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Pi3kcb Links Hippo-YAP and PI3K-AKT Signaling Pathways to Promote Cardiomyocyte Proliferation and Survival

Zhiqiang Lin[1], **Pingzhu Zhou**[1], **Alexander von Gise**[1,2], **Fei Gu**[1], **Qing Ma**[1], **Jinghai Chen**[1], **Haidong Guo**[1,3], **Pim R. R. van Gorp**[1,4], **Da-Zhi Wang**[1,5], and **William T. Pu**[1,5]

[1]Department of Cardiology, Boston Children's Hospital, Boston, MA, USA [2]Department of Pediatric Cardiology and Intensive Care, MHH-Hannover Medical School, Shanghai, China [3]Department of Anatomy, School of Basic Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China [4]Department of Cardiology, Leiden University Medical Center, The Netherlands [5]Harvard Stem Cell Institute, Harvard University, Cambridge, MA, USA.

Abstract

Rationale—YAP, the nuclear effector of Hippo signaling, regulates cellular growth and survival in multiple organs, including the heart, by interacting with TEAD sequence specific DNA-binding proteins. Recent studies showed that YAP stimulates cardiomyocyte proliferation and survival. However, the direct transcriptional targets through which YAP exerts its effects are poorly defined.

Objective—To identify direct YAP targets that mediate its' mitogenic and anti-apoptotic effects in the heart.

Methods and Results—We identified direct YAP targets by combining differential gene expression analysis in YAP gain- and loss-of-function with genome-wide identification of YAP bound loci using chromatin immunoprecipitation and high throughput sequencing. This screen identified *Pik3cb*, encoding p110β, a catalytic subunit of phosphoinositol-3-kinase (PI3K), as a candidate YAP effector that promotes cardiomyocyte proliferation and survival. YAP and TEAD occupied a conserved enhancer within the first intron of *Pik3cb*, and this enhancer drove YAP-dependent reporter gene expression. *Yap* gain- and loss-of-function studies indicated that YAP is necessary and sufficient to activate the PI3K-Akt pathway. Like *Yap*, *Pik3cb* gain-of-function stimulated cardiomyocyte proliferation, and *Pik3cb* knockdown dampened YAP mitogenic activity. Reciprocally, impaired heart function in *Yap* loss-of-function was significantly rescued by AAV-mediated *Pik3cb* expression.

Conclusion—: *Pik3cb* is a crucial direct target of YAP, through which the YAP activates PI3K-AKTpathway and regulates cardiomyocyte proliferation and survival.

Address correspondence to: Dr. William T. Pu Department of Cardiology Children's Hospital Boston Enders 1254 300 Longwood Ave. Boston, MA 02115 Tel: 617-919-2091 Fax: 617-730-0140 wpu@enders.tch.harvard.edu.

DISCLOSURES

The authors have no conflicts of interest to disclose.

Keywords

Hippo; Yap; Pik3cb; PI3 kinase; Akt; heart failure; cardiomyocyte proliferation; AAV; regeneration

INTRODUCTION

Adult mammalian cardiomyocytes largely exit from the cell cycle, thereby limiting the innate regenerative capacity of the mature heart.[1] As a result, there are currently no treatments for heart disease that reverse cardiomyocyte loss, a root cause for many cases of heart failure.

Recently, the transcriptional co-activator YAP was found to be essential for fetal cardiomyocyte proliferation, and activated YAP drove adult cardiomyocyte proliferation[2-5]. YAP is the nuclear effector of the Hippo kinase cascade, a recently defined pathway that regulates cell proliferation and survival to establish organ size.[6] Hippo pathway kinases MST1/2 and LATS1/2 phosphorylate YAP, retarding its transcriptional activity by promoting its nuclear export. Hippo kinase inactivation enhanced YAP activity and stimulated fetal and adult cardiomyocyte proliferation.[7, 8]

YAP regulates transcription of its direct target genes by binding to sequence-specific DNA binding proteins, with TEAD1-4 (TEA domain family members 1-4) being key transcriptional partners.[6] However, few direct YAP targets that are essential for its growth promoting activity are known. The phosphoinositide 3-kinase (PI3K)-AKT pathway was reported to be activated downstream of YAP,[4] although the link between these pathways was not determined. The PI3K-AKT pathway promotes cardiomyocyte proliferation, survival, and physiological hypertrophy[4, 5]. Class IA PI3Ks are composed of a p85 regulatory subunit and a p110 catalytic subunit. The p110 catalytic subunit is encoded by three different genes, *Pik3ca*, *Pik3cb*, and *Pik3cd*, with most cells expressing *Pik3ca* and *Pik3cb*[9]. Early lethality of *Pik3cb* null embryos indicates that *Pik3ca* and *Pik3cb* isoforms are not fully redundant.[10] However, most work has focused on *Pik3ca* and much less is known about *Pik3cb*.

In this study, through an unbiased whole genome screen we identified *Pik3cb* as a direct YAP target that links YAP to PI3K-AKT activation. Our data indicate that *Pik3cb* activation is sufficient to drive adult cardiomyocyte proliferation and is necessary for the mitogenic activity of YAP.

METHODS

Additional detailed materials and methods are provided in the online supplement.

Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee. Echocardiography and myocardial infarction (MI) surgery were performed blinded to genotype and treatment group.

Cell culture

We isolated ventricular cardiomyocytes (NRVMs) from 4-day-old rat hearts[2]. HL1 cells were obtained from William Claycomb and cultured as described[11].

Other procedures

Hearts were fixed, embedded, cryosectioned, and immunostained as described.[12] Antibodies are listed in Online Table I.

HL1 cells overexpressing 3FLAG-YAP[S127A] were used for ChIP-seq as described[11]. HiSeq 2000 (Illumina) sequencing data were used to identify binding peaks (Online Table II). ChIP-qPCR was performed using antibodies and primers listed in Online Tables I and III.

Gene expression was measured by qRTPCR using primers listed in Online Table III or by microarray (Mouse Gene ST 2.0 array, Affymetrix) using RNA collected from E12.5 mouse heart.

Data are available at GEO (accession number GSE57719) or the Cardiovascular Development Consortium server at https://b2b.hci.utah.edu/gnomex/.

Statistics

Values are expressed as mean ± SEM. To test for statistical significance, we used Student's *t*-test (two groups) or ANOVA with the Tukey HSD post-hoc test (more than two groups). Tests were performed using JMP10.0 (SAS).

RESULTS

YAP directly activates Pik3cb expression through TEAD

YAP plays crucial roles in regulating cardiomyocyte proliferation and survival[2-5, 7, 8], but the direct targets of YAP that mediate its effects are largely unknown. To identify candidate genes that are directly regulated by YAP and that mediate its proliferative activity, we overexpressed activated, FLAG-tagged YAP (3xFlag-YAP[S127A], in which the inhibitory Hippo phosphorylation site serine 127 is mutated to alanine) in HL1 cardiomyocyte-like cells. Chromatin immunoprecipitation (ChIP) and high throughput sequencing (ChIP-seq) identified 1340 YAP-bound chromatin regions (Fig. 1A; Online Table II). YAP bound to genes enriched for functional terms including cardiovascular system development, regulation of cell proliferation, and cell proliferation (Fig. 1B). Motif discovery using these YAP-bound regions yielded the consensus TEAD motif (P=9.7E-47), confirming the predominant interaction of YAP with TEAD and providing validation for the ChIP-seq dataset. Scanning the YAP-bound regions for known transcription factor binding motifs also identified the TEAD motif, as well as the STAT and ETS motifs, which share sequence similarity to the TEAD motif (Fig. 1C).

We used microarray gene expression profiling to identify genes downstream of YAP. We compared E12.5 mouse hearts with TNTCre-mediated YAP inactivation in cardiomyocytes ($YAP^{fl/fl}$::TNTCre) to $Yap^{fl/+}$::TNTCre littermate controls (Fig. 1A). We also compared

adenovirus-mediated YAP[S127A] overexpression in neonatal rat ventricular cardiomyocytes to adenoviral LACZ expression (Fig. 1A), which we reported previously[2]. In the murine loss-of-function dataset, we identified 2200 differentially expressed genes (1137 and 1063 up- and down-regulated in knockout, respectively; $p<0.05$; absolute $\log_2$ fold-change>0.2; n=3; Online Table III). In the neonatal rat gain-of-function dataset, there were 2091 differentially expressed genes (1030 and 1061 up- and down-regulated in Yap overexpression, respectively; $p<0.05$; absolute $\log_2$ fold-change>0.5; n=4; see ref. 2). There were 217 genes with concordant regulation by YAP in both datasets (Online Table IV). These 217 genes were enriched for functional terms encompassing heart development (P=0.00045).

The intersection of genes associated with YAP-bound chromatin regions and concordantly regulated downstream of YAP in both differential expression datasets contained 26 genes, with 13 activated by YAP, and 13 repressed by YAP (Online Table IV). Given that YAP was previously reported to activate the PI3K-AKT pathway though uncertain mechanisms,[4] we were interested to find *Pi3kcb* among the candidates for direct YAP activation. *Pik3cb* is a little studied isoform that encodes the phosphoinositol-3-kinase (PI3K) catalytic subunit (also referred to as p110β), a key kinase that regulates cell growth and metabolic activity[13, 14]. Like YAP,[2] PIK3CB proteins levels decline in the heart with increasing post-natal age (Online Fig. I). qRT-PCR of NRVMs confirmed that Ad:YAP[S127A] activated expression of *Pik3cb* compared to Ad:LacZ (Fig. 1D).

The HL1 ChIP-seq data revealed a YAP-bound sequence residing in the first intron of *Pik3cb* (Fig. 2A). We validated YAP binding to the identified sequence by ChIP-qPCR, using a pair of primers spanning the YAP bound sequence and a control pair recognizing a sequence 1.3 kb away (Fig. 2B). This YAP-bound sequence contained an evolutionarily conserved sequence (AGGAATTCGTGGAATT) containing two repeats of a motif that is similar to the TEAD, STAT, and ETS motifs (Fig. 2C-D). ChIP-qPCR to confirmed YAP and TEAD occupancy of this *Pik3cb* region but not the adjacent control region in neonatal heart (Fig. 2E). While YAP-TEAD interaction is well described, YAP has not been reported to interact with STAT or ETS. Co-IP experiments showed no detectable interaction between YAP and STAT3, STAT5a, STAT6, or ETS1 (Online Fig. II–A-C). These data suggest that YAP activates the *Pik3cb* enhancer via TEAD in cardiomyocytes.

To measure the transcriptional activity of the YAP-bound region of *Pik3cb*, which we refer to as the *Pik3cb* enhancer, we cloned a 552 bp genomic DNA fragment containing the conserved element and potential TEAD binding sites into a minimal promoter luciferase reporter construct. Co-transfection with Yap in NRVMs showed that Yap stimulates activity of the enhancer by ~5-fold. Yap stimulation was abrogated by mutation of the core conserved element (Fig. 2F). YAP[S94A], deficient in TEAD interaction,[15] failed to activate the *Pik3cb* enhancer (Fig. 2G), indicating that YAP stimulates *Pik3cb* expression through TEAD. On the other hand, S3I-201, a Stat3 inhibitor which prevents Stat3 binding DNA and suppresses Stat3 dependent transcription[16], did not affect YAP activation of the *Pik3cb* enhancer or YAP-induced cardiomyocyte proliferation (Online Fig. II-D,E).

Together, these data indicate that YAP binds to an evolutionarily conserved motif in the first intron of *Pik3cb* through TEAD to upregulate *Pik3cb* expression in cardiomyocytes.

Yap is sufficient and required to upregulate Pik3cb /Pik3ca and activate PI3K-Akt pathway in vivo

Having shown that YAP directly binds and activates the *Pik3cb* enhancer, we next asked if YAP is necessary and sufficient to stimulate *Pik3cb* expression in vivo and thereby activate the PI3K-Akt pathway. To overexpress YAP in cardiomyocytes in vivo, we used adeno-associated virus serotype 9 (AAV9), a safe, efficient and cardiotropic vector for in vivo gene transfer[17, 18] . We used a cardiomyocyte-specific chicken troponin T promoter (cTNT) to further enhance cardiac selectivity. We validated the cardiomyocyte specificity of this gene transfer system by analyzing the recombination pattern of AAV9:cTNT-iCre, in which the cTNT promoter drives expression of mammalian codon-optimized Cre recombinase. We systemically administered AAV9:cTNT-iCre by subcutaneous injection into 1-2 day old mouse pups harboring the Rosa26^{fsTRAP} allele, in which Cre recombination activates GFP expression. Seven days after virus administration, most TNNI3 positive cardiomyocytes expressed GFP, but TNNI3 negative non-cardiomyocytes did not (Online Fig. III-A). These data show that AAV9-cTNT drives cardiomyocyte-selective expression within the heart.

We then replaced the iCre gene with FLAG-tagged, activated YAP (3Flag-YAP[S127A]) to generate AAV9:cTNT-Yap virus (AAV9:Yap). As a negative control, we generated AAV9:cTNT-Luciferase (AAV9:Luci). AAV9:Yap and AAV9:Luci were injected into 1-2 day old pups. 7 days later, the expression of exogenous 3Flag-YAP[S127A] was clearly detected by western blot (Fig. 3A). Expression of activated YAP caused more than two-fold upregulation of *Pik3cb* (Fig. 3B). *Pik3ca* was also upregulated, although to a lesser degree compared to *Pik3cb* (Fig. 3B).

Xin et al. previously reported that YAP activation in cultured neonatal rat ventricular cardiomyocytes increased the levels of activated AKT (Akt[p-S473]) without changing total AKT protein level[4]. YAP was linked to AKT activation in this system by upregulation of IGF1R. In mitotic tissue (skin) and cultured cells, YAP was also shown to activate the PI3K-Akt pathway by suppressing expression of PTEN[19], an inhibitor of PI3K-Akt signaling. We found that YAP activation in cardiomyocytes in vivo increased AKT activation without changing total AKT level (Fig. 3C). YAP significantly upregulated IGF1R by ~1.5-fold, but did not alter PTEN level (Online Fig. III-B,C).

We next asked if YAP is required for normal expression of *Pik3cb* and *Pik3ca* in the heart. We generated Yap$^{fl/fl}$::Myh6-Cre mice (YAP^{cKO}), in which cardiomyocyte-specific Myh6-Cre inactivates a conditional YAP^{flox} allele. We confirmed efficient YAP inactivation by western blotting, which showed marked downregulation of cardiac YAP protein (Fig. 3D). In Yap^{cKO} hearts, *Pik3cb* and *Pik3ca* mRNA were both significantly reduced (Fig.3E). Moreover, phosphorylated but not total Akt was reduced in Yap^{cKO} mice heart, indicating that Yap is required for maintenance of the normal level of Akt activation (Fig. 3F).

The cell cycle inhibitor p27 (CDKN1B) is a direct target of Akt, and Akt-mediated p27 phosophorylation leads to p27 degradation[20]. We therefore measured p27 protein level as a

downstream readout of Akt activation. In AAV9:YAP-treated hearts, p27 protein was downregulated (Fig. 3C), while in Yap^{cKO} hearts, p27 protein was upregulated (Fig. 3F). These data further confirm that Akt activity is governed by YAP activity in cardiomyocytes.

Collectively, both gain- and loss-of-function data indicate that YAP promotes *Pik3cb* and *Pik3ca* upregulation and stimulates PI3K-Akt pathway activation in vivo.

Pik3cb overexpression activated AKT and induced cardiomyocyte proliferation

To determine if *Pik3cb* is sufficient to activate Akt and drive cardiomyocyte proliferation, we generated Ad:Pik3cb, an adenovirus that expresses *Pik3cb*. We validated over-expression of PIK3CB protein in NRVMs by Ad:Pik3cb (Fig. 4A). We then treated NRVMs with Ad:Pik3cb and measured the cardiomyocyte proliferation rate using two independent markers, phosphorylated histone H3 (pH3), an M phase marker, and BrdU uptake, an S phase marker. *Pik3cb* overexpression significantly increased the fraction of cardiomyocytes positive for BrdU and pH3 (Fig.4B-E). These data indicate that *Pik3cb* is sufficient to stimulate proliferation of cultured neonatal cardiomyocytes in vitro.

To extend these data to an in vivo context, we generated AAV9:cTNT-3Flag-Pik3cb (AAV9:Pik3cb) (Fig. 4F) and delivered it or control AAV9:Luci systemically to 1-2 day old neonatal mice. Seven days after virus administration (8-9 days of age), we collected hearts for immunoblotting and histological studies. We confirmed expression of Flag-tagged human PIK3CB protein in the heart (Fig. 4G). To determine the effect of *Pik3cb* gain-of-function on cardiomyocyte proliferation, we measured the fraction of cardiomyocytes that were positive for pH3. Compared with AAV9:Luci, AAV9:Pik3cb significantly increased the $pH3^{+}$ CM fraction (Fig. 4H-I), suggesting that PIK3CB is sufficient to increase neonatal cardiomyocyte proliferation in vivo.

We next addressed the ability of *Pik3cb* to stimulate adult cardiomyocyte proliferation in the context of disease. Myocardial infarction (MI) was induced in two month-old CFW mice by coronary artery ligation (Online Fig. IV-A). The freshly infarcted myocardium was treated with AAV9:Luci or AAV9:Pik3cb. 4 days after MI, mice were treated with one dose of EdU to label dividing cardiomyocytes. 5 days after MI, hearts were collected for analysis. In the border zone, AAV9:Pik3cb treatment resulted in higher cardiomyocyte EdU labeling index than AAV:Luci treatment (Online Fig. IV-B,C). Cardiomyocyte apoptosis was also significantly reduced in AAV9:Pik3cb treatment compared to control (Online Fig. IV-D,E). These data show that *Pik3cb* promotes cardiomyocyte cell cycle activity and enhances cardiomyocyte survival after MI.

One mechanism by which a treatment might increase cardiomyocyte proliferation is by promoting dedifferentiation. To assess whether *Pik3cb* stimulates cardiomyocyte dedifferentiation, we treated P2 neonatal hearts with AAV9-Luci or AAV9-Pik3cb. We then analyzed expression of myocardial differentiation markers at P9. We detected no change in expression of *Myh6*, *Myh7*, or *Nkx2-5* (Online Fig. IV-F), suggesting that *Pik3cb* does not promote cardiomyocyte dedifferentiation.

We next examined the effect of *Pik3cb* gain-of-function on downstream Akt signaling. Interestingly, *Pik3cb* overexpression upregulated *Pik3ca* (Online Fig. IV-G), suggesting that YAP indirectly upregulates *Pik3ca* through its direct effect on *Pik3cb*. AAV9:Pik3cb-treated hearts showed higher level of activated Akt (Akt[pS473]) than AAV9:Luci-treated hearts, while the level of total Akt was comparable between groups (Fig. 4J). Furthermore, the protein level of p27 was decreased in AAV9:Pik3cb-treated hearts (Fig. 4K; $P<0.05$; quantification in Online Fig. IV-H). These data demonstrate that overexpression of PIK3CB activates Akt and increases cardiomyocyte proliferation in vivo.

Pik3cb is required for YAP-stimulated Akt activation and cardiomyocyte proliferation

Because YAP directly regulated *Pik3cb*, which is sufficient to increase cardiomyocyte proliferation, we hypothesized that *Pik3cb* is required for YAP-induced Akt activation and cardiomyocyte proliferation. To analyze the requirement of *Pik3cb* downstream of YAP in cultured NRVMs, we synthesized three siRNAs and measured their reduction of YAP-stimulated *Pik3cb* expression. siPik3cb#2 reduced *Pik3cb* expression to 42% of control values, while the other two siRNAs were not effective (Fig. 5A). Then we stimulated NRVMs with activated YAP and treated them with siPik3cb#2 or control siRNA. siPik3cb#2 partially blocked the YAP-induced increase in cardiomyocyte proliferation markers EdU^+ and $pH3^+$ (Fig. 5B-E). These data indicate that *Pik3cb* is necessary for YAP to fully induce cardiomyocyte proliferation in vitro.

To study the requirement of *Pik3cb* downstream of YAP in cardiomyocytes in vivo, we followed a previously validated strategy[21] and embedded an shRNA directed against *Pik3cb* into the 3' UTR of AAV9:YAP to simultaneously overexpress Yap and knock down *Pik3cb* in the mouse heart. We tested four different shRNAs, and shPik3cb#3 yielded the greatest *Pik3cb* knockdown (Online Fig. V-A). We then cloned this shRNA, or a scrambled negative control shRNA, downstream of YAP or luciferase to yield AAV9:YAP-shPik3cb, AAV9:YAP-sc, and AAV9:Luci-sc (Fig. 6A). AAV9:YAP-shPik3cb was designed to overexpress activated YAP and simultaneously knock down *Pik3cb*, whereas AAV9:YAP-sc was designed to overexpress activated YAP without perturbing *Pik3cb* expression.

Compared with AAV9:Luci-sc control virus, both AAV9:YAP-shPik3cb and AAV9:YAP-sc strongly upregulated *Yap* to comparable degrees (~6.5-fold upregulation). Consistent with our earlier data, AAV9:YAP-sc upregulated *Pik3cb* by 2.5-fold and *Pik3ca* by 1.4-fold compared to AAV9:Luci-sc control (Fig. 6B). AAV9:YAP-sc robustly stimulated cardiomyocyte proliferation in the neonatal mouse heart, as reflected by pH3 staining (Fig. 6C,D). In comparison, AAV9:YAP-shPik3cb upregulation of *Pik3cb* was significantly attenuated. Reduced *Pik3cb* expression corresponded to less induction of cardiomyocyte proliferation by AAV9:YAP-shPik3cb (Fig. 6C,D). These changes were not associated with altered expression of *Myh6*, *Myh7*, or *Nkx2-5*, suggesting they were not due to altered cardiomyocyte differentiation (Online Fig. V-B). These data indicate that *Pik3cb* is required for full stimulation of cardiomyocyte proliferation by YAP.

We previously showed that several cell cycle genes including aurora A kinase *(Aurka)*, cell division cycle 20 (*Cdc20*), and cyclin A2 (*Ccna2*) are upregulated by YAP[2]. Consistent with these data, AAV9:YAP-sc upregulated these genes compared to AAV9:Luci-sc control (Fig.

6E). Reduced *Pik3cb* expression by AAV9:YAP-shPik3cb interfered with *Aurka* and *Ccna2* upregulation by YAP (Fig. 6E). Average *Cdc20* expression also decreased, although this did not reach statistical significance. Meanwhile, *Cdkn1b* (p27) mRNA level was not significantly affected, in keeping with its regulation by its post-transcriptional regulation by PI3K-AKT[20]. These results demonstrate that YAP regulation of the expression of a subset of cell cycle genes is dependent upon upregulation of *Pik3cb*.

Because YAP is sufficient to increase *Pik3cb* expression and activate AKT (Fig. 2), and AKT is a downstream target of PIK3CB, we hypothesized that PIK3CB is required for YAP to activate AKT. To test this hypothesis, we measured activated and total AKT, as well as p27 by western blotting of hearts transduced with AAV9:YAP-sc, or AAV9:YAP-shPik3cb (Fig. 6F-G). Total AKT levels were comparable between groups. YAP-sc elevated activated AKT, and this stimulation was dampened substantially by shPik3cb. Although the p27 mRNA level did not change between AAV9:YAP-sc and AAV9:YAP-shPik3cb groups (Fig. 6E), the protein level of p27 was increased by shPIK3cb (Fig. 6F), consistent with destabilization of p27 protein by activated AKT.

These data indicate that YAP stimulates AKT at least in part through upregulation of *Pik3cb*. Pik3cb upregulation improved Yap^{cKO} heart function.

Cardiac-specific depletion of *Yap* caused pathological hypertrophy and heart failure[3, 5]. Since YAP is required to activate *Pik3cb* and to maintain PI3K-AKT activity, we hypothesized that *Pik3cb* overexpression would restore function of Yap^{cKO} hearts. We tested this hypothesis by treating 1 day old Yap^{cKO} or $Yap^{fl/fl}$ pups with either AAV9:Pik3cb or AAV9:Luci (Fig. 7A). At 3 weeks of age, we administered one dose of EdU to label the cardiomyocytes traversing S phase of the cell cycle. At 4 weeks of age, we measured cardiac function by echocardiography, then we collected the hearts for analysis. Treatment of control ($Yap^{fl/fl}$) mice with AAV9:Pik3cb did not significantly affect heart function compared to AAV9:Luci, indicating that *Pik3cb* overexpression is well tolerated (Fig. 7B). YAP^{cKO} hearts had severe systolic dysfunction that was partially rescued by AAV9:Pik3cb (Fig. 7B). Due to technical difficulties, we were not able to quantitate the fraction of cardiomyocytes transduced by AAV9:Pik3cb. It is possible that the incomplete rescue was due to incomplete cardiomyocyte transduction, or to *Pik3cb*-independent YAP activities. Nevertheless, our data indicate that decreased *Pik3cb* and PI3K-AKT signaling is an important contributor to cardiac dysfunction in YAP loss of function.

Next we considered the effect of *Pik3cb* overexpression on cardiomegaly observed in YAP^{cKO}. AAV9:Pik3cb treatment of control ($Yap^{fl/fl}$) mice did not significantly affect heart size compared to AAV9:Luci (Fig. 7C,D), indicating that *Pik3cb* overexpression does not cause cardiac hypertrophy. YAP^{cKO} hearts showed significant cardiomegaly, likely secondary to cardiac dilation in heart failure. Cardiomegaly was attenuated by AAV9:Pik3cb but not AAV9:Luci (Fig. 7C,D), consistent with improvement of ventricular function (Fig. 7B). We further examined the effect of *Pik3cb* overexpression on cardiac hypertrophy caused by YAP depletion in the heart at the level of cardiomyocyte size. AAV9:Pik3cb treatment of control ($Yap^{fl/fl}$) mice did not significantly alter cardiomyocyte cross-sectional area, compared to AAV9:Luci treatment (Fig. 7E,F). Cardiomyocyte cross-

sectional area was significantly greater in YAP^{cKO} heart. *Pik3cb* overexpression by AAV9:Pik3cb significantly blunted this cardiomyocyte hypertrophy, compared to AAV9:Luci (Fig. 7E,F).

Cardiac hypertrophy is often associated with upregulation of the genes *Nppa* and *Myh7*. We assessed the effect of AAV9:Pik3cb on the expression of these hypertrophic marker genes in Yap^{cKO} heart. AAV9:Pik3cb markedly downregulated *Nppa* and *Myh7* compared to AAV9:Luci (Fig. 7G), consistent with attenuation of cardiomyocyte hypertrophy. These data indicate that AAV9:Pik3cb partially mitigated hypertrophic marker gene activation caused by cardiac YAP depletion.

Together these data demonstrate that *Pik3cb* overexpression attenuates cardiac dysfunction and pathological cardiac hypertrophy of Yap^{cKO} mice.

AAV9:Pik3cb increased cardiomyocyte proliferation and decreased cardiomyocyte apoptosis in Yap^{cKO} mice

We investigated potential mechanisms through which AAV9:Pik3cb rescued dysfunction and hypertrophy of Yap^{cKO} hearts. Yap^{cKO} mice were reported to have more cardiomyocyte apoptosis[3], and their cardiomyocyte regenerative capacity was impaired[5]. Because the PI3K-AKT pathway promotes both cell proliferation and survival[22], we hypothesized that *Pik3cb* overexpression ameliorates the Yap^{cKO} phenotype by increasing cardiomyocyte proliferation, decreasing apoptosis, or both.

To investigate the effect on cardiomyocyte proliferation, we measured the fraction of cardiomyocytes that incorporated EdU, administered at 3 weeks of age (experimental timeline shown in Fig. 7A). Staining of tissue sections showed that cardiomyocyte EdU uptake was higher in Yap^{cKO} mice treated with AAV9:Pik3cb, compared to those treated with AAV9:Luci (Fig. 8A,B). We further confirmed this finding by staining dissociated cardiomyocytes (Fig. 8C,D), which obviates potential artifacts that can occur in tissue sections. Moreover, AAV9:*Pik3cb* increased the fraction of mononuclear cardiomyocytes (Fig. 8E), which contains the proliferative cardiomyocyte subset.[23], and it increased the frequency that we observed cardiomyocytes in cytokinesis, as marked by staining with Aurora B kinase (Fig. 8F).[23] Together, these data demonstrated that *Pik3cb* stimulated cardiomyocyte proliferation in YAP-deficient hearts.

To study the effect of *Pik3cb* overexpression on apoptosis in YAP-deficient cardiomyocytes, we measured the fraction of cardiomyocytes positive for TUNEL-staining, a marker of apoptosis. $TUNEL^{+}$ cardiomyocytes were less frequent in Yap^{cKO}, treated with AAV9:Pik3cb compared AAV9:Luci, indicating that *Pik3cb* rescues cardiomyocyte apoptosis caused by YAP-deficiency (Fig. 8G,H).

Yap activation increased the expression of both *Pik3ca* and *Pik3cb* (Fig. 3B), and *Pik3cb* was sufficient to induce the expression of *Pik3ca* in wild type mouse heart (Online Fig. IV-G). These data suggest that *Pik3cb* functions downstream of *Yap* to regulate *Pik3ca* expression, AKT activation, and p27 levels. Indeed, *Pik3cb* overexpression in the absence of

YAP rescued AKT activation without changing total AKT level (Fig. 8I). AKT activation corresponded with decreased p27 protein but not mRNA (Fig. 8I-J).

Together, our data support a model in which YAP directly activates *Pik3cb* expression through TEAD binding to an enhancer in the first *Pik3cb* intron. PIK3CB subsequently promotes expression of Pik3ca and activation of AKT, which regulates cardiomyocyte apoptosis and proliferation, in part through p27 (Fig. 8K).

DISCUSSION

Emerging studies have revealed the critical role of Hippo-YAP signaling in heart development, growth, and homeostasis.[2-5, 7, 8]. One major pathway through which Hippo-YAP signaling regulates cardiomyocyte growth and survival is the PI3K-AKT signaling axis[3, 4]. This pathway has well established, pleiotropic effects on cardiomyocyte proliferation, growth, survival, and function[24]. However, the mechanistic link between YAP and the PI3K-AKT pathway was not previously known. Our genome-wide screen for directly activated YAP target genes showed that YAP, through its transcriptional partner TEAD, directly activates *Pik3cb* expression via an enhancer in the first intron of *Pik3cb*. Our functional analyses demonstrate that YAP requires *Pik3cb* to promote cardiomyocyte proliferation and activate the AKT pathway. Together these findings establish *Pik3cb* as a regulator of cardiac growth, serving as a direct link between Hippo-YAP and PI3K-AKT signaling pathways.

In mammals, most cells express both *Pik3ca* and *Pik3cb*, isoforms of the p110 catalytic subunit of Class IA PI3K. These isoforms each have unique functions, as germline inactivation of either *Pik3ca* or *Pik3cb* caused embryonic lethality before E10.5[10, 25]. Compared to *Pik3ca*, relatively less is known about *Pik3cb* and how it differs from *Pik3ca*. Interestingly, *Pik3cb* is unique among PI3-kinases in signaling downstream of both receptor tyrosine kinases and G-protein coupled receptors[26]. Adult, cardiac-specific inactivation of *Pik3cb* did not cause a baseline cardiac phenotype, while similar inactivation of *Pik3ca* caused cardiac dysfunction[27]. These data showing that *Pik3cb* is dispensable for adult heart homeostasis are compatible with our observations, as it is likely that *Pik3cb* regulates physiological heart growth and stress responses downstream of YAP. Indeed, both YAP and PIK3CB protein levels in the heart decline with postnatal age, and our data point to a role of *Pik3cb* in promoting heart growth downstream of *Yap*. Additional studies will be required to interrogate further the roles of *Pik3cb* in heart development, stress responses, and regeneration.

Our work demonstrates that YAP functions directly upstream of *Pi3kcb* to enhance its expression. However, *Pik3cb* expression is likely regulated by other factors as well, as its expression was reduced but not completely abrogated by YAP knockout. Recently, it was reported that growth factors such as EGF signal through receptor tyrosine kinases to PI3K, which then promotes disassembly of the Hippo kinase complex, relieving its inhibition of YAP transcriptional activity[28]. Combined, these studies suggest reciprocal, mutually stimulatory cross-talk between YAP and PI3K that establish a feed forward regulatory

circuit, in which Yap increases the expression of the PI3K subunit *Pik3cb*, and PI3K stimulates YAP activity.

Yap^{cKO} mice had heart dysfunction[3, 5]. Several targets of Yap have been identified, such as *Ctgf*, *Birc3* and *Birc5*, but none have been shown to be essential for the Yap^{cKO} phenotype. By activating *Pik3cb* in 1-day old Yap^{cKO} pups, we largely restored heart function of Yap^{cKO} mice, indicating that *Pik3cb* is a major target of Yap and is important for preserving heart function. This result differs from the observation that *Pik3cb* is dispensible for normal heart homeostasis[27]. We reasoned that the different experimental contexts (normal adult heart versus failing juvenile heart) likely account for the different results. YAP is normally downregulated in the mature adult heart[2], and it is possible that YAP and *Pik3cb* play more vital roles during heart development and postnatal growth. Furthermore, the Yap^{cKO} mouse has systolic dysfunction at birth that progresses over ~3 months to death. Thus, the Yap^{cKO} represents a stressed heart, which may have a different requirement for Pik3cb than a normal heart.

The heart failure phenotype of Yap^{cKO} is likely multifactorial. Consistent with published data, we found less proliferation and more apoptosis in the Yap^{cKO} mice[3], indicating that the heart dysfunction of Yap^{cKO} mice is at least partially due to cumulative effect of cardiomyocyte insufficiency. *Pik3cb* rescued Yap^{cKO} heart function and our data indicate that this is through both mitogenic and pro-survival activities, in keeping with known roles of PI3K-AKT signaling.The cell cycle inhibitor p27, which is normally down-regulated at the protein level by AKT phosphorylation-triggered degradation[20], was upregulated in Yap^{cKO}. P27 heterozygous inactivation enhanced cardiomyocyte proliferation[29], suggesting that its aberrant expression in Yap^{cKO} heart contributes to reduced cardiomyocyte proliferation seen in these mutants (Fig.8E). At present, we are unable to determine the relative contribution of *Pik3cb*'s proproliferative versus pro-survival effects to its overall beneficial activity.

Other cardiomyocyte functions are also likely to be regulated by YAP. For instance, TEAD transcription factors are implicated in regulating sarcomere gene expression through its recognition sequence, known as the MCAT motif[30], suggesting a role for YAP in regulation of sarcomere assembly and function. Consistent with this idea, YAP-bound genes in our ChIP-seq data were more highly enriched for functional terms related to cardiovascular system development than terms related to cell proliferation. YAP has also been reported to regulate cell metabolism, a function that intersects with a well-described function of PI3K-AKT signaling. A subset of these YAP activites are likely to be independent of PIK3CB. This, in combination with incomplete transduction of all cardiomyocytes by AAV9:Pik3cb, likely accounts for incomplete *Pik3cb* rescue of Yap^{cKO} hearts.

In summary, we identified *Pik3cb* as a crucial direct target of YAP that links Hippo-YAP and PI3KAKT signaling pathways (Fig. 8K). YAP, through its transcriptional partner TEAD, increases *Pik3cb* expression, which further activates AKT. *Pik3cb* activation downstream of YAP promotes cardiomyocyte proliferation and survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

YAP	Yes-associated protein
MST1/2	Macrophage Stimulating 1/2
LATS1/2	Large tumor suppressor kinase 1
TEAD1-4	TEA-domain family members 1-4
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
AKT	protein kinase B
MI	myocardial infarction
ChIP-seq	Chromatin immunoprecipitation followed by high throughput sequencing
ChIP-qPCR	Chromatin immunoprecipitation followed by quantitative PCR
STAT	Signal transducers and activators of transcription ETS v-ets avian erythroblastosis virus E26 oncogene homolog
p27	cyclin-dependent kinase inhibitor 1B
NRVM	neonatal rat ventricular cardiomyocyte
EdU	5-ethynyl-2 ´-deoxyuridine
BrdU	bromodeoxyuridine
cTNT	cardiac troponin T
AAV9	adeno-associated virus serotype 9 AAV9:cTNT-3Flag-Pik3cb (AAV9:Pik3cb) AAV9 with cTNT promoter expressing Pik3cb with 3 FLAG epitope tags
AAV9:cTNT-Luci	(AAV9:Luci) AAV9 with cTNT promoter expressing luciferase
AAV9:YAP-shPik3cb	AAV9 with cTNT promoter expressing YAP[S127A] and a Pik3cb-directed shRNA
AAV9:YAP-sc	AAV9 with cTNT promoter expressing YAP[S127A] and a scrambled shRNA

AAV9:Luci-sc	AAV9 with cTNT promoter expressing luciferase and a scrambled shRNA
YAP^{cKO}	cardiac specific YAP knockout (Myh6-Cre::Yap$^{fl/fl}$)

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Novelty and Significance

What Is Known?

- Loss of cardiomyocytes is associated with increased mortality and morbidity.
- There is no effective means to replace the lost cardiomyocytes.
- The transcriptional co-activator YAP is essential for heart growth and for normal adult heart systolic function. Inactivation of YAP causes lethal dilated cardiomyopathy with reduced cardiomyocyte proliferation and increased apoptosis.
- YAP activation increases cardiomyocyte proliferation, albeit modestly.
- The direct targets of Yap that convey its mitotic signal have not been defined.
- YAP is known to activate the phosphoinositide 3-kinase (PI3K)-AKT pathway, a key regulator of cell proliferation and survival. However, the molecular link is not known.

What New Information Does This Article Contribute?

- Through an unbiased, genome-wide screen, we found that YAP directly activates expression of *Pik3cb* through a conserved enhancer within its first intron. *Pik3cb* encodes a less-studied isoform of the catalytic subunit of PI3K.
- *Pik3cb* promotes cardiomyocte proliferation and survival by stimulating *Pik3ca* expression and AKT activation.
- YAP-induced cardiomyocyte proliferation requires upregulation of *Pik3cb*.
- Heart failure resulting from inactivation of YAP was mitigated by activation of *Pik3cb*. Forced expression of *Pik3cb* normalized cardiomyocyte proliferation and survival in the absence of YAP, pointing out the key role of *Pik3cb* and the PI3K-AKT pathway in the physiological function of YAP.

This study focused on delineating the mechanism by which YAP regulates cardiomyocyte proliferation and survival. By employing unbiased whole genome profiling methods and AAV-mediated gain- and loss-of-function studies, we confirmed that *Pik3cb* is a major downstream target of YAP, being both sufficient and necessary for YAP activation of AKT. These data show that *Pik3cb* is a crucial link between Hippo-YAP and PI3K-AKT pathways.

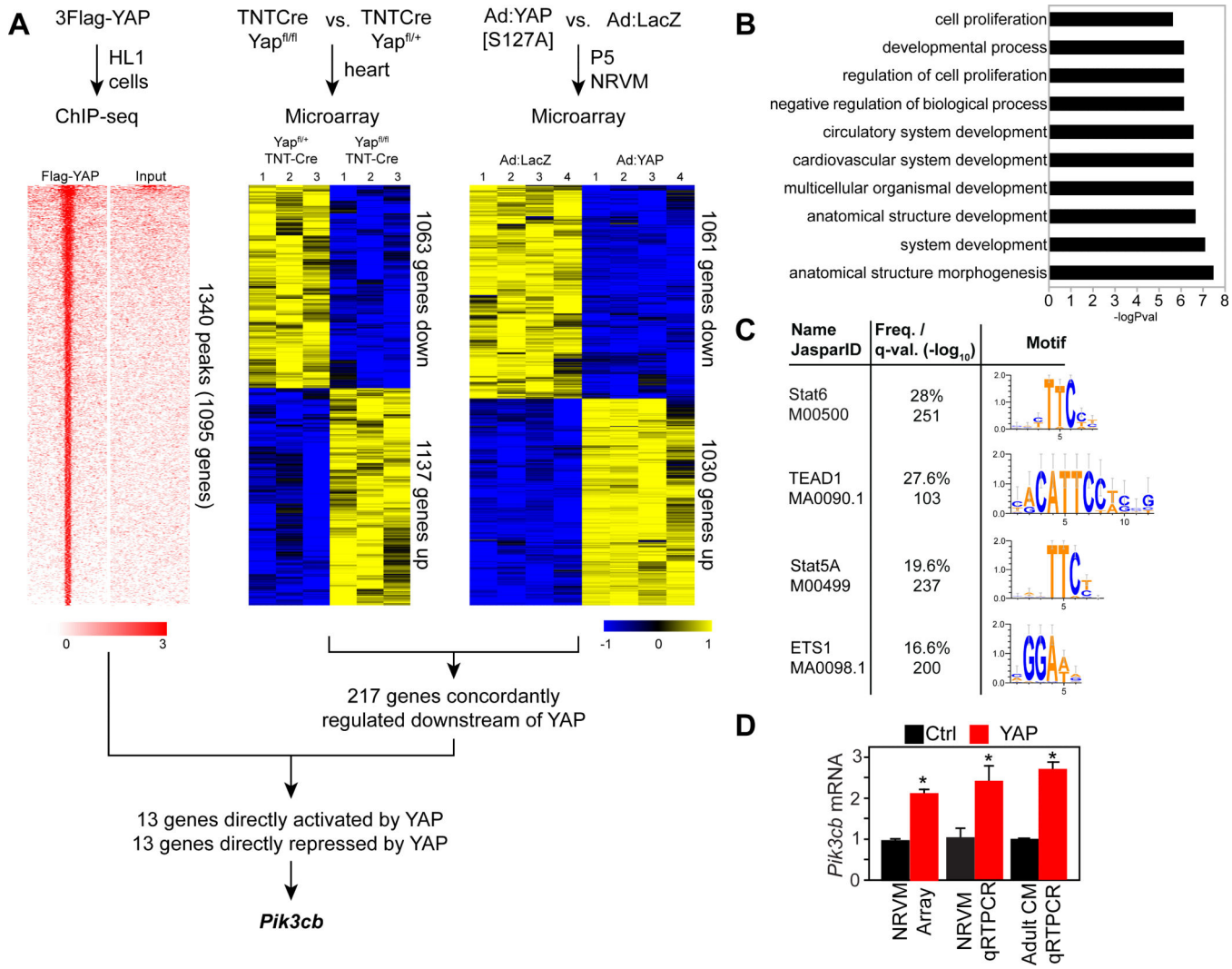

Figure 1. Genome-wide screen to identify genes directly regulated by YAP in cardiomyocytes

A. Strategy to identify genes directly bound by YAP and differentially expressed in YAP gain- and loss-of-function. ChIP-seq was performed using Flag-tagged YAP in HL1 cells. Tag heatmap shows signal intensity in ChIP and input sample in a 4 kb window centered on YAP peaks. Differential gene expression in YAP loss-of-function and gain-of-function were determined by microarray analysis of E12.5 hearts with cardiomyocyte-restricted YAP knockout, and NRVMs that overexpressed activated YAP. Heatmap shows the row-scaled expression z-score. **B**. Gene Ontology term analysis of YAP- bound genes. **C**. Motif analysis of the top 1000 YAP bound regions. **D**.YAP overexpression induced upregulation of *Pik3cb*. *, P<0.05.

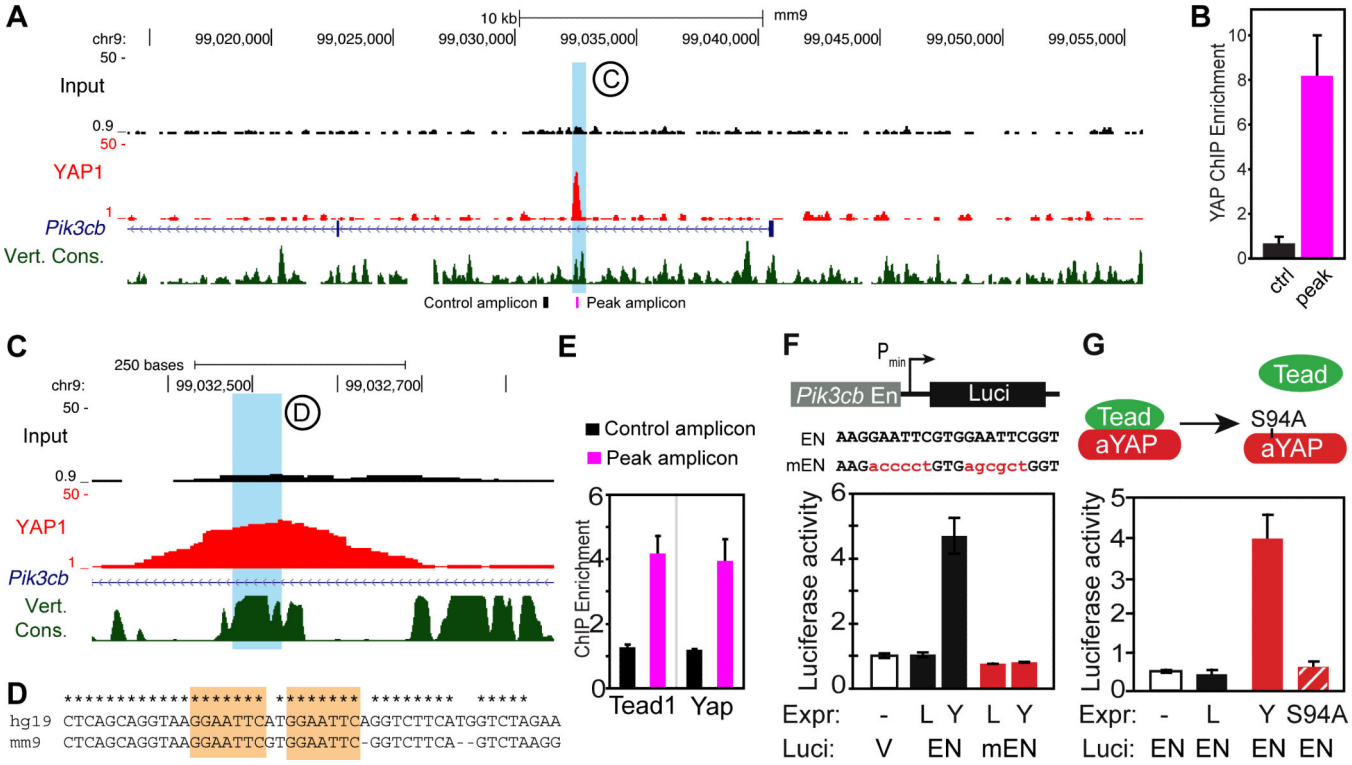

Figure 2. YAP binds and activates an enhancer of Pik3cb

A. Genome browser view showing YAP ChIP-seq signal around *Pik3cb*. HL1 cells overexpressing FLAG-tagged YAP were processed for FLAG ChIP-seq. Blue shaded area highlights a YAP peak in the first intron on *Pik3cb*. **B.** ChIP-qPCR measurement of FLAG-YAP occupancy of the peak region and an adjacent control region. Locations of amplicons are indicated by the black and purple labels in the bottom of panel (A). **C-D.** Magnified view of the YAP-occupied region of *Pik3cb*, also showing vertebrate conservation. The sequence of the region in blue is shown in (D). * indicates identity between mouse and human genomes. Orange shading indicates two conserved binding motifs. **E.** ChIP-qPCR measurement of TEAD1 and YAP occupancy of the peak region and adjacent control region in 8-day-old mouse heart. Amplicons indicated by lack and purple labels in the bottom of panel (A). **F.** A 552 bp region encompassing the YAP ChIP-seq peak (En) was cloned into a minimal promoter-luciferase construct. A mutant version (mEN) contained the base substitutions in the TEAD motif indicated in red. NRVMs were transfected with YAP (Y) or LacZ (L) expression constructs and wild-type (En) or mutant (mEN) *Pik3cb* enhancer-luciferase reporter constructs. Luciferase activity was normalized to an internal transfection control. **G.** NRVMs were transfected with the *Pik3cb* intronic enhancer-luciferase reporter construct (EN) and either YAP (Y), YAP[S94A] (YS94A), or LacZ (L). Luciferase activity was normalized to an internal transfection control.

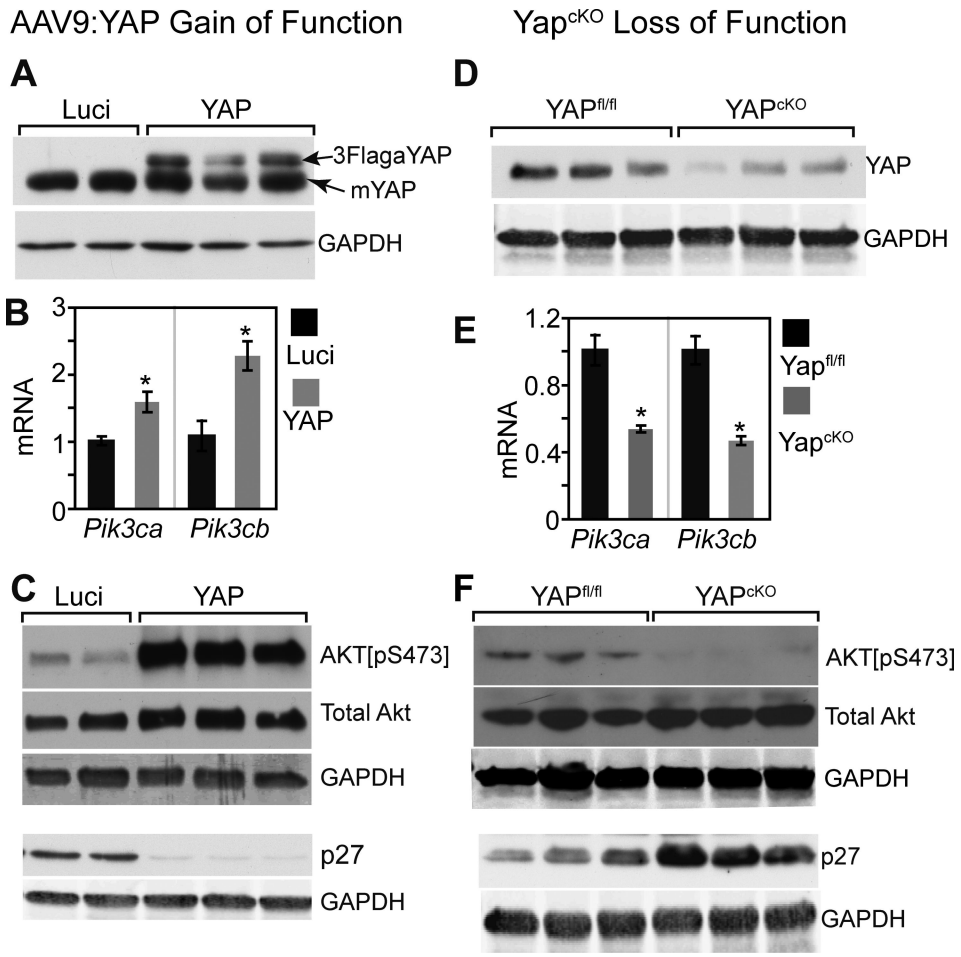

Figure 3. YAP is sufficient and required for activating the PI3K-AKT pathway

A. AAV9:YAP-mediated overexpression of activated YAP. AAV9:YAP was subcutaneously injected into P3 pups. 7 days later, hearts were collected for western blotting. AAV9:Luci served as a negative control. **B.** qRTPCR measurement of *Pik3cb* and *Pik3ca* mRNA level in hearts of mice treated with AAV9:YAP or AAV9:Luci. *, P<0.05. n=4. **C.** Western blot assessment of AKT pathway activation state in hearts of AAV9:YAP or AAV9:luci-treated mice. Primary antibodies were directed against AKT[pS473] (activated AKT), total AKT, GAPDH (internal loading control), or p27. **D.** YAP protein level in hearts from 4-week-old Yap^{cKO} mice and their littermate controls ($Yap^{fl/fl}$). **E.** qRT-PCR measurement of *Pik3cb* and *Pik3ca* mRNA level in Yap^{cKO} and $Yap^{fl/fl}$ heart. *, P<0.05, n=3. **F.** Western blot assessment of AKT pathway activation state in Yap^{cKO} and $Yap^{fl/fl}$ heart.

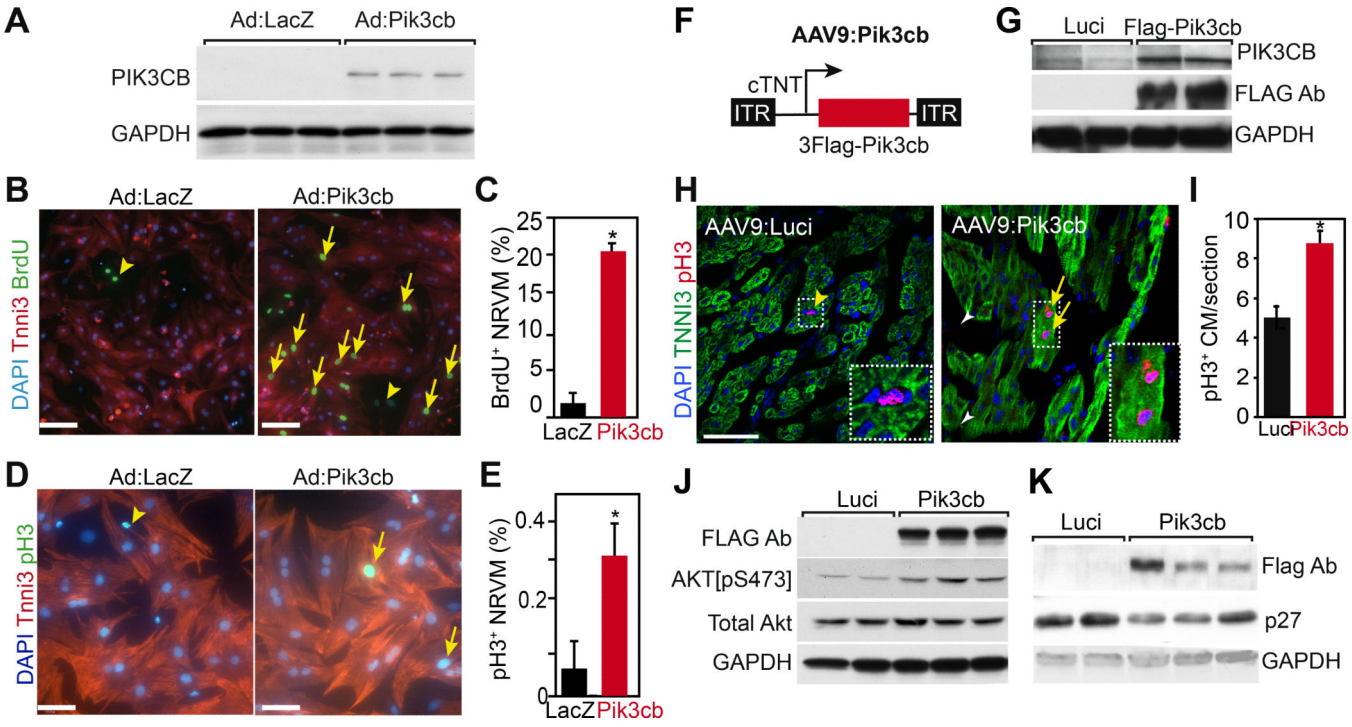

Figure 4. *Pik3cb* overexpression increased cardiomyocyte proliferation

A. Adenovirus-mediated *Pik3cb* overexpression in cultured NRVMs isolated on postnatal day 4 (P4). PIK3CB protein was detected by immunoblotting. **B-E.** Effect of *Pik3cb* overexpression on P4 NRVM proliferation in vitro was measured by BrdU incorporation rate (B, C) and phosphohistone H3 positive (pH3+) cardiomyocyte frequency (D,E). Bar = 50 μm. Arrows indicate BrdU and pH3 positive cardiomyocytes. Arrowheads indicate non-myocytes. *, P<0.05. n=3. **F.** Schematic of the AAV9:Pik3cb construct. 3Flag epitope-tagged *Pik3cb* was expressed from the cardiac troponin T promoter. **G.** AAV9:Luci and AAV9:Pik3cb were injected subcutaneously into P2 neonatal mice. 8 days later, hearts extract western blots were probed with FLAG, PIK3CB, or GAPDH antibodies. **H, I.** Effect of *Pik3cb* overexpression on neonatal cardiomyocyte proliferation in vivo. AAV9:Luci or AAV9:Pik3cb were administered subcutaneously to P2 neonatal mice. Hearts were analyzed by immunofluorescent staining at P9. Arrows and arrowheads indicate $pH3^+$ cardiomyocytes and non-myocytes, respectively. Boxed area is enlarged in inset. Bar = 50 μm. *, P<0.05. n=4. **J,K.** Immunoblot analysis of P9 heart lysates. Mice were treated with AAV:Luci or AAV9:Pik3cb at P2.

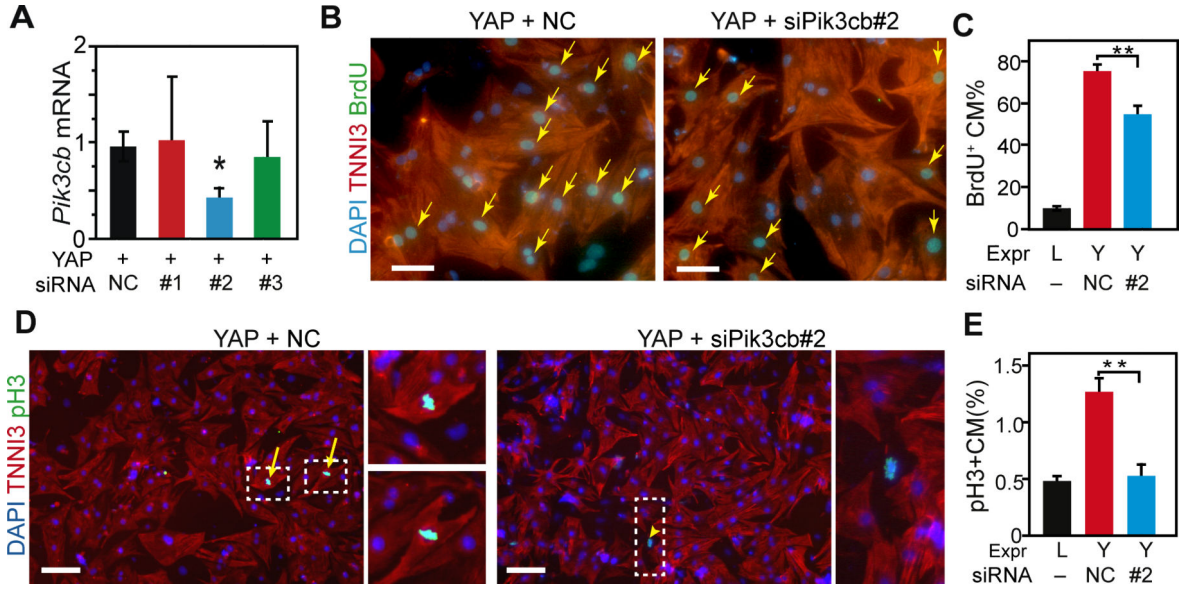

Figure 5. Yap stimulation of NRVM proliferation requires Pik3cb

A. Validation of siRNAs directed against *Pik3cb*. NRVMs were transfected with the indicated siRNAs. After 2 days, *Pik3cb* mRNA was measured by qRT-PCR. NC, negative control. **B-E.** Effect of *Pik3cb* knockdown on YAP-stimulated NRVM proliferation. P4 NRVMs were treated with control (NC) or *Pik3cb* siRNA and adenovirus expressing LacZ (L) or YAP (Y). Proliferation was measured 1 day later by BrdU uptake (B,C) or pH3 staining (D,E). Dashed rectangles indicate areas enlarged in insets. Arrows and arrowheads indicate positive cardiomyocytes and non-myocytes, respectively. Bar = 50 μm (B) or 100 μm (D). *, P<0.05. n=3.

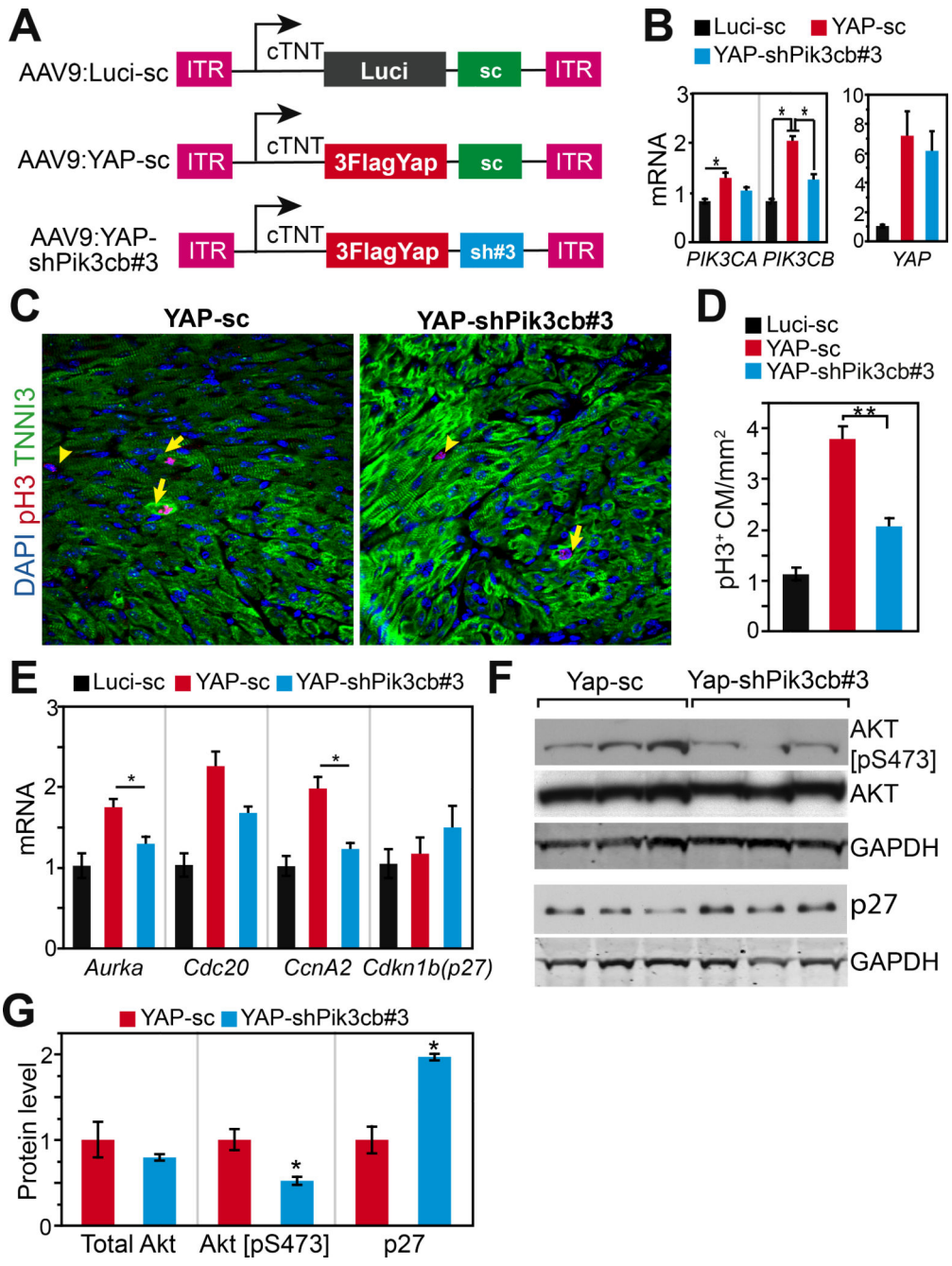

Figure 6. Yap stimulation of mouse cardiomyocyte proliferation requires *Pik3cb* in vivo

A. Schematic view of AAV plasmids. shRNA effective for *Pik3cb* knockdown (shPik3cb#3) was cloned into AAV ITR plasmid downstream of YAP[S127A] (human) to simultaneously express YAP and knockdown *Pik3cb*. Control constructs contained Luci (luciferase) or scrambled control shRNA (sc). **B.** qRT-PCR measurement of *Pik3cb*, *Pik3ca* and *Yap* mRNA levels from P9 mouse heart. AAV was injected into mice at P2. Primers that amplified both mouse and human *Yap* were used for measuring *Yap* expression level. **C, D.** pH3 immunofluorescence staining of heart sections. AAV was injected into 2-dayold mouse pups and hearts were analyzed at P9. **E.** qRT-PCR measurement of cell cycle genes from P9

hearts after AAV treatment at P2. **F, G.** Effect of *Pik3cb* knockdown in the presence of activated YAP on activated Akt and p27 protein levels. B, D, E, n=4. *, P<0.05.

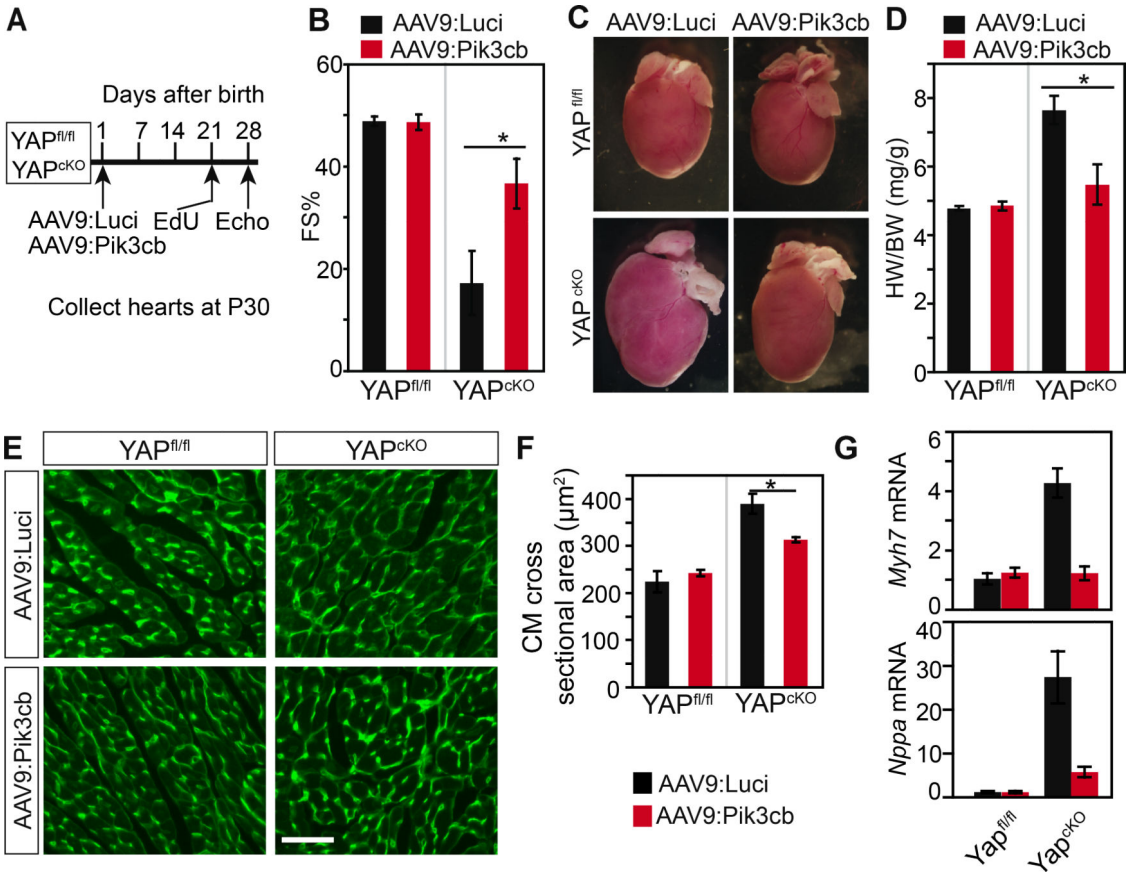

Figure 7. AAV9:Pik3cb mitigated heart dysfunction in Yap^{cKO} mice

A. Experimental design. AAV9:Luci or AAV9:Pik3cb were administered to Yap^{cKO} or control (Yap$^{fl/fl}$) mice at P1. Echocardiography and heart collection were performed at P30. **B.** Effect of *Pik3cb* overexpression on heart dysfunction of Yap^{cKO} mice. Fractional shortening (FS) was measured by echocardiography. *, $P<0.05$. $n=4$. **C,D.** Effect of *Pik3cb* overexpression on cardiomegaly of Yap^{cKO} mice. *, $P<0.05$. $n=4$. **E,F.** Effect of *Pik3cb* overexpression on Yap^{cKO} cardiomyocyte size. Wheat germ agglutinin (WGA) stained heart cross-sectional area. Bar=50 μm. *, $P<0.05$. $n=3$. **G.** Effect of *Pik3cb* overexpression on expression of hypertrophic marker genes *Nppa* and *Myh7* in Yap^{cKO} heart.

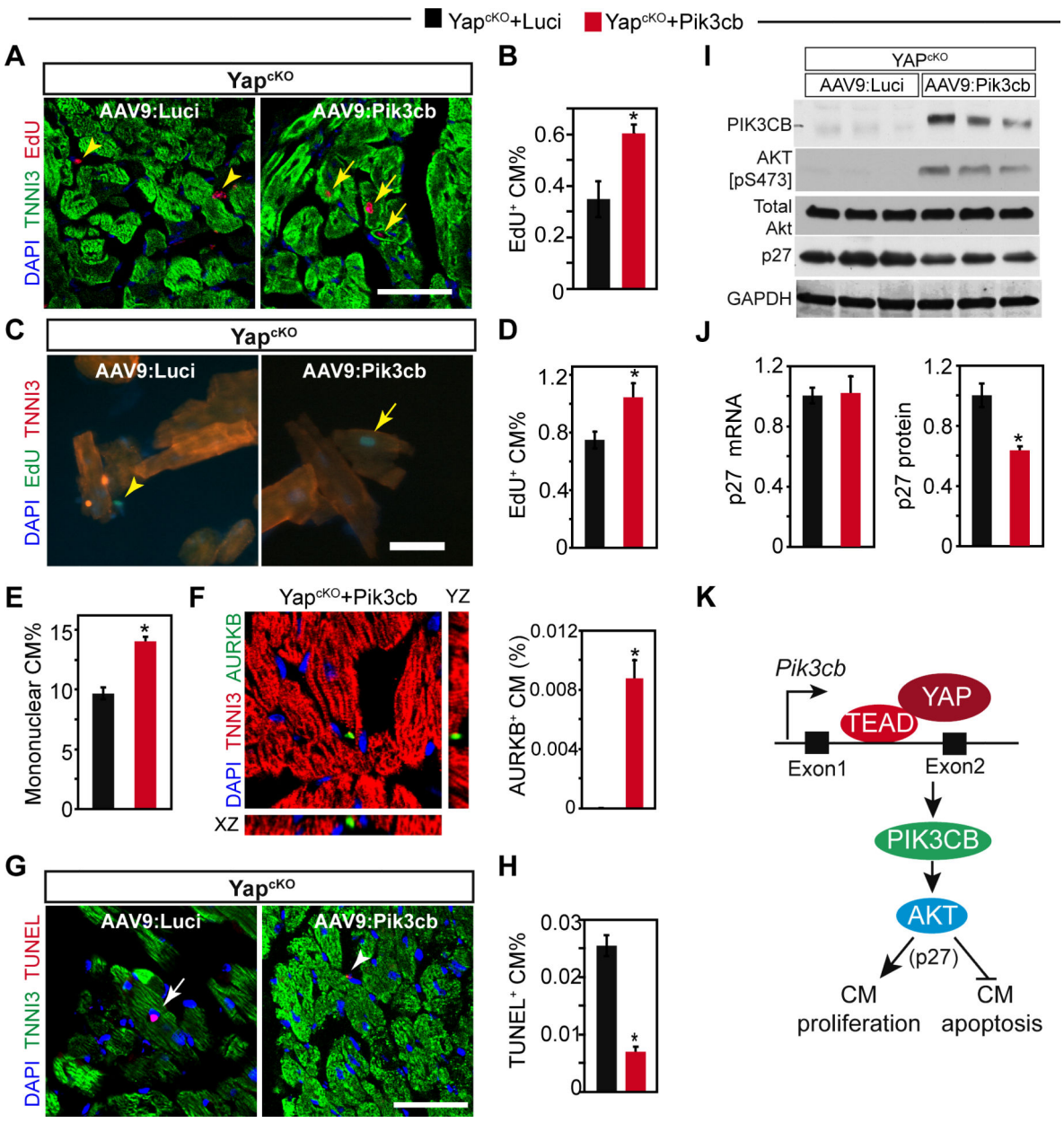

Figure 8. AAV9:Pik3cb increased cardiomyocyte proliferation and decreased cardiomyocyte apoptosis in the absence of YAP

Yap^{cKO} mice were treated with AAV9:Pik3cb or AAV9:Luci as indicated in Fig. 7A. **A-F**. Effect of *Pik3cb* overexpression on depressed cardiomyocyte proliferation seen in Yap^{cKO} heart. Cardiomyocyte proliferation was measured by uptake of EdU (A-D) 4 weeks after birth. **A-B**. EdU staining and quantification. EdU was administered once at P21. **C-D**. EdU quantification with dissociated cardiomyocytes. EdU was administered at P21 and P24. Arrows and arrowheads indicate EdU positive cardiomyocytes and non-myocytes, respectively. **E.** Percentage of mononuclear cardiomyocytes. **F.** Measurement of cytokinesis. Aurora kinaseB antibody was used to detect cardiomyocytes undergoing cytokinesis. **G-H.** Effect of *Pik3cb* overexpression on cardiomyocyte apoptosis in Yap^{cKO} heart, as measured

by TUNEL assay. Arrows and arrowheads indicate $TUNEL^+$ cardiomyocytes and nonmyocytes, respectively. **I.** Effect of *Pik3cb* overexpression on AKT activation and p27 expression in Yap^{cKO} heart. Lysates collected from one-month old mouse hearts were analyzed by western blotting. **J.** Effect of *Pik3cb* overexpression on p27 mRNA and protein levels in Yap^{cKO} heart. **K.** YAP-TEAD stimulated *Pik3cb* transcription through an enhancer in the first intron. *Pik3cb* activates AKT, which suppresses cardiomyocyte proliferation and increases cardiomyocyte apoptosis, in part through increased p27 protein. Bar = 50 μm. *, $P<0.05$. $n=3$.