Published in final edited form as:

Hepatology. 2017 December ; 66(6): 2029–2041. doi:10.1002/hep.29373.

YAP suppresses gluconeogenic gene expression via PGC1 α

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Abstract

Cell growth and proliferation are tightly coupled to metabolism, and dissecting the signaling molecules which link these processes is an important step towards understanding development, regeneration and cancer. The transcriptional regulator Yes-associated protein 1 (YAP) is a key regulator of liver size, development and function. We now show that YAP can also suppress gluconeogenic gene expression. *Yap* deletion in primary hepatocytes potentiates the gluconeogenic gene response to glucagon and dexamethasone, whereas constitutively active YAP suppresses it. The effects of YAP are mediated by the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1 α). YAP inhibits the ability of PGC1 α to bind to and activate transcription from the promoters of its gluconeogenic targets and the effects of YAP are blunted upon knockdown of PGC1 α . *In vivo*, constitutively active YAP lowers plasma glucose levels and increases liver size. YAP therefore appears to reprogram cellular metabolism, diverting substrates away from the energy consuming process of gluconeogenesis, and towards the anabolic process of growth.

Keywords

Hippo pathway; Gluconeogenesis; Development; Hepatocellular carcinoma; Glucagon signaling

Introduction

Yes associated protein 1, or YAP, is a key effector of the Hippo signaling pathway, triggering growth in response to a loss of cell-cell contacts (1, 2). YAP regulates gene expression to coordinate proliferation, differentiation and apoptosis pathways (1, 2). In the liver, YAP activation produces hepatomegaly, whereas *Yap* deletion leads to the development of smaller, dysfunctional livers (3). YAP is also required for the regenerative response to hepatic injury (4) and frequently activated in pathological growth, such as hepatocellular carcinoma (1, 2).

Growth and proliferation require adenosine triphosphate (ATP), reducing equivalents and metabolic building blocks (5). Genes that induce growth appear to rewire metabolic pathways to produce or acquire these necessary substrates (6). Consistent with this, YAP has recently been shown to drive expression of glutamine synthetase, which supports the biosynthesis of nucleotide building blocks (7).

The liver, due to its central role in maintaining whole body homeostasis, faces a unique metabolic challenge. In the fasted state, the liver supplies glucose to the brain and other tissues, to prevent the development of severe hypoglycemia and death. This altruistic response of the liver comes at a cost: each molecule of glucose generated by the hepatocyte consumes six ATP equivalents, two nicotinamide adenine dinucleotide hydride (NADH), and two molecules of pyruvate. We would thus expect gluconeogenesis to be tightly regulated in the proliferating hepatocyte, allowing it to balance the needs of the whole organism for glucose with its own needs for anabolic substrates.

At the hormonal level, gluconeogenesis is activated by several factors, including glucagon. Glucagon acts by binding its receptor, a G-protein coupled receptor (GPCR), to activate adenylate cyclase and increase cAMP levels, which in turn activate protein kinase A (PKA). This ultimately activates a transcriptional network involving numerous transcription factors and coactivators, including peroxisome proliferator-activated receptor gamma coactivator 1 (PPARGC1 α , or PGC1 α), to induce the gluconeogenic genes.

Interestingly, YAP is inhibited by glucagon (8, 9). Glucagon, via PKA, activates the large tumor suppressor homolog (LATS) 1/2 (8), a core component of the Hippo signaling pathway. LATS1/2 phosphorylates YAP on S127, which promotes the retention of YAP in the cytoplasm, where it is unable to regulate transcription (10–12).

We show here that YAP also suppresses expression of the gluconeogenic genes, at least in part by suppressing the transcriptional coactivator PGC1 α . This is associated *in vivo* with decreased plasma glucose levels, and suggests that YAP shunts substrates away from the energy consuming processes of gluconeogenesis and towards anabolic pathways.

Experimental Procedures and Materials

Please also see Extended Experimental Procedures and Materials in the online Supplement.

Animals and Treatments

All mice were male, fed a standard chow diet, maintained on a 12-h light/dark cycle with free access to food and water unless otherwise indicated, and sacrificed at 2:00 pm in the non-fasted state. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children's Hospital Boston.

YAP^{flox/flox} mice and rtTA^{flox/flox}::TetO-YAP mice have been described previously (13, 14). To knockout YAP, five- to seven-week-old YAP^{flox/flox} mice were injected with adeno-associated viral vectors (AAV, purchased from the University of Pennsylvania Vector Core) expressing Cre recombinase (AAV8-*TBG* Cre, or AAV-Cre) or green fluorescent protein (AAV8-*TBG* GFP, or AAV-GFP) under the control of a liver specific thyroxine binding globulin (*TBG*) promoter. Adeno-associated viruses were administered via retro-orbital injection at a dose of 1.5×10^{11} genome copies per mouse. Two weeks after AAV injection, mice were subjected to an intraperitoneal glucose tolerance test (IGTT) as previously described (15). Mice were fasted for 16 hours and then given 1 mg glucose/kg body weight intraperitoneally at time zero; blood glucose levels were measured using a glucometer. Five weeks after AAV injection mice were sacrificed. To induce the expression of YAP S127A, rtTA^{flox/flox}::TetO-YAP mice were crossed with mice expressing Cre driven by an *albumin* (*Alb*) promoter, yielding *Alb*-Cre::rtTA^{flox/flox}::TetO-YAP mice. At seven to nine weeks of age, the drinking water was supplemented or not with doxycycline (1 mg/ml), and administered ad libitum for the remainder of the experiment. Mice were subjected to an IGTT six days after beginning doxycycline supplementation and sacrificed nine days after beginning doxycycline supplementation.

Primary Hepatocyte Studies

Primary mouse hepatocytes were isolated from eight- to twelve-week-old male YAP^{flox/flox} or *Alb*-Cre::rtTA^{flox/flox}::TetO-YAP, as described previously (15). After plating the cells, YAP S127A was induced in primary hepatocytes from *Alb*-Cre::rtTA^{flox/flox}::TetO-YAP mice by treating with 100 ng/mL doxycycline overnight.

For adenoviral mediated overexpression, adenoviruses were added to cell culture media at MOI 10 to 50 for 24 hours prior to harvest. Alternatively, for adenoviral mediated knockdown of PGC1 α , hepatocytes from *Alb*-Cre::rtTA^{flox/flox}::TetO-YAP were isolated, incubated with Ad-shPGC1 α or Ad-shLamin for 48 hours prior to harvest, and treated with Ad-GFP or Ad-YAP S127A for 24 hours prior to harvest. Adenovirus expressing YAP S127A (Ad-YAP S127A) was previously described (16); adenoviruses expressing PGC1 α (Ad-PGC1 α), LacZ (Ad-LacZ), and an shRNA against PGC1 α (Ad-shPGC1 α) were gifts from Dr. Pere Puigserver (Dana Faber Cancer Institute) (17); adenovirus against human lamin (Ad-shLamin), used as a control, was previously described (15). Adenoviruses expressing GFP (Ad-GFP) and Cre (Ad-Cre) were purchased from ViraQuest. All viruses were amplified in 293A cells per manufacturer's instructions.

The day of harvest the media was replaced with fresh Williams' E medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin and 10% FBS (without doxycycline or adenovirus). Cells were treated with or without 10 nM glucagon (Sigma) for 1.5 hours, 1 μ M 8-Br-cAMP (Sigma) for 1.5 hours, 10 μ M forskolin (Sigma) for 1.5 hours, or 200 nM dexamethasone (Sigma) for 6 hours. All cells were harvested at the same time, at the conclusion of the experiment.

Chromatin Immunoprecipitation (ChIP)

ChIP assays using primary hepatocytes and liver tissues were performed as previously described with minor modifications (18, 19). The immunoprecipitated DNA as well as 10% of the pre-cleared chromatin (input DNA) were subjected to real-time PCR using Power SYBR Green PCR Master Mix (Life Technologies), in triplicate. For each immunoprecipitate, we calculated the relative enrichment as 2^{-Ct} where Ct was calculated as the average Ct value obtained from the immunoprecipitate DNA minus the average Ct value obtained from the input DNA. The relative enrichment of replicate immunoprecipitates (2–4 per group) was averaged, and the results expressed in Arbitrary Units, with the IgG control for each primer pair set to 1. Representative results of two to four independent experiments are shown. The antibodies used in the ChIP assays are listed in Supplemental Table 2 and real-time PCR primers are listed in Supplemental Table 3.

Microarray Studies

Generation of the YAP Target Gene Set.—To identify hepatic YAP targets in a unbiased manner, we analyzed microarray data obtained from the livers of control mice, and mice expressing YAP S127A in their livers for 1 or 6 weeks (GEO accession GSE55559 (2)). For each probeset, expression at each time point was compared to expression in the control using moderated t-tests from the limma package (9); p-values from both time points were then combined using the z.transform method in the combine.test function of the survcomp package (10).

Gene Set Enrichment Analysis.—Microarray data from liver samples obtained from patients with hepatocellular carcinoma were retrieved (GSE64041, (20)). We removed the unpaired normal samples and used the paired biopsies of tumor and surrounding non-tumor regions from the livers of 60 patients with hepatocellular carcinoma. We calculated $\log_2$ -ratios of the expression in the tumor versus the surrounding non-tumor region for each probeset. To determine whether the YAP target gene set (generated as described above) was significantly upregulated and the gluconeogenic genes (based on the gluconeogenic gene set defined in HumanCyc (21)) were significantly altered in the tumors, we used the fry function implementing the ROAST method in the limma package, which can exploit the paired nature of the dataset (22). We examined the p-value of the gluconeogenic gene set using the mixed (non-directional) test, whereas we confirmed that the YAP target gene set was significantly upregulated.

Correlation Analysis.—For each patient, we averaged the $\log_2$ -ratios of the expression in the tumor versus the surrounding non-tumor region for all of the genes in the YAP target gene set as well as all of the genes in the gluconeogenic gene set, and plotted them against

one another; the correlation coefficients and their p-values were calculated using the `cor.test` function. All bioinformatics analyses were done in the R software (23) and are available upon request.

Statistical Analysis

In vitro Studies—Gene expression studies were performed with triplicate wells. Bars and error bars correspond to the mean and SEM, respectively. Representative results of two to five independent experiments are shown. Differences between groups were assessed by a two-tailed unequal variance Student's t-test.

Mouse Studies—Animals were randomized to control and experimental groups. Bars and error bars correspond to the mean and SEM, respectively. Representative results of two to four independent experiments are shown. Differences between groups were assessed by a two-tailed unequal variance Student's t-test.

Results

YAP suppresses the induction of the gluconeogenic enzymes

To determine how YAP affects the gluconeogenic gene response, we studied primary hepatocytes with either constitutively active YAP or knockdown of YAP. For constitutive activation of YAP, a mutant form of YAP, resistant to phosphorylation on serine 127 (YAP S127A), was expressed under a doxycycline inducible-promoter. These hepatocytes were isolated from the livers of *Alb-Cre::rtTA^{flox/flox}::TetO-YAP* mice, which harbor a transgene encoding a mutant form of human YAP, in which serine 127 is mutated to alanine, under the control of the Tetracyclin-On (TetO) promoter (14): Cre recombinase, under the *albumin* (*Alb*) promoter, drives the reverse tetracycline transactivator (rtTA); rtTA, in the presence of doxycycline, drives YAP S127A (Supplemental Fig. S1A). Thus, doxycycline treatment of these hepatocytes increased total YAP levels (Fig. 1A).

In control cells, glucagon induced the gluconeogenic genes, glucose-6-phosphatase catalytic subunit (*G6pc*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*) more than 500-fold (Fig. 1B). In cells treated with doxycycline to induce YAP S127A, however, the effects of glucagon were almost entirely abolished (Fig. 1B). Consequently, YAP S127A suppressed *G6pc* and *Pck1* more than 99% in the presence of glucagon. In parallel, YAP S127A abolished the ability of the cAMP analogue, 8Br-cAMP, and activator of PKA, forskolin, to induce the gluconeogenic genes (Fig. 1C, D).

Glucocorticoids also induce expression of *G6pc* and *Pck1*. Thus, treatment of hepatocytes with dexamethasone, a synthetic glucocorticoid that activates the glucocorticoid receptor, also strongly induced the gluconeogenic genes. Interestingly, YAP S127A was able to abolish the induction of the gluconeogenic genes in response to dexamethasone, and YAP S127A reduced *G6pc* and *Pck1* by 80–90% in dexamethasone treated cells (Fig. 1E). Similar effects of YAP S127A on the gluconeogenic gene response to glucagon, forskolin and dexamethasone were obtained using an adenovirus encoding YAP S127A, rather than the doxycycline inducible transgene, to express YAP (Supplemental Fig. S1B, C).

Conversely, we examined the ability of glucagon to activate transcription of *G6pc* and *Pck1* in hepatocytes with knockdown of YAP. In this case, cells from *Yap^{flox/flox}* mice treated *in vitro* with adenovirus encoding either GFP or Cre recombinase. Knockdown of YAP was confirmed by reduced YAP protein and mRNA levels after Ad-Cre infection (Fig. 2A). We found that glucagon stimulation of *G6pc* and *Pck1* was significantly potentiated by the knockdown of YAP. Thus, in cells treated with glucagon (Fig. 2B), 8Br-cAMP (Fig. 2C), forskolin (Fig. 2D), or dexamethasone (Fig. 2E), YAP deletion increased expression of *G6pc* and *Pck1* by 25–300%.

YAP suppresses *Pck1* and *G6pc* via PGC1 α

PGC1 α is a powerful activator of the gluconeogenic genes and is induced by glucagon. Thus, glucagon increased *Pgc1a* mRNA levels ten- to twenty-fold in control hepatocytes. This effect was abolished by YAP S127A and potentiated by YAP knockdown (Fig. 3A). Taken together, these results indicate that YAP can suppress *Pgc1a* in the presence of glucagon.

To determine whether constitutive expression of PGC1 α could overcome the effects of YAP S127A on the gluconeogenic genes, we treated cells with adenovirus encoding either PGC1 α or LacZ. Adenoviral treatment markedly increased *Pgc1a* mRNA levels (Fig. 3B), as well as protein levels (Supplemental Fig. S2A). Interestingly, the ability of PGC1 α to activate expression of the gluconeogenic genes was blunted by YAP S127A (Fig. 3B). Thus, YAP S127A impaired the ability of PGC1 α to activate the gluconeogenic genes even when *Pgc1a* mRNA levels were not different (Fig. 3B).

PGC1 α is also known to activate transcription of the genes involved in oxidative phosphorylation (including cytochrome c, or *Cyts*, and cytochrome c oxidase subunit 5b, or *Cox5b*); the tricarboxylic acid (TCA) cycle (including isocitrate dehydrogenase 3 α , or *Idh3a*); and fatty acid oxidation (including very long chain acyl-CoA dehydrogenase, or *Vlcad*) (24). Consistent with this, PGC1 α overexpression increased these targets by three- to ten-fold in control cells (Fig. 3B). Interestingly, YAP S127A had no effect on the ability of PGC1 α to induce *Cyts* and reduced the other non-gluconeogenic targets of PGC1 α by only 10–20%. Thus, YAP S127A appeared to preferentially impair PGC1 α 's activation of its gluconeogenic targets.

Conversely, the deletion of YAP preferentially potentiated PGC1 α activation of its gluconeogenic targets. Thus, PGC1 α increased expression of *G6pc* and *Pck1* by two- to four-fold in control cells but six to ten-fold in hepatocytes with knockout of YAP (Fig. 3C). On the other hand, with the exception of *Idh3a*, the deletion of YAP had a modest effect on the non-gluconeogenic targets of PGC1 α (Fig. 3C).

PGC1 α induces expression of the gluconeogenic genes by coactivating numerous transcription factors, including hepatic nuclear factor 4 alpha (HNF4 α). YAP did not alter levels of HNF4 α (Supplemental Fig. S2B), but did appear to reduce the ability of PGC1 α to coactivate HNF4 α on the *PCK1* promoter: luciferase reporters in HepG2 cells showed that PGC1 α in combination with HNF4 α was able to stimulate *PCK1* transcription by three-fold, but that this was reduced 50% by YAP S127A (Fig. 3D). Similarly, PGC1 α in combination

with glucocorticoid receptor, which is also coactivated by PGC1 α , and dexamethasone, was able to induce *PCK1* transcription six-fold, but this was again reduced 50% by YAP S127A (Fig. 3D).

To further examine the effects of YAP on the ability of PGC1 α to activate transcription, we performed chromatin immunoprecipitation (ChIP) assays using control antibodies (IgG) or antibodies against PGC1 α . Thus, primary hepatocytes from *Alb-Cre::rtTA^{flox/flox}::TetO-YAP* were infected with adenovirus encoding PGC1 α or LacZ and treated with or without doxycycline to induce YAP S127A. PGC1 α was associated with regions within the *G6pc* (–310 to –231 and –215 to –111) and *Pck1* promoters (–700 to –599 and –615 to –517) but not regions outside of the promoter (*Pck1*, –3678 to –3564). The association of PGC1 α with the *G6pc* promoter, as well as the *Pck1* promoter, was reduced 75% in the presence of YAP S127A (Fig. 3E). Conversely, the association of PGC1 α with the *G6pc* and *Pck1* promoters was slightly enhanced by knockdown of *Yap* (Supplemental Fig. S2D). Importantly, YAP appeared to alter the ability of PGC1 α to bind to the promoters of the gluconeogenic genes without altering PGC1 α protein levels (Supplemental Fig. S2C and S2E).

Finally, to determine the extent to which PGC1 α was required for the effects of YAP S127A, we knocked down *Pgc1a* in forskolin-treated cells. Thus, control hepatocytes were treated with adenovirus encoding an shRNA against *Pgc1a*, a control shRNA, YAP S127A, and/or GFP. In control cells, YAP S127A suppressed *G6pc* and *Pck1* thirteen- and eight-fold, respectively. Upon knockdown of *Pgc1a*, YAP S127A suppressed *G6pc* and *Pck1* by only four- and two-fold, respectively (Fig. 3F).

Overexpression of YAP is sufficient to suppress hepatic gluconeogenic gene expression and lower plasma glucose levels in vivo

To determine the effects of YAP deletion *in vivo*, YAP^{flox/flox} mice were infected with AAV expressing Cre recombinase or GFP under the control of a liver specific thyroxine binding globulin (*TBG*) promoter: AAV8-*TBG*Cre (or AAV-Cre) or AAV8-*TBG*GFP (or AAV-GFP), and sacrificed five weeks later. Despite the fact that AAV-Cre reduced *Yap* mRNA levels by 85% (Supplemental Fig. S3A), no changes were observed in body weight (Supplemental Fig. S3B), liver weight (Supplemental Fig. S3C), or the expression of the YAP targets connective tissue growth factor (*Ctgf*) and cysteine rich angiogenic inducer 61 (*Cyr61*) (Supplemental Fig. S3D). Moreover, the knockdown of *Yap* had no significant effect on gluconeogenic gene expression, glucose levels or glucose tolerance (Supplemental Fig. S3E-G). These data indicate that YAP has little contribution to glucose homeostasis in the normal adult liver.

To determine the effects of constitutively active YAP *in vivo*, *Alb-Cre::rtTA^{flox/flox}::TetO-YAP* mice were administered water with or without doxycycline supplementation for nine days prior to sacrifice. YAP S127A did not alter the body weights of the mice (Fig. 4A), but increased expression of *Ctgf* and *Cyr61* (Supplemental Fig. S4A) and doubled the weight of the liver (Fig. 4B, Supplemental Fig. S4B). Interestingly, mRNA levels of hepatic *G6pc* and *Pck1*, but not the fatty acid oxidation or mitochondrial genes, were decreased 75 to 90% in doxycycline treated mice (Fig. 4C). PGC1 α trended lower at both the mRNA and protein levels (Fig. 4C and Supplemental Fig. S4D), but no changes were noted in HNF4 α or cAMP

responsive element binding protein (CREB) (Supplemental Fig. S3C, D). Nonetheless, chromatin immunoprecipitation assays showed that binding of PGC1 α to *G6pc* and *Pck1* promoters was markedly decreased in the presence of YAP S127A (Fig. 4G). These data suggest that *in vivo*, as *in vitro*, YAP S127A was sufficient to suppress gluconeogenic gene expression. Consistent with this, the induction of YAP S127A decreased random fed blood glucose levels (Fig. 4D) and improved glucose tolerance (Fig. 4E, F).

YAP activation is associated with the suppression of the gluconeogenic genes in hepatocellular carcinoma

Since YAP is frequently induced in human hepatocellular carcinomas, we examined the relationship between YAP activity and gluconeogenic gene expression using liver microarray data obtained from paired biopsies taken from tumor and surrounding non-tumor regions in 60 patients with hepatocellular carcinoma (20). To assess YAP activation, we generated, in an unbiased manner, a set of genes (referred to as the YAP target gene set) induced by YAP S127A expression in the livers of mice (14). Gene set enrichment analysis revealed that the YAP target gene set was significantly induced in the tumors compared to the surrounding non-tumor regions ($p = 4 \times 10^{-7}$; $FDR = 8 \times 10^{-7}$), consistent with YAP activation in the tumors. We then plotted the average change in the expression of the YAP target genes against the average change in the expression of the gluconeogenic genes for each patient (Fig. 5A). We found a strong inverse correlation between YAP activation and gluconeogenic gene expression ($r = -0.57$, $p = 2.3 \times 10^{-6}$). Similar strong, inverse correlations were found between *G6PC* and *PCK1* expression and the canonical YAP targets *CTGF*, baculoviral IAP repeat containing 5 (*BIRC5*) and secreted phosphoprotein 1 (*SPPI*) (Supplemental Fig. S5A-F).

To determine whether the suppression of PGC1 α might be important for the growth promoting effects of YAP, we infected Hepa1c1c7 cells with adenoviruses encoding green fluorescent protein (GFP), YAP S127A, and/or PGC1 α , and measured cell mass at several time points afterwards, using a sulforhodamine B (SRB) assay (7). We found that Ad-YAPS127A increased cell mass, as expected, but that Ad-PGC1 α either alone, or in addition to Ad-YAPS127A, lowered cell mass (Fig. 5B). Thus, the suppression of PGC1 α appears to be required for the growth promoting effects of YAP.

Discussion

Here, we show that YAP effectively suppresses the gluconeogenic response to glucagon in a PGC1 α dependent manner. Thus, the ability of glucagon, as well as dexamethasone, to induce the expression of the gluconeogenic genes *Pck1* and *G6pc* is abolished in the presence of YAP S127A. Conversely, the inactivation of YAP leads to a de-repression of these genes. The effect of YAP on *G6pc* and *Pck1* is mediated in large part by PGC1 α , as the effects of YAP are blunted by the knockdown of PGC1 α . Mice expressing YAP S127A in their livers show reduced expression of the gluconeogenic genes and lower levels of plasma glucose.

Several pieces of data point to PGC1 α as a key mediator of YAP's effects on the gluconeogenic genes. First, the effects of YAP on the gluconeogenic genes are observed only

under conditions in which PGC1 α is induced: glucagon stimulation, dexamethasone stimulation, and PGC1 α overexpression (Fig. 1B-E, 2B-E, 3B-C). In contrast, no effects of YAP are observed in the basal state. Second, PGC1 α is sufficient to confer sensitivity of the gluconeogenic genes to YAP (Fig. 3B, C). Third, YAP reduces the accumulation of PGC1 α on the promoters of the gluconeogenic genes (Fig. 3E, 4G). And, finally, the effects of YAP are largely, but not entirely, diminished after knockdown of PGC1 α (Fig. 3F). The residual ability of YAP to suppress the gluconeogenic genes after PGC1 α knockdown may be due to additional PGC1 α -independent effects of YAP on these genes.

Importantly, YAP appears to preferentially impair the ability of PGC1 α to activate its gluconeogenic targets (Fig. 3B, C). Thus, YAP almost entirely suppresses gluconeogenic gene expression but only modestly reduces the fatty acid oxidation or mitochondrial gene targets of PGC1 α . These data suggest that YAP modulates PGC1 α , preventing PGC1 α from driving the consumption of ATP by gluconeogenesis while largely preserving its ability to drive the production of ATP through fatty acid oxidation and oxidative phosphorylation.

Exactly how YAP regulates PGC1 α is not yet clear. In contrast to PGC1 α , YAP does not appear to be present on the promoters of the gluconeogenic genes (Supplemental Fig. S2F, S4E). Moreover, we were unable to detect PGC1 α in immunoprecipitates of YAP (data not shown). Taken together, these data suggest that the effects of YAP on PGC1 α may be indirect. Indeed the literature suggests S6 kinase as one potential indirect mediator: YAP overexpression activates S6 kinase (25); S6 kinase phosphorylates PGC1 α on its arginine/serine-rich domain (24); and PGC1 α phosphorylation by S6 kinase selectively inhibits the ability of PGC1 α to coactivate its gluconeogenic, but not mitochondrial, gene targets (24).

YAP suppresses *Pgc1a* mRNA levels under some, but not all, conditions. PGC1 α transcription is driven, in part, by a positive feedback loop, in which PGC1 α coactivates transcription of its own promoter (26). Thus, YAP could impair the ability of PGC1 α to coactivate the *Pgc1a* promoter, as it does the *G6pc* and *Pck1* promoters. Consistent with this, YAP suppressed endogenous *Pgc1a* mRNA in the presence of glucagon (Fig. 3A), but did not suppress exogenous *Pgc1a* mRNA, driven by the CMV promoter (Fig. 3B).

Our data also show that YAP must be inactivated for the full gluconeogenic gene response to glucagon. The regulation of the gluconeogenic response is highly complex, involving multiple transcription factors and coactivators. The canonical pathway by which glucagon activates gluconeogenesis is via the cAMP/PKA signaling pathway, which ultimately leads to the activation of CREB and CREB regulated transcription coactivator 2, in addition to PGC1 α . Our data reveal the existence of yet another layer of regulation, in which PKA signaling also inhibits YAP, permitting PGC1 α activation of the gluconeogenic targets.

In the hepatocytes of normal adult livers, YAP expression is very low (14), which most likely renders the deactivation of YAP by glucagon superfluous. Consistent with this, knockout of YAP in the livers of adult mice had little effect on either gluconeogenic gene expression or plasma glucose levels (Supplemental Fig. S3). However, in the post-natal developing liver and the regenerating liver, YAP is present (3). Under these conditions, glucagon regulation of YAP may become important in balancing the hepatocyte's need to

meet the anabolic demands of growth with the opposing needs of the organism to prevent hypoglycemia. For example, in the perinatal period, the organism is highly susceptible to hypoglycemia and glucagon plays an important role in maintaining blood glucose levels by driving gluconeogenesis (27). During this critical period, the ability of glucagon to override YAP inhibition of PGC1 α and gluconeogenesis may be necessary for the maximal response to gluconeogenesis. It is also interesting to note that two other hormones involved in the hypoglycemic response, epinephrine and insulin, could act in parallel with glucagon to suppress YAP: epinephrine, which also acts via a G-coupled protein receptor to inhibit YAP (9), is increased with hypoglycemia, whereas insulin, which may activate YAP (28), is decreased with hypoglycemia.

YAP is also activated in many forms of liver cancer. Our data show that the activation of YAP targets is correlated with the suppression of gluconeogenic genes in the liver. YAP suppression of the gluconeogenic genes could explain data accumulated over the last five decades showing that gluconeogenesis is inhibited in tumor cells (29–32). It could also potentially explain, in part, the hypoglycemia associated with some forms of liver cancer (33, 34). The ability of YAP to suppress gluconeogenesis may very well play an important part in its ability to drive tumorigenesis (Fig. 5A). Indeed, patients with mutations in *G6pc* are prone to the development hepatic adenomas (29), and therapies designed to induce gluconeogenesis appear to be beneficial in the treatment of hepatic carcinoma (35, 36).

In summary, our data identify PGC1 α and the gluconeogenic genes as important targets of YAP in the liver. By preventing the diversion of ATP, reducing equivalents and building blocks to gluconeogenesis, YAP, a master regulator of growth and proliferation, appears to rewire cellular metabolism to support the anabolic demands of growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Financial Support

This project was supported by NIH grants DK094162 to S.B.B. and R00DK100539 to J.M., a fellowship from China Scholarship Council to Y.H., and AHA grant 15SDG25590001 to Z.L. The Joslin Bioinformatics Core is supported by NIH P30DK036836 (Diabetes Research Center).

Abbreviations

AAV	adeno-associated virus
Ad	adenovirus
Alb	Albumin
ATP	adenosine triphosphate
AUC	area under the curve
BIRC5	baculoviral IAP repeat containing 5

cAMP	cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation
COX5b	cytochrome c oxidase subunit 5b
CREB	cAMP responsive element binding protein
CTGF	connective tissue growth factor
CYCS	cytochrome c somatic
CYR61	cysteine-rich angiogenic inducer 61
Dex	dexamethasone
Dox	doxycycline
FBS	fetal bovine serum
G6PC	glucose-6-phosphatase catalytic subunit
GFP	green fluorescent protein
GR	glucocorticoid receptor
GPCR	G-protein coupled receptor
HNF4α	hepatocyte nuclear factor 4 alpha
GTT	intraperitoneal glucose tolerance test
IHD3α	isocitrate dehydrogenase 3 alpha
ATS	large tumor suppressor
mRNA	messenger RNA
mTORC1	mammalian target of rapamycin complex 1
ADH	nicotinamide adenine dinucleotide
BS	phosphate-buffered saline
PCK1	phosphoenolpyruvate carboxykinase 1
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1
PKA	protein kinase A
rtTA	reverse tetracycline transactivator
shRNA	short hairpin RNA
SPP1	secreted phosphoprotein 1
TBG	thyroxine binding globulin

TCA	tricarboxylic acid
TEAD	TEA domain family member
VLCAD	very long chain acyl-CoA dehydrogenase
TetO	tetracyclin On
YAP	yes-associated protein 1

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Fig. 1.

YAP S127A blocks the induction of gluconeogenic genes by glucagon and dexamethasone. Primary hepatocytes were isolated from inducible *A/b-Cre::rtTA^{flox/flox}::TetO-YAP* mice and incubated in the presence or absence of doxycycline (Dox). (A) Protein levels were measured by immunoblotting whole cell lysates. (B-E) Cells were treated with 10 nM glucagon for 1.5 hours (B), 1 μ M 8Br-cAMP for 1.5 hours (C), 10 μ M forskolin for 1.5 hours (D), or 200 nM dexamethasone (Dex) for 6 hours (E) and gene expression was measured by real-time RT-PCR. Data are presented as the mean and SEM ($n = 3$ technical replicates per condition), and are representative of two to four independent experiments. Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus untreated cells and # $p < 0.05$ versus similarly treated cells incubated in the absence of doxycycline.

Fig. 2.

YAP knockdown potentiates the induction of gluconeogenic genes by glucagon and dexamethasone. Primary hepatocytes were isolated from YAP^{flax/flax} mice and infected with Ad-Cre or Ad-GFP. (A) YAP levels were measured by immunoblotting whole cell lysates and real-time RT-PCR. (B-E) Cells were treated with glucagon (B), 8Br-cAMP (C), forskolin (D), or dexamethasone (E) as in Fig. 1, and gene expression was measured by real-time RT-PCR. Data are presented as the mean and SEM (n = 3 technical replicates per condition), and are representative of three or more independent experiments. Statistical significance was assessed by Student's t-test; * p < 0.05 versus untreated cells infected with the same adenovirus and [#] p < 0.05 versus similarly treated cells infected with Ad-GFP.

Fig. 3.

YAP suppresses gluconeogenic gene expression via PGC1 α . (A) mRNA levels of *Pgc1a* were measured using real-time RT-PCR in primary hepatocytes treated with or without glucagon, as described in Fig. 1 and 2. In the left panel, cells were isolated from inducible *Alb-Cre::rtTA^{flx/flx}::TetO-YAP* mice and incubated without or with doxycycline to induce YAP S127A. In the right panel, cells were isolated from *Yap^{flx/flx}* mice and infected with Ad-GFP, or Ad-Cre to knockdown *Yap*. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; *

$p < 0.05$ versus untreated cells and $^{\#} p < 0.05$ versus glucagon treated cells incubated in the absence of doxycycline (left panel) or infected with Ad-GFP (right panel).

(B) Primary hepatocytes were isolated from inducible *Alb-Cre::rtTA^{flox/flox}::TetO-YAP* mice, treated with Ad-PGC1 α and Ad-LacZ and incubated in the presence or absence doxycycline; gene expression was measured by real-time RT-PCR. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus Ad-LacZ infected cells and $^{\#} p < 0.05$ versus Ad-PGC1 α infected cells incubated in the absence of doxycycline.

(C) Primary hepatocytes were isolated from *YAP^{flox/flox}* mice and infected with the indicated adenoviruses; gene expression was measured by real-time RT-PCR. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus Ad-LacZ treated cells and $^{\#} p < 0.05$ versus Ad-PGC1 α /Ad-GFP infected cells.

(D) HepG2 cells were transfected and treated as indicated. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus basal and $^{\#} p < 0.05$ versus cells transfected without YAP S127A.

(E) Primary hepatocytes were isolated from inducible *Alb-Cre::rtTA^{flox/flox}::TetO-YAP* mice, incubated with or without doxycycline, infected with Ad-PGC1 α and stimulated for 1.5 hours with 10 μ M forskolin. ChIP assays were performed using an antibody against PGC1 α or control IgG. Relative enrichment of PGC1 α was accessed by real-time PCR using primers against the indicated regions of the *G6pc* and *Pck1* genes. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus control IgG and $^{\#} p < 0.05$ versus cells without doxycycline treatment.

(F) Control hepatocytes were treated with Ad-shPGC1 α , Ad-shLamin, Ad-YAP S127A and/or Ad-GFP, and treated for 1.5 hours with 10 μ M forskolin. Gene expression was measured by real-time RT-PCR. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus Ad-GFP infected cells and $^{\#} p < 0.05$ versus shLamin infected cells.

Fig. 4.

YAP suppresses hepatic gluconeogenic gene expression *in vivo*. Seven- to nine-week-old *Alb-Cre::rtTA^{lox/lox}::TetO-YAP* mice were sacrificed after nine days with or without doxycycline treatment. Body weight (A), liver to body weight ratio (B) and blood glucose levels (D) were measured at the time of sacrifice. Gene expression (C) was measured by real-time RT-PCR. Glucose tolerance test was performed after six days of doxycycline treatment (E) and glucose area under the curve (AUC) was calculated (F). Data are presented as the mean and SEM (n = 4 – 7). Statistical significance was assessed by Student's t-test; *p < 0.05 versus mice not administered doxycycline. (G) ChIP assays were performed using an antibody against PGC1α or control IgG. Relative enrichment of PGC1α was assessed by real-time PCR using primers against the indicated regions of the *G6pc* and *Pck1* genes. Data are presented as the mean and SEM (n = 3 technical replicates per condition). Statistical significance was assessed by Student's t-test; * p < 0.05 versus without doxycycline treatment and # p < 0.05 versus control IgG.

Fig. 5.

(A) Gene expression in paired biopsies of tumor and non-tumor regions obtained from the livers of patients with hepatocellular carcinoma ($n = 60$) (20). The average log-ratio of the gluconeogenic genes (Y-axis) was plotted against the average log-ratio of the YAP target genes (X-axis), with each point representing data from a single patient. (B) Proliferation of mouse hepatoma cells over five days in the presence of adenoviruses expressing GFP, YAP S127A, and/or PGC1 α . Data are presented as the mean and SEM ($n = 4$). Statistical significance was assessed by Student's t-test; * $p < 0.05$ versus Ad-GFP treated and # $p < 0.05$ versus both Ad-YAP S127A and Ad-PGC1 α treated.