Pi3kcb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival

Supplemental Material

A. Detailed Materials and Methods.

Mice

$Yap^{fl/fl}$ [1], TNT-Cre[2], and MHCα–Cre[3] alleles were previously described. 5-ethynyl-2'deoxyuridine (EdU) was administered at 5 μg/g bodyweight IP. Echocardiography was performed on a VisualSonics Vevo 2100 with Vevostrain software. To induce MI, mice aged 8 weeks were subjected to LAD ligation as described previously.[4]

Cell culture

4-day-old rat pups were used for cardiomyocyte isolation. Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured using the Neomyts isolation kit (Cellutron, cat#: nc-6031). NRVM culture and proliferation studies were carried out as described previously.[5]

HL1 cells were obtained from William Claycomb and cultured as described.[6]

Pik3cb enhancer cloning and Luciferase activity measurements

A 552 bp fragment of mouse *Pik3cb* genomic DNA was amplified with the following primers: 5'-AGTTTCCAATTTCCCCGTGG-3' and 5'-CTTAAATGTCAGTTGTTCAGA-3'. The PCR product was then cloned into pGL basic vector. NRVMs were cultured in 24-well plates for luciferase assay. 500 ng/well of the indicated plasmids and 500 ng pRLTK internal control vector (Promega) were transfected with 1.25 μl Lipofectamine 2000 (Invitrogen), and medium was changed 4 hours after transfection. Luciferase activity was measured 24 hours after transfection using the Dual-Luciferase reporter assay system (Promega).

siRNA and shRNA

A TriFECTaTM Dicer-Substrate RNAi kit (IDT) containing three siRNAs was used to knock down *Pik3cb* in NRVM. Four independent shRNAs against mouse *Pik3cb* were designed using a published algorithm[7]. The *Pik3cb* shRNAs were cloned into CAG-miR30-GFP plasmid to test the *Pik3cb* knock down efficiency in cultured MES13 cells. We then used a previously described method[8] to make AAV that simultaneously expressing Yap and *Pik3cb* shRNA. The sequences of the rat *Pik3cb* siRNAs and mouse *Pik3cb* shRNAs are listed in Online Table III.

AAV and adenovirus

Adenovirus was generated using the AdEasy system.

3Flag-hYAP, Luciferase and 3Flag tagged human *Pik3cb* were separately cloned into ITR-containing AAV plasmid (Penn Vector Core P1967) harboring the chicken cardiac TNT promoter, to yield pAAV.cTnT::3Flag-hYAP and pAAV.cTnT::Luciferase, pAAV.cTnT::Pik3cb, respectively. The human Yap used in this study is a constitutive active version, which contains a Serine 127 Alanine mutation.[9] AAV was packaged using AAV9:Rep-Cap and pAd:deltaF6 (Penn Vector Cre) as described.[10]

AAV9 was packaged in 293T cells with AAV9:Rep-Cap and pHelper (pAd deltaF6, Penn Vector Core) and purified and concentrated by gradient centrifugation. AAV9 titer was determined by quantitative PCR.

Histology

EdU was detected with the Click-iT EdU imaging kit (Life Technologies). Imaging was performed on a Fluoview 1000 confocal or a Nikon TE2000 epifluorescent microscope.

Informatics

Reads were mapped using Bowtie[11] and peaks were called with Homer[12]. Motif analysis was performed with CompleteMotifs[13].

B. Supplemental References

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C. Online Tables

Online Table I. Antibodies used in this study

Primary antibodies

Antigen	Source	Species	Working dilution
Cardiac troponin I (TNNI3)	Abcam	Goat	1 to 200, IF
Flag	Sigma	Rabbit	1 to 200, IF
Luciferase	Abcam	Rabbit	1 to 200, IF
GAPDH	Sigma	Mouse	1 to 200000, IB
WGA-555	Invitrogen	NA	1 to 25, IF
Pik3cb	Santa Cruz	Rabbit	1 to 1000, IB
Phospho AKT S473	CST	Rabbit	1 to 1000, IB
AKT	CST	Rabbit	1 to 1000, IB
p27	Santa Cruz	Rabbit	1 to 1000, IB
YAP	CST	Rabbit	ChIP
TEAD1	BD transduction laboratories	Mouse	ChIP
FLAG	Sigma	Mouse	ChIP
Aurora B kinase	Abcam ab2254	Rabbit	1 to 200, IF

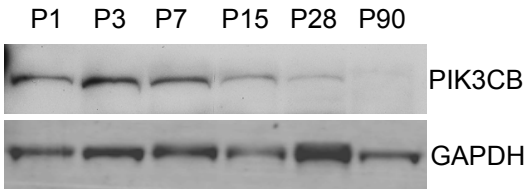
Secondary antibodies

anti-goat Alexa488	Invitrogen	Donkey	1 to 500, IF
anti-goat Alexa647	Invitrogen	Donkey	1 to 500, IF
anti-rabbit Alexa555	Invitrogen	Donkey	1 to 500, IF
anti-rabbit HRP	Invitrogen	Goat	1 to 10000, IB

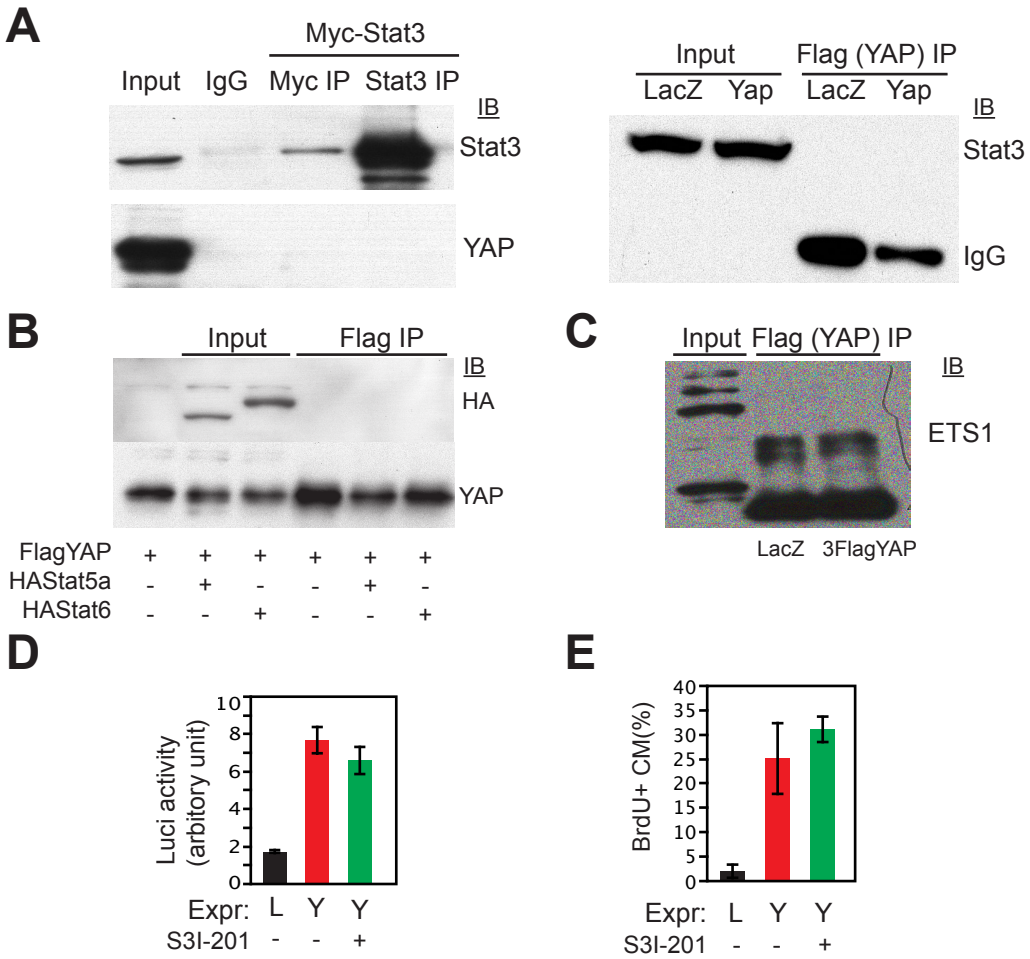
Online Table III. Primers and DNA oligo sequences used in this study

Gene	Species	Forward	Reverse
		Syber green primers	
Ccna2	Mouse	GCCTTCACCATTCATGTGGAT	TTGCTCCGGGTAAAGAGACAG
CDC20	Mouse	TTCGTGTTCGAGAGCGATTTG	ACCTTGGAACTAGATTTGCCAG
Aurka	Mouse	GGGTGGTCGGTGCATGCTCCA	GCCTCGAAAGGAGGCATCCCCACTA
Myh6	Mouse	CTCTGGATTGGTCTCCCAGC	GTCATTCTGTCACTCAAACTCTGG
Yap	Mouse/Human	GACCCTCGTTTTGCCATGAA	ATTGTTCTCAATTCCTGAGAC
Cdkn1b	Mouse	GGCCTTCGACGCCAGACGTAA	GCGCAATGCTACATCCAATGCTT
Igf1r	Mouse	CTTTGCGAGAACCATGCCAG	TAGACGGTTGAGTTTGGCCC
Pik3cb	Mouse/rat	GGGGAAGCGTGGGGCACATG	AGGTCAGAGAGCGCCTCCCG
GAPDH	Mouse	CAGGTTGTCTCCTGCGACTT	GGCCTCTCTTGCTCAGTGTC
Pik3ca	Mouse	AAAATGACAAGGAACAGCTCCG	GCAGTACATCTGGGCCACTTC
Nkx2-5	Mouse	CCAAGTGCTCTCCTGCTTTCC	CGCGCACAGCTCTTTTTTATC
		ABI Taqman probes accession number	
GAPDH	Mouse	4352339E	
NPPA	Mouse	Mm01255747_g1	
Myh7	Mouse	Mm00600555_m1	
		ChIP Primers (Mouse)	
Pik3cb	Enhancer	ACCTGCATTGCCCACATAAT	AGTGGCTCAGCAGGTAAGGA
Pik3cb	control	CCTTGGCTGGCATTACTGAT	GCACTTAGCACAGCCTGACA
		siRNA and shRNA target sequences	
Neg.ctrl	Rat	CGTTAATCGCGTATAATACGCGTAT	siRNA
Pik3cb-1	Rat	GGAAGCAAGTTCACAATTACCCAAT	siRNA
Pik3cb-2	Rat	ACAAGAAATGATTGCCATAGAGGCT	siRNA
Pik3cb-3	Rat	CGATAAGATCATTGAGAAGGCAGCT	siRNA
Scramble	Mouse	GCATAGTACGCATCGTGTAACAA	Target for shRNA
Pik3cb-1	Mouse	CTGTGAAGATGCGTATCTGATTT	Target for shRNA
Pik3cb-2	Mouse	TGTCGCATGGGTAAATACCATGG	Target for shRNA
Pik3cb-3	Mouse	TCACACAGTACGGAAAGACTACA	Target for shRNA

D. Online Figures



Online Figure I. PIK3CB protein levels in normal postnatal mouse heart. Total protein was extracted from wild type mouse hearts of different ages and analyzed for PIK3CB content by immunoblotting. GAPDH was used as loading control. P, postnatal day.



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Online Figure II. YAP does not interact with ETS or STAT3a/Stat5/Stat6.

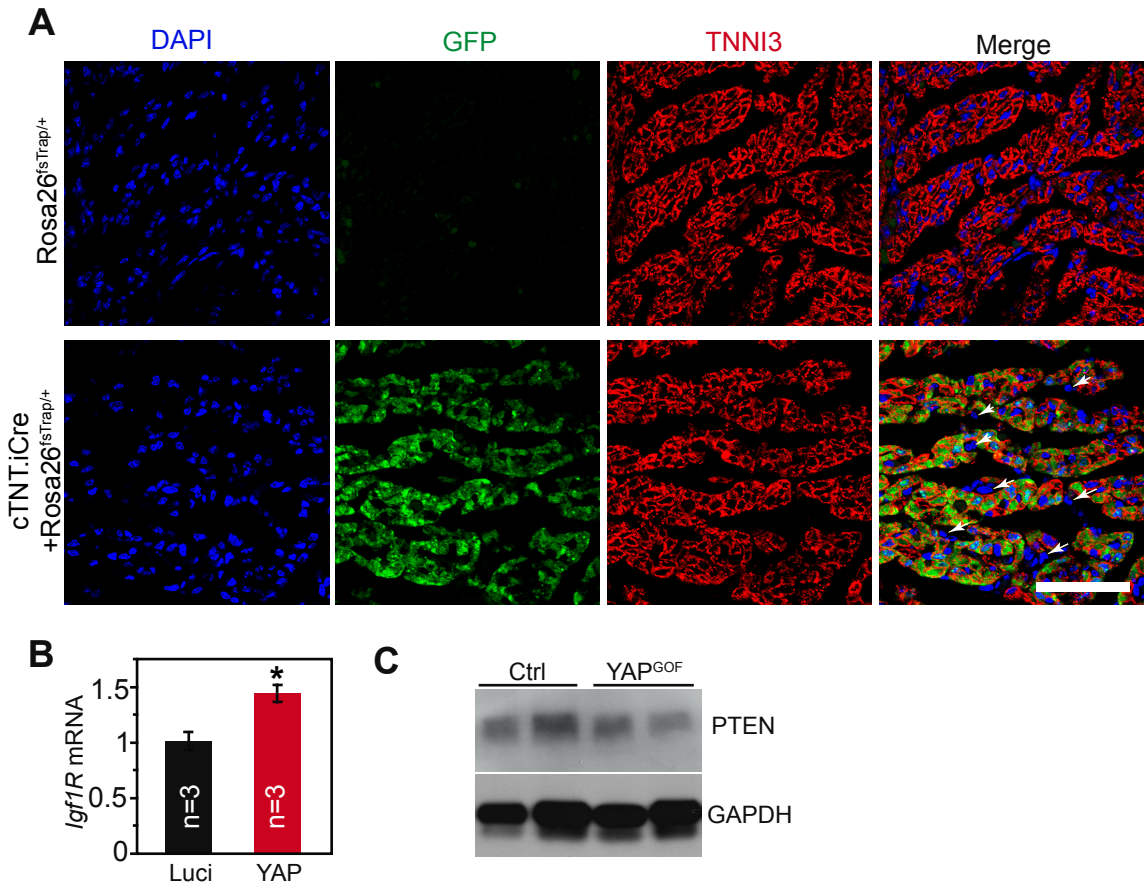
A-B. Co-immunoprecipitation assay did not detect interaction between YAP and Stat3a, Stat5 or Stat6. Myc-Stat3, HA-Stat5a, HA-Stat6, orFLAG-YAP were overexpressed in 293 cells.

C. Flag-YAP does not interact with ETS in HL1 cells.

D. Luciferase assay. S3I-201, a Stat3 inhibitor, did not block YAP activation of the *Pik3cb* enhancer. NRVMs were transfected with LacZ (L) or YAP (Y) expression constructs and Pik3cb enhancer-luciferase reporter constructs.

E. BrdU incorporation assay. S3I-201 did not block YAP-induced cardiomyocyte DNA synthesis. NRVMs were transfected with adenovirus expressing LacZ (L) or YAP (Y).

D-E, n=3 for each group.



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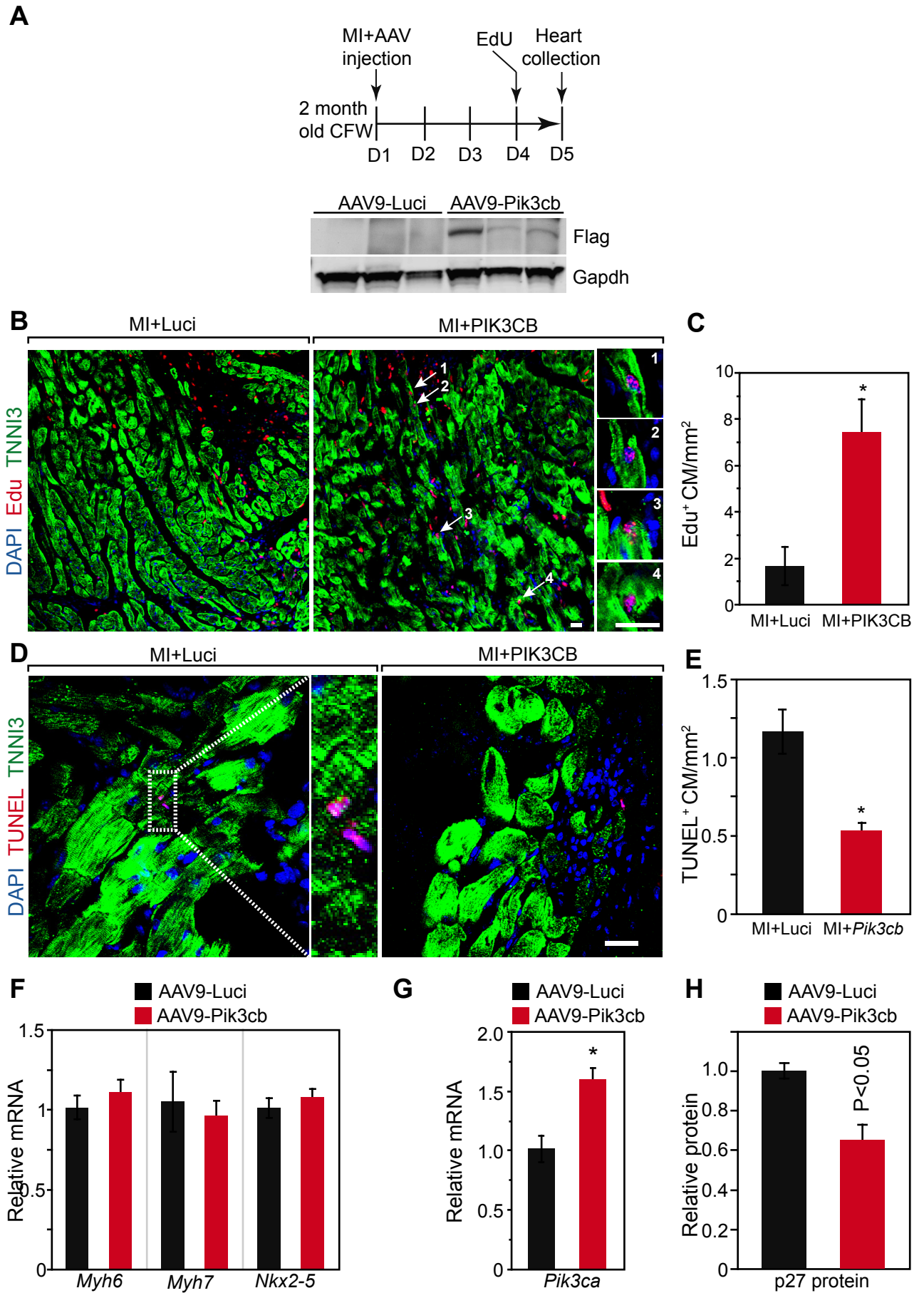
Online Figure III. AAV9-mediated overexpression of YAP.

A. AAV9.cTNT selectively drives cargo expression in cardiomyocytes. Immunofluorescent staining of heart sections from Rosa26$^{fsTRAP/+}$ mice were treated at postnatal day 2 with AAV9.Luci or AAV9.cTNT.iCre. 6.5 days later, hearts were collected for analysis. AAV9:cTNT.iCre-activated GFP signals were detected in TNNI3 positive cardiomyocytes, but not in the TNNI3 negative non-cardiomyocytes (white arrows). Bar = 50 μm.

B. qRT-PCR measurement of IGF1R expression level. Heart RNA from AAV9:Luci and AAV9:YAP transduced mice were used for testing IGF1R expression. **C.** Western blot of PTEN. Heart protein from adult Yap gain of function (YAP^{GOF}) animals were used to test PTEN protein level. GAPDH was used as internal control.



Online Figure IV

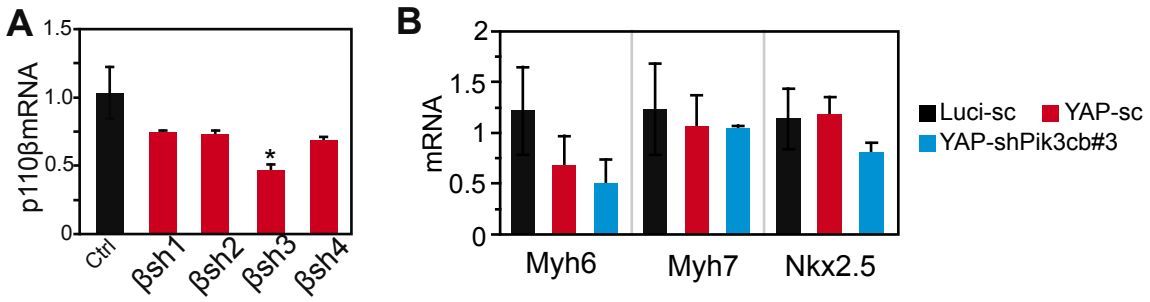
Online Figure IV.

A-E. *Pik3cb* gain-of-function in adult cardiomyocyte proliferation in the context of myocardial infarction. A, upper panel, shows the experimental timeline. 2-month-old CFW mice underwent left anterior coronary artery ligation to produce an MI. AAV was injected into the myocardium immediately after coronary artery ligation. One dose of EdU was administered by intraperitoneal injection 4 days after MI. Lower panel shows immunoblot of Flag-PIK3CB expression in myocardium. GADPH served as the loading control. B-C**.** Cardiomyocyte proliferation was measured by uptake of EdU. Arrows indicate EdU positive cardiomyocytes. Representative examples are magnified on the right. n=3. *, P<0.05. D-E. Cardiomyocyte apoptosis was measured by TUNEL assay. Magnification shows representative TUNEL+ cardiomyocyte nuclei. n=3. *, P<0.05. Bar=25 μm.

F. qRT-PCR measurement of expression of sarcomere and cardiac progenitor gene expression. AAV9:Luci or AAV9:Pik3cb were administered subcutaneously to P2 neonatal mice. Total heart RNA were analyzed by qRT-PCR at P9. N=4. *, P<0.05.

G. qRT-PCR measurement of *PIK3CA* mRNA. Samples were prepared as in F.

H. Quantitation of p27 protein levels, normalized to GAPDH. Samples were prepared as in F. The western blot is shown in Fig. 4K. n=3.



Online FIgure V. *Pik3cb* in vivo knockdown with shRNA. A.qRT-PCR validation of mouse Pik3cb shRNA. MES13 cell line was transfected with indicated Pik3cb shRNAs. 3 days later, cells were collected for qRT-PCR analysis. **B**. qRT-PCR measurement of expression of sarcomere and cardiomyocyte progenitor gene expression. P1 mouse pups were transduce with indicated AAV. 9 days later, hearts were collected for qRT-PCR analysis. n=4. Groups were not significantly different.