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

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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.**

Monitoring Editor
Asma Nusrat
University of Michigan

Received: Apr 10, 2019
Revised: Oct 8, 2019
Accepted: Oct 29, 2019

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; Low *et al.*, 2014). When Hippo is on, YAP and its closely related paralogue TAZ become phosphorylated. Phosphorylated 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; Low *et al.*, 2014). In the nucleus, YAP interacts with several transcription factors, particularly members of the TEA 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

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E19-04-0205) on December 6, 2019.

The authors declare no conflict of interest.

Author contributions: S.S., A.C.H., and D.B.S. designed the study; S.S. and A.C.H. conducted experiments; S.S., A.C.H., and D.B.S. analyzed data; S.S., A.C.H., and D.B.S. wrote the manuscript.

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Abbreviations used: AMOTL2, angiomotin-like 2; AMPK, AMP-activated protein kinase; chFBS, charcoal-stripped fetal bovine serum; CTGF, connective tissue growth factor; EGF, epidermal growth factor; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; IGF-1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositide-3-kinase; PLA, proximity ligation assay; pYAP, phosphorylated YAP; TEAD, TEA domain family member; TGFβ, transforming growth factor β; YAP, yes-associated protein.

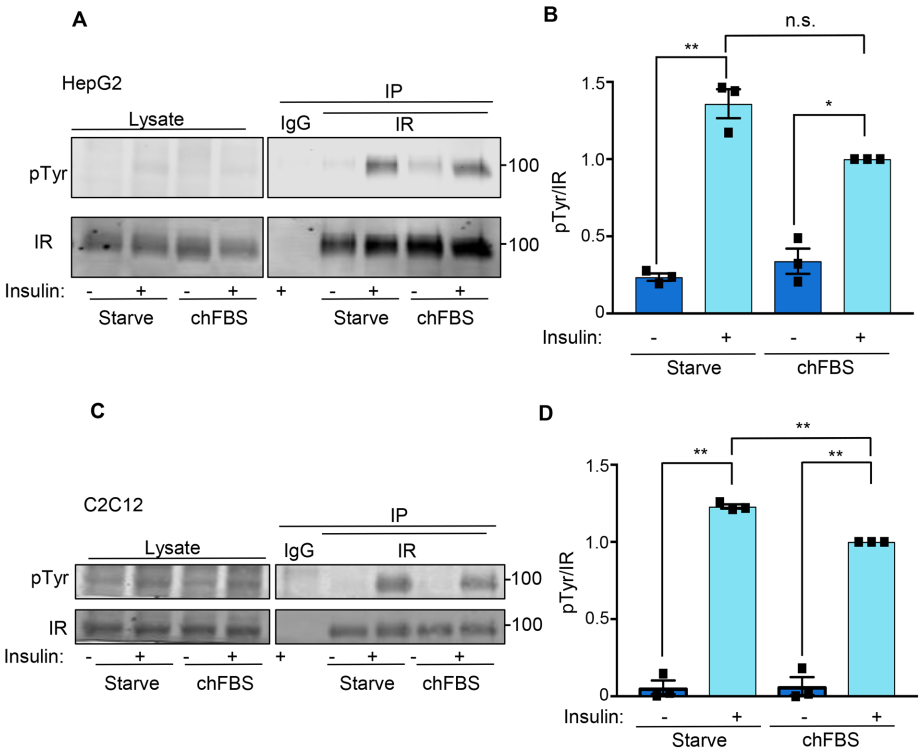

FIGURE 1: Insulin activates IR in chFBS-containing medium. (A) HepG2 cells were cultured in serum-free medium (starve) or medium containing charcoal-stripped FBS (chFBS) for 16 h and then incubated without (–) or with (+) 100 nM insulin for 5 min. IR was immunoprecipitated (IP) from cell lysates, and samples were analyzed by SDS–PAGE and Western blotting with anti-IR and anti-phosphotyrosine (pTyr) antibodies. IgG was used as a negative control for IP. An aliquot of lysate not subjected to immunoprecipitation was loaded as control. The positions of migration of molecular weight markers are indicated on the right. (B) The phosphotyrosine bands were quantified with Image Studio 2.0 (LI-COR Biosciences) and corrected for the amount of IR immunoprecipitated in the corresponding sample. Data are expressed as means ± SEM (error bars; $n = 3$) with insulin-treated samples in chFBS set as 1. (C, D) C2C12 cells were cultured in serum-free or chFBS-containing medium for 16 h and stimulated without (–) or with (+) 100 nM insulin for 5 min. Samples were processed as described for HepG2 cells by Western blotting (C) and quantification (D) ($n = 3$). *$p < 0.05$, **$p < 0.01$, ANOVA; n.s., not significant.

critical for its function (Zhao *et al.*, 2007, 2010b; Sudol *et al.*, 2012). Proteins, such as angiomotin, interact with YAP in a phosphorylation-independent manner, sequestering YAP in the cytoplasm and inhibiting its function (Zhao *et al.*, 2011). In the phosphorylation-dependent mechanism, selected stimuli enhance phosphorylation of YAP (notably on Ser^{127}), which promotes YAP cytoplasmic retention and inhibits its transcriptional functions. Recent findings suggest that YAP can be regulated by metabolic and nutrient-sensing pathways (Santinon *et al.*, 2016). Nutrient deprivation promotes Ser^{127} phosphorylation and ubiquitination of YAP (Adler *et al.*, 2013) and also inhibits YAP function (Miller *et al.*, 2012; Yu *et al.*, 2012). For example, YAP is suppressed when phosphorylated by AMP-activated protein kinase (AMPK) in response to glucose starvation (Mo *et al.*, 2015; Wang *et al.*, 2015). The status of nutrition, therefore, is an important factor 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) (Urtasun *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 10% 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 Ser^{127}. C2C12 cells were

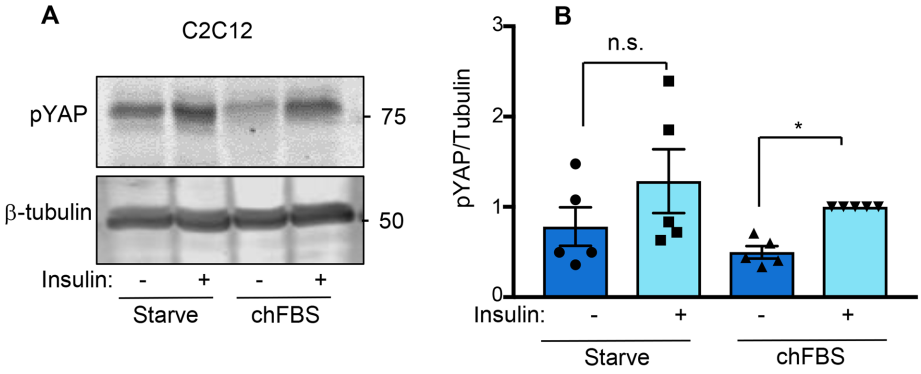

FIGURE 2: Insulin promotes YAP phosphorylation in chFBS-containing medium. (A) C2C12 cells were incubated in serum-free (starve) or chFBS-containing medium for 16 h and stimulated without (–) or with (+) 100 nM insulin for 5 min. Cell lysates were resolved by SDS–PAGE and Western blots were probed with antibodies to phospho-YAP Ser^{127} (pYAP) and to β-tubulin. (B) The pYAP bands were quantified with Image Studio 2.0 (LI-COR Biosciences) and corrected for β-tubulin (loading control) in the corresponding sample. Data are expressed as means ± SEM (error bars; $n = 5$) with insulin-treated samples in chFBS set as 1, *$p < 0.05$, ANOVA; n.s., not significant.

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 differences 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} to 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 hnRNA by 70% in HepG2 cells cultured in chFBS after 24 h (Figure 5C). Similarly, AMOTL2 and CTGF hnRNA 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.

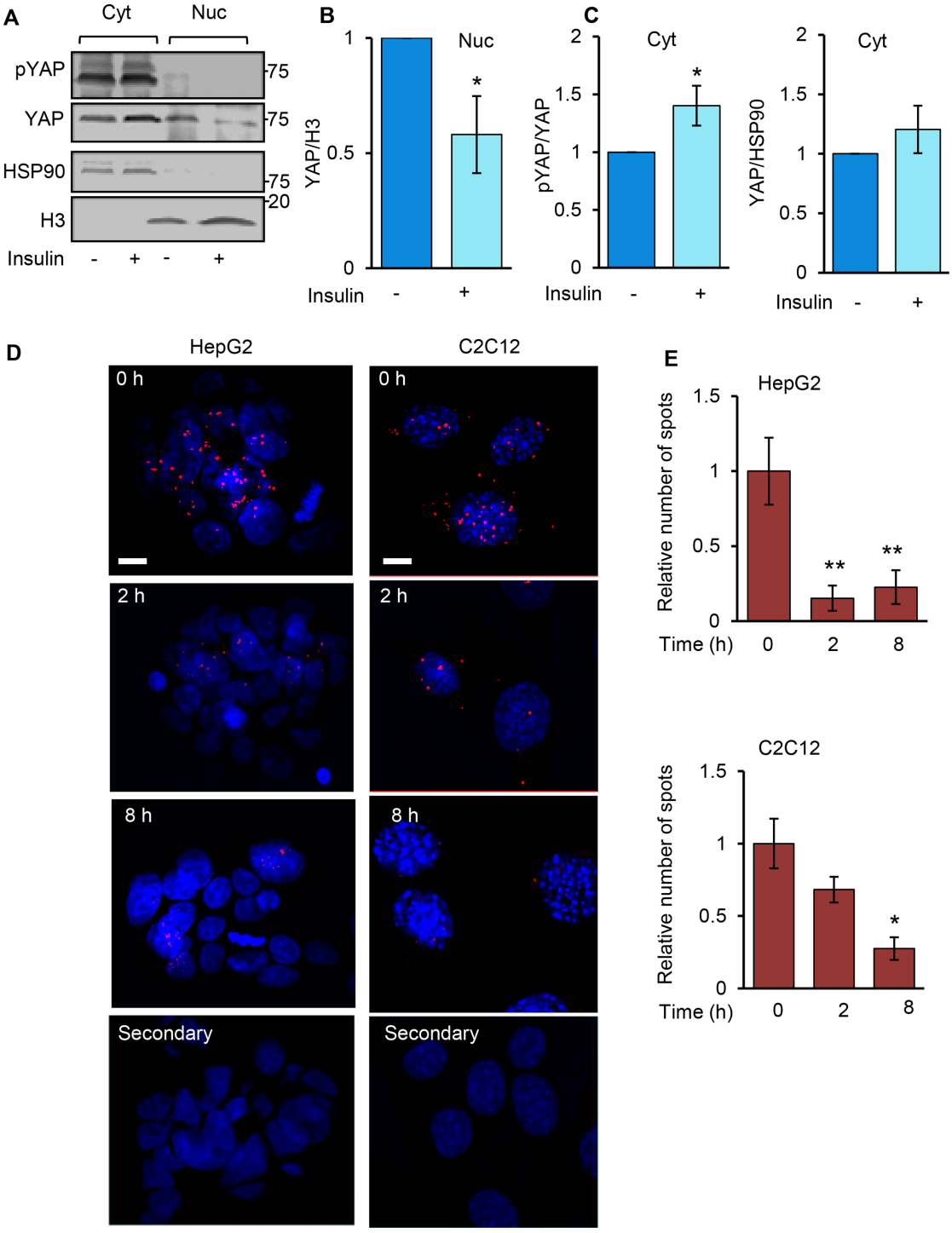

FIGURE 3: Insulin modulates YAP subcellular localization. (A) HepG2 cells, cultured in chFBS medium, were treated without (–) or with (+) 100 nM insulin for 2 h. Cell lysates were separated into cytoplasmic (Cyt) and nuclear (Nuc) fractions as described under *Materials and Methods*. Equal amounts of 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 $n = 8$, pYAP/YAP; $n = 5$, YAP/HSP90; $n = 7$, YAP/H3) 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*. Red spots indicate positive PLA. 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 ($n \geq 117$) and C2C12 ($n \geq 45$) 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.

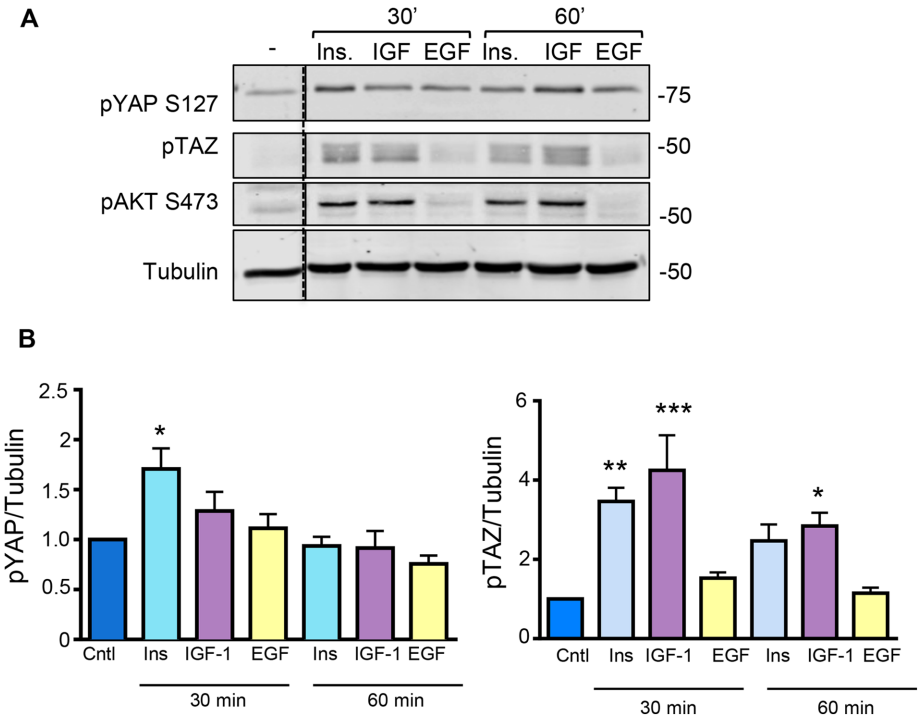

FIGURE 4: Insulin increases YAP/TAZ phosphorylation in C2C12 cells. (A) C2C12 cells were cultured in chFBS-containing medium for 16 h and stimulated with either 100 nM insulin (Ins), 100 ng/ml IGF-1, or 100 ng/ml EGF for 30 or 60 min. Unstimulated cells were used as controls. Equal amounts of protein lysate from cells were analyzed by Western blotting with antibodies that recognize pAKT S^{473} or both phospho-YAP Ser^{127} (pYAP) and phospho-TAZ Ser^{89} (pTAZ). β-Tubulin is the loading control. Representative Western blots are shown. The dotted line indicates where irrelevant lanes were removed from the blot. The positions of migration of molecular weight markers are indicated at the right. All lanes were scanned at the same intensity. (B) The graphs are derived from quantification of Western blots from four and six independent experiments for pYAP and pTAZ, respectively, and represent means ± SEM with vehicle control (Cntl) set as 1. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$, 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 Ser^{127} 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 Ser^{127}, 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

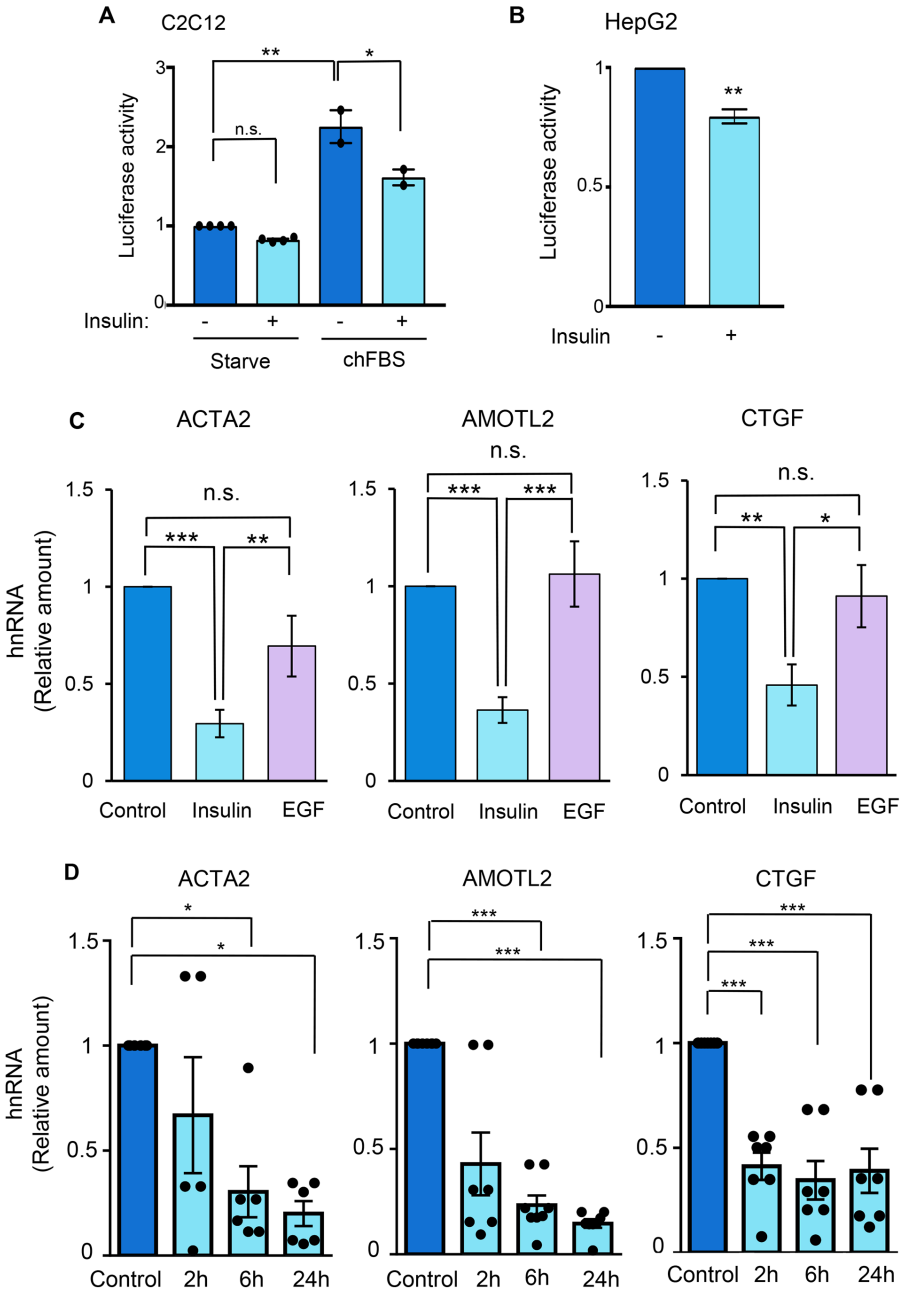

FIGURE 5: Insulin suppresses YAP-mediated gene transcription. (A) C2C12 cells were transfected with TEAD reporter luciferase plasmid and *Renilla* luciferase–Pol III constructs. After 24 h, cells were cultured in serum-free (starve) or chFBS-containing medium and incubated without (–) or with (+) 100 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. *$p \leq 0.01$, **$p \leq 0.0001$, 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), $n = 9$. **$p \leq 0.0001$, Student's *t* test. (C) 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. (D) HepG2 cells, cultured in chFBS-containing medium, were incubated for 0, 2, 6, or 24 h with 100 nM insulin. 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 Ser^{397}, Ser^{94}, and Ser^{61} 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 Ser^{127} 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 Ser^{127} 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

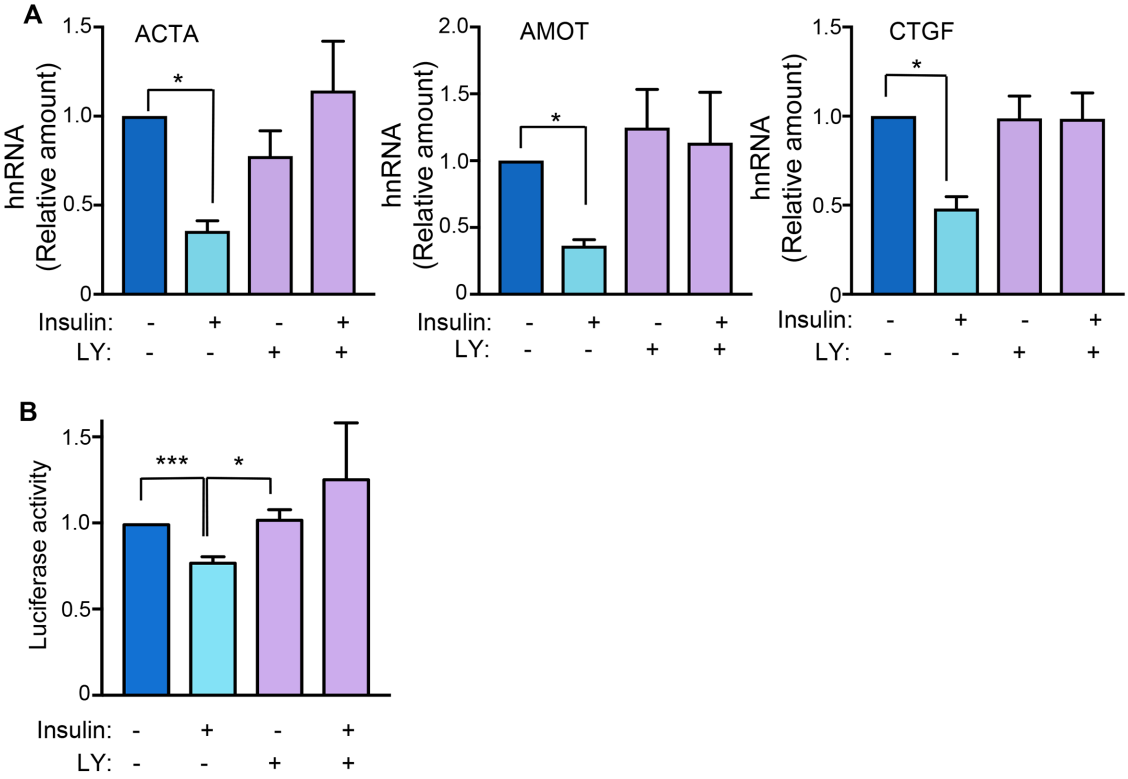

FIGURE 6: Inhibition of the PI3K pathway prevents insulin from inhibiting YAP. (A) HepG2 cells, cultured in chFBS-containing medium, were incubated without (–) or with (+) 100 nM insulin and/or 10 μM PI3K inhibitor LY294002 (LY) for 24 h. ACTA, AMOTL2, and CTGF hnRNA were measured by quantitative RT-PCR. The amount of hnRNA was corrected for β-actin hnRNA in the same sample. hnRNA in vehicle-treated cells was set as 1. The data represent the means ± SEM (error bars) of at least four independent experiments, each performed in triplicate. *$p \leq 0.05$, ANOVA. (B) HepG2 cells, cultured in chFBS-containing medium, were transfected with TEAD reporter luciferase plasmid and *Renilla* luciferase–Pol III constructs. Cells were incubated without (–) or with (+) 100 nM insulin and/or 10 μM PI3K inhibitor LY294002 (LY) for 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 vehicle-treated cells. Data are expressed as mean ± SEM (error bars) of at least six independent experiments, each performed in duplicate. *$p \leq 0.05$, ***$p \leq 0.001$, ANOVA.

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.

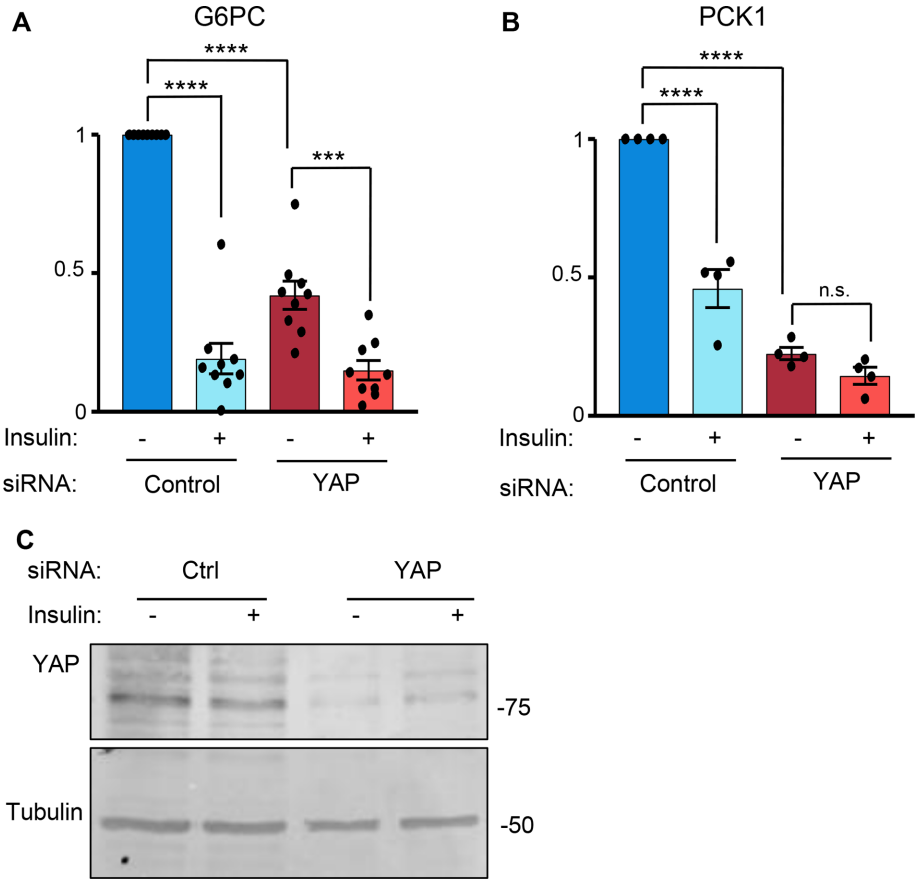

FIGURE 7: YAP regulates expression of gluconeogenesis genes. HepG2 cells were transfected with control siRNA or YAP-targeted siRNA. At 48 h posttransfection, cells were incubated without (–) or with (+) 100 nM insulin for 6 h and RNA was harvested. RT-PCR was performed to analyze changes in gluconeogenesis genes G6PC (nine independent experiments performed in triplicate) (A) and PCK1 (four independent experiments in triplicate) (B). 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). ***$p \leq 0.0001$, ****$p \leq 0.0003$, ANOVA. n.s., not significant. (C) A representative Western blot showing expression of YAP and β-tubulin (loading control).

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[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[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[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[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 Biosciences. Anti-IRβ (sc-711) and anti-YAP (sc-101199) were from Santa Cruz Biotechnology. Hoechst 33342 (#561908) was purchased from BD Pharmingen. Blocking buffer and infrared dye–conjugated (IRDye) antibodies, both anti-mouse and anti-rabbit, were obtained from LI-COR Biosciences. Insulin (#I9278-5ML) 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 Selleckchem. Charcoal-stripped 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.

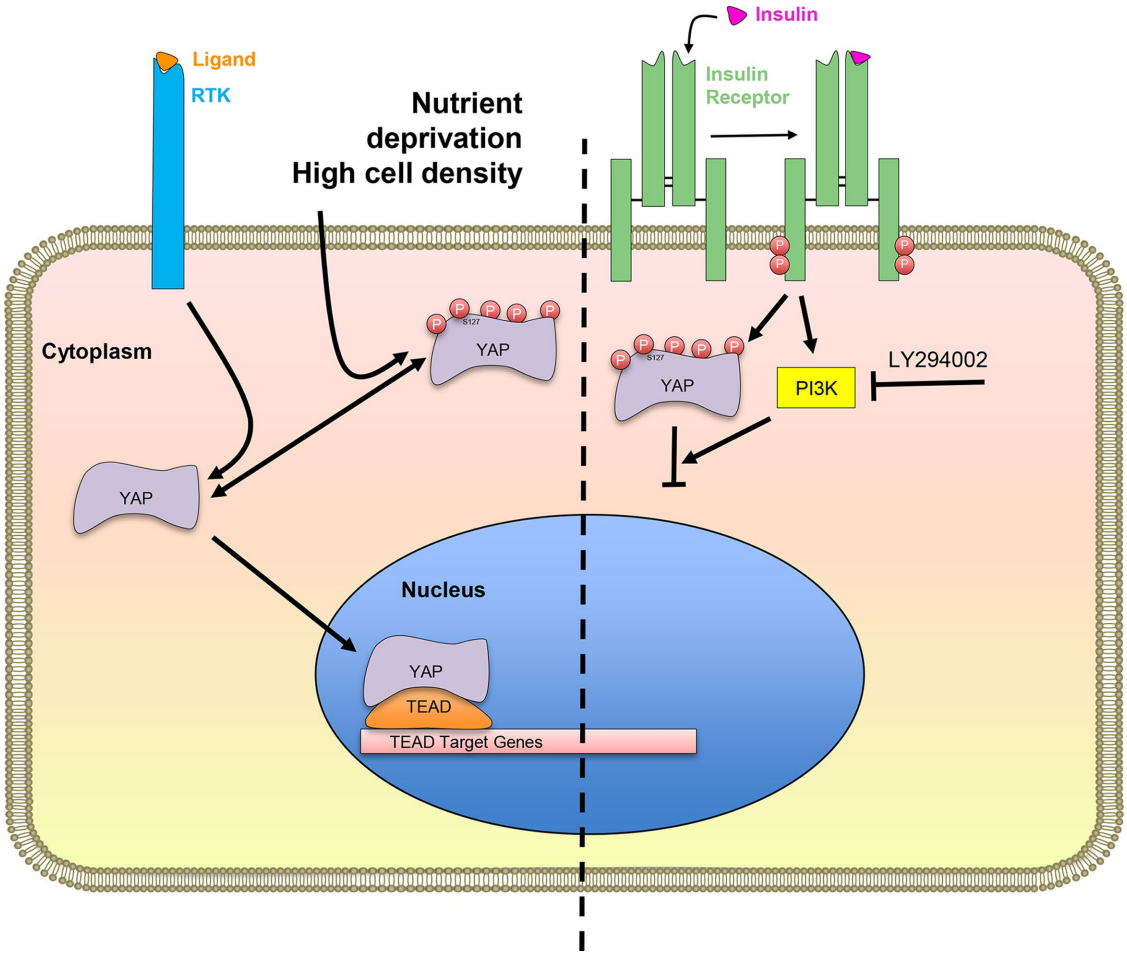

FIGURE 8: Model depicting the regulation of YAP transcriptional activity by growth factors and insulin. Nutrient deprivation and high cell density promote YAP phosphorylation and cytoplasmic retention. Under these conditions, activation of receptor tyrosine kinases (RTK; e.g., EGF) by growth factors induces dephosphorylation of YAP and its translocation to the nucleus. In the nucleus, YAP interacts with the TEAD family of transcription factors to drive gene expression. In contrast, in the presence of nutrients, insulin enhances YAP phosphorylation in the cytoplasm and prevents its nuclear translocation and YAP-mediated transcription. Inhibition of PI3K attenuates the suppressive effect of insulin on YAP function.

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 electrochemiluminescense 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 for 0 or 5 min, washed with phosphate-buffered saline (PBS), and then lysed in 500 µl lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% (vol/vol) Triton X-100 with protease and phosphatase inhibitor added. Lysates were subjected to sonication, followed by centrifugation at 16,000 × *g* at 4°C. Lysates were precleared with glutathione–Sepharose for 1 h. Equal concentrations of protein lysate were incubated with 0.5 µg anti-IR antibody or normal rabbit immunoglobulin G (IgG) for 2 h, protein A-Sepharose was added for 4 h, and beads were washed five times. Immunoprecipitates were eluted in sample buffer. Lysate samples were taken before addition of antibody. Samples were resolved by SDS–PAGE and Western blotting with anti-phosphotyrosine and anti-IR antibodies.

Yes-associated protein phosphorylation

C2C12 cells were grown to 80% confluence and cultured in DMEM without serum or with 10% chFBS 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 (0.5×10^6) of cells were cultured in six-well plates for 24 h at 80% confluence 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 (Sayedyahossein *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) 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 (Sayedyahossein *et al.*, 2016). The primers used were AMOTL2 hnRNA, forward primer, 5-AGAGATTGGAATCGGCAAAC-3, and reverse primer, 5-TTCTCCTGTTCCTGTTGCTG-3; CTGF forward primer, 5-CAAGGACCGCACAGCAGTT-3, and reverse primer, 5-AGAACAGGCGCTCCACTCTG-3; ACTA2 forward primer, 5-AGGGCTGTTTTCCCATCCATCG-3, and reverse primer, 5-TCTCTTGCTCTGGGCTTCATCC-3; β actin, forward primer, 5-TGCGTGACATTAAGGAGAAG-3, and reverse primer, 5-GCTCGTAGCTCTTCTCCA-3; G6PC forward primer, 5-CCAAGACTCCCAGGACTGGTTC-3, and reverse primer, 5-CCATGGCATGGCCAGAGGG-3; and PCK1 (Honma *et al.*, 2017) forward primer, 5-GGTCAGTGAGAGCCAACCAG-3, and reverse primer, 5-AGATGGAGGAAGAGGGCATT-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 63× 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 (Sayedyahossein *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.

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