1 (Hatting et al., 2018). To determine whether YAP has a functional role, we examined insulin-regulated transcription of gluconeogenesis genes after siRNA-mediated knockdown of YAP. We observed that, as anticipated, insulin decreased the levels of G6PC and PCK1 in HepG2 cells. In addition, G6PC and PCK1 were significantly reduced following knockdown of YAP. While insulin further reduced G6PC and slightly decreased PCK1 in these cells, the magnitude of inhibition by insulin was attenuated when YAP was knocked down. The molecular mechanism by which YAP decreases basal gluconeogenesis requires further investigation.
On the basis of previous studies by others investigating growth factor signaling after serum starvation, and data in this study, we propose a model of the regulation of YAP by growth factors and insulin (Figure 8). Low nutrient levels and increased cell–cell contact result in Ser\textsuperscript{127} phosphorylation of YAP and its retention in the cytoplasm. Under these conditions, stimulation with growth factors, such as EGF, may promote YAP nuclear localization by inducing its dephosphorylation (Figure 8). In contrast, in the presence of nutrients, insulin inhibits YAP in classic insulin target cells by promoting YAP phosphorylation and cytoplasmic retention, thereby impairing nuclear translocation and transcriptional activation. Insulin activates PI3K signaling, which is required for inhibition of YAP (Figure 8).

To the best of our knowledge, this study is the first to demonstrate that insulin is a negative regulator of YAP. To avoid interference from enhanced phosphorylation and degradation of YAP in the absence of serum, we paid stringent attention to the experimental conditions. Investigations were conducted in cells cultured in serum, but lacking insulin and IGF-1. Also, we used physiologically relevant insulin concentrations and two classic insulin target cell lines derived from skeletal muscle and liver. The YAP transcriptional function was analyzed by two complementary methods, both of which revealed that insulin suppresses YAP function. Extrapolating from our data, it seems reasonable to postulate that insulin resistance would result in enhanced YAP activity. Patients with type 2 diabetes have insulin resistance and >70% of these individuals develop nonalcoholic fatty liver disease (Zanconato et al., 2015). Consistent with our hypothesis, YAP nuclear localization and function are significantly increased in non-alcoholic fatty liver disease (Tan et al., 2015). More recently, reduced YAP Ser\textsuperscript{127} phosphorylation was reported in the liver in a mouse model of diabetes (Zhang et al., 2017). Additionally, several types of cancer are linked to insulin resistance (Orgel and Mittelman, 2013; Matyszewski et al., 2015) and many of these exhibit increased YAP activity (Plouffe et al., 2015). Liver carcinomas from patients with diabetes had less Ser\textsuperscript{127} phosphorylation of YAP than those from nondiabetic individuals (Zhang et al., 2017). These findings suggest that reduced insulin signaling may modulate YAP activity in vivo.

Our study provides insight into a previously uncharacterized mechanism for regulating the Hippo signaling cascade. Insulin modulation of YAP transcriptional function has potential relevance in metabolism, development, and cancer biology. Targeting insulin signaling may be an unexplored therapeutic approach to diseases in which the Hippo pathway is disrupted.

**MATERIALS AND METHODS**

**Materials**

HepG2 and C2C12 cells were obtained from the American Type Culture Collection. All reagents for tissue culture were from Life Technologies. Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore Corp. Anti-YAP (#12395 and #14074), anti-pYAP Ser\textsuperscript{127} (#13008), anti-histone H3 (#9715), anti-phospho-tyrosine (#8954), anti-IR (#3020), and anti-HSP90 (#4877) antibodies were purchased from Cell Signaling. Anti-TEAD1 antibody (#610922) was from BD Pharmingen. Blocking buffer and infrared dye–conjugated (IRDye) antibodies, both anti-mouse and anti-rabbit, were obtained from LI-COR Biosciences. Insulin (#9278-SML) and EGF (#SRP3027-500UG) were purchased from Sigma. IGF-1 was purchased from Fisher Scientific (291G1200). Control (AM4635) and YAP (107951) siRNA were from ThermoFisher. LY294002 (#S1105) was from Fisher Scientific (291G1200). Control (AM4635) and YAP (107951) siRNA were from ThermoFisher. LY294002 (#S1105) was from Selleckchem. Charcoal-striped fetal bovine serum (chFBS) was purchased from Thermo Fisher Scientific (#12676029).

**Cell culture and transfection**

HepG2 and C2C12 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). All the experiments were conducted on cells at ~70–80% confluence. HepG2 were transfected with 10 μl of 10-μM control or YAP siRNA using Lipofectamine RNAiMAX (Thermo Fisher) following the manufacturer’s instructions.
Insulin treatment
Serum starvation induces ubiquitination of YAP and inhibits its transcriptional activity in several cell lines, including HepG2 (Adler et al., 2013). To avoid the suppressive effects of serum starvation on YAP function, instead of serum-free medium, in this study we used 10% charcoal-stripped FBS (chFBS). chFBS contains very low concentrations of hormones and growth factors (Cao et al., 2009). In the chFBS we used, the insulin concentration measured by electrochemiluminescence immunoassay using a Roche COBAS 6000 system was <0.2 μU/ml (1.38 pM) and the concentration of IGF-1 was <25 ng/ml, as measured by a Siemens Immulite assay.

Immunoprecipitation for insulin receptor phosphorylation
C2C12 or HepG2 cells were cultured in 10% chFBS or serum-free DMEM for 16 h. Cells were stimulated with 100 nM insulin, 100 ng/ml IGF-1, or 100 ng/ml EGF for 30 and 60 min, washed in PBS, and lysed in lysis buffer. Lysates were examined by SDS–PAGE and Western blotting using the antibodies indicated in the figure legends.

Dual luciferase-based assay
Equal numbers (0.5 × 10⁶) of cells were cultured in six-well plates for 24 h at 80% confluency in serum-containing media. Cells were transfected using TransIT-2020 transfection reagent (Mirus Bio LLC)
according to the manufacturer's instructions. Briefly, cells were transfected with 2.5 μg of plasmid containing a synthetic YAP/TAZ-responsive promoter driving luciferase expression (8xGTIIC-luciferase, Addgene #34615) and 2.5 μg Renilla luciferase-Pol III (Addgene #37380) using TransIT-2020 transfection reagent (Mirus Bio LLC). After 24 h, samples were incubated without or with 100 nM insulin in the presence of absence of 10 μM LY294002 in chFBS for 16 h. Cells were lysed using passive lysis buffer, and luciferase (YAP/TAZ) and Renilla (control) signals were measured using a dual luciferase reporter assay kit (Promega E1910), essentially as previously described (Sayyedhossein et al., 2016). Luminescence was measured using a 1420 Victor-3 microplate reader (Perkin Elmer). Data are expressed as the ratio of luciferase to the Renilla signal in the same sample.

Quantitative RT-PCR
AMOTL2, ACTA2, and CTGF hnRNA were measured in cells incubated for 24 h without or with 100 nM insulin in chFBS. Where indicated, cells were incubated with 100 ng/ml EGF or 10 μM LY294002 for 24 h. Total RNA was isolated from the cells using an RNA isolation kit (QIAGEN) following the manufacturer's protocol. DNA was stained formed using Duolink In Situ Detection Reagents Red (DUO92008) and 0.25% Triton PBS, and blocked overnight in Sigma) following the manufacturer's protocol. DNA was stained using Hoechst 33342 and coverslips were mounted. Cells were examed using a confocal microscope (LSM880; Carl Zeiss Micros) using a dual luciferase reporter assay kit (Promega E1910), essentially as previously described (Sayyedhossein et al., 2016). Fluorescent images of cells were collected using a confocal microscope (LSM880; Carl Zeiss Micros) and 2 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, essentially as previously described (Sayyedhossein et al., 2016). The primers used were AMOTL2 hRNA, forward primer, 5-AGAGGCGAGTGCTCTCTCAGCTG-3, and reverse primer, 5-GGTCAGTGAGAGCCAACCAG-3; ACTA2 forward primer, 5-AGGGCTGTTTCCCCATCCATCG-3, and reverse primer, 5-TCTCTTGCTCTGGGCTCTAATC-3; β actin, forward primer, 5-TGGTGTGACATATTAGAGGAAAG-3, and reverse primer, 5-GGTCGTAGTCTCTTCCAACAG-3; and reverse primer, 5-GATGGAAGAGGAGGGCTT-3. RT-PCR enzyme activation was initiated for 10 min at 95°C and then amplified for 40 cycles (15 s at 95°C and 1 min at 60°C). All samples were assayed in triplicate, and β-actin was used as an internal control. Results were analyzed using the ΔΔCT method with StepOnePlus software (Applied Biosystems).

Proximity ligation assay
HepG2 and C2C12 cells were plated on coverslips in 24-well dishes at 80% confluence. Insulin (100 nM) in chFBS was added and 0, 2, and 8 h later 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 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. Cells were examined using a confocal microscope (LSM880; Carl Zeiss Microscopy). Fluorescent images of cells were collected using a 63x objective lens (N.A. 1.4), X-Y pixel size 0.07 μm, and an optical slice thickness of 2.0 μm. The number of PLA spots in each cell nucleus was counted using Imaris (v. 8.1.2) image analysis software (Bitplane USA) essentially as described (Sayyedhossein et al., 2016). Briefly, for C2C12 cells, the ImarisCell software module was used to segment individual nuclei based on Hoechst staining and the PLA spots were segmented as individual objects based on the red fluorescence intensity above background. For HepG2 cells, where nuclear segmentation was more difficult, an image mask was established to detect the nuclear blue staining and the number of nuclei was counted manually. The total number of individual PLA objects included within the mask was counted and divided by the number of nuclei to give the average number of PLA spots per nucleus. The same image processing and analysis parameters were used to detect PLA spots for all of the images in the data set.

Miscellaneous methods
Statistical analysis was performed by Student's t test or analysis of variance (ANOVA) as indicated in individual experiments using Prism 6 (Graphpad). Subcellular fractionation was conducted on cells incubated with chFBS-DMEM for 16 h before stimulation with or without 100 nM insulin for 2 h. An NE-PE nuclear and cytoplasmic extraction kit (ThermoFisher; # 78833) was used to obtain cytoplasmic and nuclear fractions following the manufacturer's instruction. Samples were resolved with SDS-PAGE and subjected to Western blotting. All Western blots 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).

ACKNOWLEDGMENTS
We thank members of the Sacks laboratory for helpful discussions and Michael Kruhlak (Experimental Immunology Branch, National Institutes of Health) for expert assistance with confocal microscopy. This work was supported by the National Institutes of Health Intramural Research Program.

REFERENCES
Adler JJ, Johnson DE, Heller BL, Bringman LR, Ranahan WP, Corwell MD, Sun Y, Hudson A, Wells CD (2013). Serum deprivation inhibits the transcriptional co-activator YAP and cell growth via phosphorylation of the 130-kDa isoform of Angiomotin by the LATS1/2 protein kinases. Proc Natl Acad Sci USA 110, 17368–17373.
Avruch J (2007). MAP kinase pathways: the first twenty years. Biochim Biophys Acta 1773, 1150–1160.
Boucher J, Kleinridders A, Kahn CR (2014). Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb Perspect Biol 6, a009191.
Cao Z, West C, Norton-Wenzel CS, Rej R, Davis FB, Davis PJ, Rej R (2009). Effects of resin or charcoal treatment on fetal bovine serum and bovine calf serum. Endoc Res 34, 101–108.
Chen J, Harris RC (2016). Interaction of the EGF receptor and the Hippo pathway in the diabetic kidney. J Am Soc Nephrol 27, 1689–1700.
Choi HJ, Zhang H, Park H, Choi KS, Lee HW, Agrawal V, Kim YM, Kwon YG (2015). Yes-associated protein regulates endothelial cell contact-mediated expression of angiopeptin-2. Nat Commun 6, 6943.
De Meys P (2000). The insulin receptor and its signal transduction network. In: Endotext [Internet], ed. KR Feingold, B Anawalt, A Boyce, G Chrousos, K Dungan, A Grossman, JM Hershman, G Kaltsas, C Koch, P Kopp, et al., South Dartmouth, MA: MDText.com, Inc. www.ncbi.nlm.nih.gov/pubmed/27512793.
DeRani M, Yang J, Shen CH, Peters EC, Fitamant J, Chan P, Hsieh M, Zhu S, Asara JM, Zheng B, et al. (2014). Energy stress regulates hippo-YAP signaling involving AMPK-mediated regulation of angiomotin-like 1 protein. Cell Rep 9, 495–503.
Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, et al. (2011). Role of YAP/TAZ in mechanotransduction. Nature 474, 179–183.
Fernandez R, Tabarini D, Azpiazu N, Frasch M, Schlessinger J (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO J 14, 3373–3384.
Hao F, Xu Q, Zhao Y, Stevens JV, Young SH, Sinnett-Smith J, Rozengurt E (2017). Insulin receptor and GPCR crosstalk stimulates YAP via PI3K and PKD in pancreatic cancer cells. Mol Cancer Res 15, 929–941.

Hatting M, Tavares CDJ, Sharabi K, Rivas AK, Puigserver P (2018). Insulin regulation of glucose homeostasis. Ann NY Acad Sci 1411, 21–35.

Honma K, Kamikubo M, Mochizuki K, Goda T (2017). Insulin-inhibited induction of glucose homeostasis genes, including glutamic pyruvic transaminase 2A, is associated with reduced histone acetylation in a human liver cell line. Metabolism 71, 118–124.

Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ (2008). Essential roles of PI3K-p110beta in cell growth, metabolism and tumorigenesis. Nature 454, 776–779.

Joyal JL, Burks DJ, Pons S, Matter WF, Vlahos CJ, White MF, Sacks DB (1997). Calmodulin activates phosphatidylinositol 3-kinase. J Biol Chem 272, 28183–28186.

Li W, Cooper J, Zhou L, Yang C, Erdjument-Bromage H, Zaggag D, Snuderl M, Ladanyi M, Hanemann CO, Zhou P, et al. (2014). Merlin/NF2 loss-driven tumorigenesis linked to CRL4(CAF1)-mediated inhibition of the hippo pathway kinases Lats1 and 2 in the nucleus. Cancer Cell 26, 48–60.

Li XJ, Leem SH, Park MH, Kim SM (2013). Regulation of YAP through an Akt-dependent process by 3,3’-diindolylmethane in human colon cancer cells. Int J Oncol 43, 1992–1998.

Liu-Chittenden Y, Huang B, Shim JS, Chen Q, Lee SJ, Anders RA, Liu JO, Pan DJ (2012). Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. Gene Dev 26, 1300–1305.

Low BC, Pan CQ, Shivashankar GV, Bershadsky A, Sodul M, Sheetz M (2014). YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth. FEBS Lett 588, 2663–2670.

Matyszewski A, Czamecka A, Kawecki M, Korzen P, Safer IJ, Lukwa W, Szczepanski AM (2015). Impaired glucose metabolism treatment and carcinogenesis. Oncol Lett 10, 591–597.

Miller E, O’ Neill E (2016). TGF-beta targets the Hippo pathway scaffold RASSF1A to facilitate YAP/SMAD2 nuclear translocation. Mol Cell 63, 156–166.

O’ Neill E (2016). TGF-beta targets the Hippo pathway scaffold RASSF1A to facilitate YAP/SMAD2 nuclear translocation. Mol Cell 63, 156–166.

Federico Y, Stambolic V (2015). Obesity and cancer, a case for insulin signaling. J Cell Biochem 116, 2724–2731.

Pan DJ (2012). Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. Gene Dev 26, 1300–1305.

Pefani DE, Pankova D, Abraham AG, Grawenda AM, Vlahov N, Scrace S, Orgel E, Mittelman SD (2013). The links between insulin resistance, diabetes, and cancer. Curr Diab Rep 13, 213–222.

Yu FX, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, Zhao J, Yuan H, Tumaneng K, Li H, et al. (2012). Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. Cell 150, 780–791.

Zhang X, Qiao Y, Chen Y, Zou S, Liu X, Zhu G, Zhao Y, Chen Y, Yu Y, et al. (2017). The essential role of YAP O-GlcNAcylation in high-glucose-stimulated liver tumorigenesis. Nat Commun 8, 15280.

Zhou B, Li, L, Guan KL (2010a). Hippo signaling at a glance. J Cell Sci 123, 237–242.

Zanconato F, Forcaro M, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Bricciato S, Cardonensis M, Piccolo S (2015). Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. Nat Cell Biol 17, 1218–1227.

Zhang X, Qiao Y, Wu Q, Chen Y, Zou S, Liu X, Zhu G, Zhao Y, Chen Y, Yu Y, et al. (2017). The essential role of YAP O-GlcNAcylation in high-glucose-stimulated liver tumorigenesis. Nat Commun 8, 15280.

Zhou B, Li, L, Guan KL (2010b). The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev 24, 862–874.

Zhou B, Li, L, Tumaneng K, Wang CY, Guan KL (2011). Angiogenin is a novel Hippo pathway component that inhibits YAP oncoprotein. Genes Dev 25, 51–63.

Zhou B, Li, L, Tumaneng K, Wang CY, Guan KL (2010c). A coordinated phosphorylation by Lats and CLK1 regulates YAP stability through SCF(betatorcPC). Genes Dev 24, 72–85.

Zhu G, Cao X, Dai Q, Zhang B, Huang J, Xiong S, Zhang Y, Chen W, Yang J, Li H (2015). A novel role for microRNA-129–5p in inhibiting ovarian cancer cell proliferation and survival via direct suppression of transcriptional co-activators YAP and TAZ. Oncotarget 6, 8676–8686.

Zhao B, Li, L, Wang CY, Guan KL (2010a). Hippo signaling at a glance. J Cell Sci 123, 237–242.

Zhou B, Li, L, Guan KL (2010b). The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev 24, 862–874.

Zhou B, Li, L, Wang CY, Guan KL (2011). Angiogenin is a novel Hippo pathway component that inhibits YAP oncoprotein. Genes Dev 25, 51–63.

Zhou B, Li, L, Tumaneng K, Wang CY, Guan KL (2010c). A coordinated phosphorylation by Lats and CLK1 regulates YAP stability through SCF(betatorcPC). Genes Dev 24, 72–85.

Zhu G, Cao X, Dai Q, Zhang B, Huang J, Xiong S, Zhang Y, Chen W, Yang J, Li H (2015). A novel role for microRNA-129–5p in inhibiting ovarian cancer cell proliferation and survival via direct suppression of transcriptional co-activators YAP and TAZ. Oncotarget 6, 8676–8686.