YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy

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Heart growth is tightly controlled so that the heart reaches a predetermined size. Fetal heart growth occurs through cardiomyocyte proliferation, whereas postnatal heart growth involves primarily physiological cardiomyocyte hypertrophy. The Hippo kinase cascade is an important regulator of organ growth. A major target of this kinase cascade is YAP1, a transcriptional coactivator that is inactivated by Hippo kinase activity. Here, we used both genetic gain and loss of YAP1 function to investigate its role in regulating proliferative and physiologic hypertrophic heart growth. Fetal YAP1 inactivation caused marked, lethal myocardial hypoplasia and decreased cardiomyocyte proliferation, whereas fetal activation of YAP1 stimulated cardiomyocyte proliferation. Enhanced proliferation was particularly dramatic in trabecular cardiomyocytes that normally exit from the cell cycle. Remarkably, YAP1 activation was sufficient to stimulate proliferation of postnatal cardiomyocytes, both in culture and in the intact heart. A dominant negative peptide that blocked YAP1 binding to TEAD transcription factors inhibited YAP1 proliferative activity, indicating that this activity requires YAP1–TEAD interaction. Although YAP1 was a critical regulator of cardiomyocyte proliferation, it did not influence physiological hypertrophic growth of cardiomyocytes, because postnatal YAP1 gain or loss of function did not significantly alter cardiomyocyte size. These studies demonstrate that YAP1 is a crucial regulator of cardiomyocyte proliferation, cardiac morphogenesis, and myocardial trabeculation. Activation of YAP1 in postnatal cardiomyocytes may be a useful strategy to stimulate cardiomyocyte expansion in therapeutic myocardial regeneration.

Heart development | physiological hypertrophy

Between the early heart tube stage and adulthood, the murine heart increases by >300-fold in mass (Fig. S1) (1). The ∼18-fold increase in mass achieved during fetal life occurs mainly through cardiomyocyte proliferation, whereas the remaining ∼18-fold growth achieved postnataally largely involves increased cardiomyocyte size (physiological hypertrophy), plus expansion of nonmyocyte populations. Growth of the heart is precisely regulated so that there is little variability in the final size of the adult heart. Derangements of these regulatory pathways likely contribute to congenital heart malformations, the leading form of heart defects. Moreover, understanding of these pathways will be highly relevant for regenerative approaches to postnatal heart disease.

The Hippo signaling cascade was discovered in Drosophila as a potent mechanism that regulates cell proliferation and organ size (2, 3). The core kinases of this signaling pathway, Hippo (Hpo) and Warts (Wts), and their regulatory subunits Salvador (Sav) and Mats, phosphorylate Yorkie (Yki) (4–7). In the absence of phosphorylation by the Hpo kinases cascade, Yki coactivates transcription in conjunction with specific transcription factors, such as the TEAD family transcription factor Scalloped (Sd), stimulating organ growth by increasing cell proliferation and reducing apoptosis (8, 9). Incompletely understood upstream molecular signals activate the Hpo kinase cascade, leading to Yki phosphorylation, nuclear export, and reduced organ growth. The core of the Hippo pathway is highly conserved in mammals, where Mst1/2, Sav1, Lats1/2, Yap1, and Tead1-4 are the orthologs of Hpo, Sav, Wts, Yki, and Sd, respectively (3).

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(Yap1 mutant) heart at embryonic day (E) 12.5. In these embryos, Tnnt2−Cre activated membrane-localized GFP (mGFP) from the Cre-activated Rosa26mTmG reporter and inactivated the conditional Yap1 allele specifically in cardiomyocytes. By quantitative RT-PCR (qRT-PCR), Yap1 transcripts were depleted by >95% at E12.5 in mutant compared with heterozygous control cardiomyocytes (Fig. 1A), confirming effective gene inactivation.

Yap1fl/fl::Tnnt2−Cre (abbreviated Yap1Tnnt2) embryos were present at a sub-Mendelian ratio by E12.5 and were not recovered beyond E16.5. The embryos displayed peripheral edema and pericardial effusion, consistent with heart failure (Fig. 1B and Fig. S3A). Overall cardiac patterning was preserved, but ventricular chamber size was severely reduced (Fig. 1C and Fig. S3B). In some cases, the hypoplasia affected both ventricles equally, whereas in other cases the left ventricle was more severely affected. Histological sectioning demonstrated a four-chambered heart with two atrioventricular and two outflow tract valves (Fig. 1D and Fig. S3C).

To investigate the cellular mechanism underlying the myocardial hypoplasia, we performed immunostaining for markers of proliferation and apoptosis. Staining for histone H3 phosphorylated on serine 10 (pH3), a marker of M phase of the cell cycle, showed substantially reduced cardiomyocyte proliferation. Quantitative analysis showed that the fraction of cardiomyocytes positive for pH3 was reduced by twofold (P < 0.05; Fig. 1E and F). This result was confirmed by analysis of cardiomyocyte uptake of 5-ethyl-2’-deoxyuridine (EdU), a nucleotide analog that labels cells passing through S phase. EdU+ cardiomyocytes were reduced by twofold in Yap1Tnnt2 mutant heart (P < 0.05; Fig. 1G and H). Although YAP1 regulates apoptosis in other settings (14), apoptosis was not elevated in Yap1Tnnt2 cardiomyocytes, as assessed by TUNEL staining (Fig. S3E).

Collectively, these data indicate that Yap1 is essential for fetal cardiomyocyte proliferation.

To confirm that reduced cardiomyocyte proliferation led to decreased cardiomyocyte number, we used FACS to quantitate cardiomyocyte number in dissociated fetal hearts (Fig. S3 F and G). We took advantage of the Rosa26mTmG (15) Cre reporter allele, in which Cre activity switches off baseline mRFP (membrane-bound RFP) and activates mGFP. GFP+ cardiomyocyte number was reduced in Yap1fl/fl::Tnnt2−Cre::Rosa26mTmG/+ heterozygous and mutants compared with Yap1fl/fl::Tnnt2−Cre::Rosa26mTmG/+ heterozygous controls. On the other hand, cardiomyocyte size, as measured by FACS forward scatter, did not differ between groups, suggesting that Yap1 mutation did not affect cardiomyocyte size regulation (Fig. S3F).

Collectively, these data indicate that Yap1 is required to promote normal cardiomyocyte proliferation in the fetal heart.

**Cardiomyocyte Hypertrophic Growth Does Not Require Yap1.**

The fetal heart primarily grows through cardiomyocyte proliferation, but by postnatal day (P) 4, cardiomyocytes stop increasing in number and postnatal heart growth occurs by increased cardiomyocyte size (physiological hypertrophy) (16). Loss of Yap1 in the fetal heart impaired cardiomyocyte proliferation but did not alter size. Therefore, we also hypothesized that Yap1 is dispensable for physiological cardiomyocyte hypertrophy.

To test this hypothesis, we asked whether loss of Yap1 has a cell-autonomous effect on postnatal cardiomyocyte growth. We sought to inactivate Yap1 postnatally in a small fraction of cardiomyocytes, to avoid potentially triggering secondary effects that may result from widespread myocardial Yap1 inactivation. A previous report indicated that retro-orbital administration of adenovirus to newborn pups leads to mosaic cardiomyocyte gene transfer (17). Retro-orbital delivery of Ad:Tnnt2−Cre to Yap1fl/fl::Tnnt2−Cre::Rosa26mTmG/+ heterozygous control pups activated the Cre-dependent mGFP reporter (and inactivated the baseline mRFP reporter) in a small fraction of cardiomyocytes, confirming that this technique is an effective means to achieve mosaic Cre-mediated recombination in the heart (Fig. 2A). We did not observe GFP expression outside of the heart.

To investigate the cell-autonomous role of Yap1 in postnatal cardiomyocyte growth, we delivered Ad:Tnnt2−Cre to Yap1fl/fl::Rosa26mTmG/+ mutant cardiomyocytes at 3 d of life. At 4–6 wk of life, we compared the size of GFP+ (Yap1-deficient) and GFP− (control) cardiomyocytes. In tissue sections, the cross-sectional areas of GFP+ cardiomyocytes were larger than GFP− (control) cardiomyocytes by twofold (P < 0.05; Fig. 2B). This result was confirmed by analysis of cardiomyocyte hypertrophic growth in postnatal hearts (Fig. 2C).

Thus, we conclude that Yap1 is not required in a cell-autonomous manner for physiological cardiac hypertrophy.

Cardiomyocytes also increase in size in response to biomechanical stress, a process known as pathological hypertrophy. To determine whether YAP1 is essential for pathological hypertrophy, we performed ascending aortic constriction (AAC) on adult mice with mosaic inactivation of Yap1. The cross-sectional area of GFP− control cardiomyocytes was larger in AAC compared with unoperated hearts, consistent with activation of the hypertrophic response (Fig. S4). GFP+ mutant cardiomyocytes were not significantly different in size compared with GFP− cardiomyocytes in either unoperated or AAC hearts (Fig. S4). Thus, Yap1 was dispensable for pathological cardiomyocyte hypertrophy.
Yap1 Gain of Function Stimulates Proliferation of Cultured Cardiomyocytes. Given the essential role of Yap1 in sustaining normal cardiomyocyte proliferation, we next asked whether increased Yap1 is sufficient to drive cardiomyocyte proliferation. We developed an adenoviral vector to express activated Yap1 with an N-terminal triple FLAG epitope (Ad:FLAG–aYap1), where “activated Yap1” contained a serine 127 to alanine substitution that was sufficient to drive cardiomyocyte proliferation. We used this tool to ask whether Yap1 gain of function is sufficient to enhance proliferation of fetal cardiomyocytes, which are normally blocked Hippo pathway-mediated Yap1 inactivation (18). We prepared cardiomyocyte cultures from P4 neonatal rats. As expected, control neonatal rat ventricular cardiomyocytes treated with LacZ adenovirus rarely stained for pH3 or Aurora B, markers of M phase and cytokinesis, respectively, confirming that nearly all neonatal cardiomyocytes had exited the cell cycle (Fig. 3A). A small fraction (2%) of control neonatal cardiomyocytes were labeled with BrdU, consistent with low-level, persistent DNA synthesis activity (Fig. 3A). In contrast to control neonatal cardiomyocytes, aYAP1 neonatal cardiomyocytes exhibited far greater cell-cycle activity, with labeling indexes of 31%, 2.7%, and 2.1% for BrdU, pH3, and Aurora B, respectively (Fig. 3A). aYAP1 neonatal cardiomyocytes were observed at all stages of the cell cycle (Fig. S5C). Sequential imaging of the same fields over time demonstrated completion of cytokinesis in a subset of aYAP1-expressing but not control cardiomyocytes (Fig. 3B and Fig. S5D). We detected cell division events in 0/366 control cells, 0/267 LacZ cells, and 8/566 aYAP1 cells (P = 0.015; Fig. 3B). However, there was ongoing apoptosis in these cultures, which was reduced, but not eliminated, with FLAG–aYap1 expression (Fig. S5E and F). As a result, there was an overall decline in cardiomyocyte number over the course of the experiment, although this decline was least in the aYAP1 group as a result of both increased proliferation and reduced apoptosis (Fig. S5G). Collectively, these data indicate that activated Yap1 continues to drive the cell cycle in postnatal cardiomyocytes, which normally have very little cell-cycle activity.

Activated Yap1 Stimulated Fetal Cardiomyocyte Proliferation in Vivo. Next, we asked whether Yap1 gain of function stimulates cardiomyocyte proliferation in intact fetal heart. We used a report-positive transgene (TetO–aYap1) (10) that expresses activated Yap1 (Yap1–S127A) from a doxycycline (Dox)-dependent promoter. In Tnnt2–Cre::Rosa26mTmG::TetO–aYap1 (abbreviated Yap1GOF) mice, Dox induced expression of activated Yap1 selectively in cardiomyocytes (Fig. S6A and B). Administration of Dox to Yap1GOF fetuses starting at E8.5 resulted in fetal demise by E15.5. At E12.5, mutant fetuses exhibited peripheral hemorrhage, hepatic congestion, and cardiomegaly (Fig. S6C). Histological examination revealed dramatic myocardial overgrowth with moderate thickening of the compact myocardium and marked expansion of the trabecular myocardium, causing near chamber obliteration (Fig. 4A, arrowheads).

To evaluate the cellular mechanism for myocardial expansion, we performed immunostaining for proliferation markers. Cardiomyocyte proliferation was significantly increased in Yap1GOF.
fetal heart and control mice exposed to Dox starting at E8.5, as measured by the M-phase marker pH3 (Fig. 4 C and D). The S-phase marker EdU also increased by twofold, which approached, but did not achieve, statistical significance (P = 0.07, n = 3; Fig. 4 B and D). Thus, Yap1 gain of function in fetal heart was sufficient to enhance cardiomyocyte proliferation, causing marked cardiomyocyte hyperplasia.

In control heart, the proliferation rate of cardiomyocytes was substantially lower in trabecular compared with compact myocardium (Fig. 4 B and C), indicating that, even in fetal heart, cardiomyocytes in specific compartments are exiting the cell cycle. This finding was particularly evident by pH3 staining, where it was very rare to observe pH3+ trabecular cardiomyocytes. In contrast, proliferating mutant trabecular cardiomyocytes were readily observed in Yap1GOF (Fig. 4 B and C). Quantitation of EdU uptake showed that Yap1GOF stimulated trabecular myocardial proliferation fivefold, to levels comparable with compact myocardium (Fig. 4E). These data indicate that Yap1 gain of function is sufficient to sustain cell-cycle activity in trabecular cardiomyocytes, causing tremendous expansion of trabecular myocardium.

Cardiomyocyte proliferation is often linked to differentiation. Trabecular and compact cardiomyocytes have distinct gene expression programs. One marker expressed in trabecular, but not compact, myocardium is Nppa. In situ hybridization showed that Nppa expression was strongly down-regulated in trabecular myocardium expressing activated YAP1 (Fig. S6D), consistent with impaired activation of the trabecular myocardial gene program.

Activated YAP1 Stimulated Postnatal Cardiomyocyte Proliferation in Vivo. In both P4 neonatal cardiomyocytes and fetal trabecular cardiomyocytes, YAP1 gain of function was sufficient to drive proliferation of cardiomyocytes that normally have exited the cell cycle. Therefore, we asked whether YAP1 gain of function was sufficient to drive proliferation of cardiomyocytes in the intact infant heart, in which cardiomyocytes are normally exiting from the cell cycle. We treated Yap1GOF mice with Dox from P5 and analyzed hearts at P15. Heart weight was increased in Yap1GOF mice compared with the controls (heart weight/body weight, 9.0 ± 0.1 vs. 7.5 ± 0.5 mg/g; P = 0.03; n = 3). This result was not due to cardiomyocyte hypertrophy, because cardiomyocyte size did not differ significantly between Yap1GOF and control groups (Fig. 5A). EdU labeling index was increased nearly twofold in Yap1GOF cardiomyocytes, indicative of a higher fraction of cardiomyocytes passing through S phase during the 24-h EdU pulse (Fig. 5B). This finding was corroborated by staining for the M-phase marker pH3, which showed 15-fold increased cell-cycle activity in Yap1GOF cardiomyocytes (Fig. 5C). Collectively, these data indicate that YAP1 gain of function increased postnatal cardiomyocyte proliferation.

YAP1 Regulates Expression of Cell-Cycle Genes in Cardiomyocytes. To begin to identify genes downstream of Yap1 that stimulate cardiomyocyte proliferation, we performed microarray expression profiling in Yap1GOF mice. We used the Affymetrix Rat Gene 1.0 ST microarray to compare gene expression between P4 neonatal cardiomyocyte transduced with FLAG-aYap1 or LacZ (control). Gene Set Enrichment Analysis (19) of the expression profiles using gene sets for manually curated canonical pathways indicated that the large majority of the sets significantly enriched by activated YAP1 related to cell proliferation and DNA synthesis (Fig. S7 A and B). Hierarchical clustering using cell cycle-related genes clustered samples into control or aYAP1 groups (Fig. 6A and Fig. S7C). Comparison of individual gene expression levels between groups identified 1,263 differentially expressed probe sets corresponding to 1,057 known genes (n = 4; P < 0.005 and fold change >50%; Dataset S1). We validated differential expression of a subset of cell cycle-regulated genes by qRT-PCR. Of eight genes tested that were differentially expressed by

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Fig. 4. Fetal YAP1 gain of function stimulated cardiomyocyte proliferation in vivo. (A) Histological sections of Yap1GOF heart at E12.5 revealed marked hypertrabeculation that nearly obliterated the cardiac chambers. Black arrowheads indicate trabecular myocardium. (B and C) pH3 and EdU staining showed elevated cardiomyocyte proliferation, particularly in trabecular myocardium (white arrowheads). (D) Quantitation of B and C for ventricular myocardium (compact and trabecular pooled). (E) Quantitation of cardiomyocyte EdU uptake in compact vs. trabecular myocardium. (Scale bars: 100 μm.)

Fig. 5. Postnatal YAP1 gain of function stimulated cardiomyocyte proliferation in vivo. YAP1GOF or control mice were treated with Dox from P5 to P15, when they were analyzed for cardiomyocyte proliferation. (A) The size of YAP1 gain-of-function cardiomyocytes was indistinguishable from control. Wheat germ agglutinin (WGA) staining outlined the edges of cardiomyocytes. (B and C) Cardiomyocyte proliferation was increased based on EdU labeling index (B, deconvolution) and pH3 staining (C, confocal). Arrowheads indicate cardiomyocytes and arrows nonmyocytes. Insets show magnifications of boxed regions, with cardiomyocyte borders highlighted by WGA staining. n = 3. *P < 0.05. (Scale bars: 20 μm.)
microarray, six were validated by qRT-PCR (Fig. 6B). Among up-regulated cell-cycle genes were Cyclin A2 (CcnA2), Cyclin B1 (CcnB1), and Cyclin-dependent kinase 1 (Cdc2), which have been shown to be sufficient to drive limited cardiomyocyte proliferation in postnatal hearts (20–22). Cyclins D1 and D2, which also promote cardiomyocyte proliferation (23, 24), approached but did not reach our cutoff thresholds for differential expression by microarray (P = 0.003–0.004 and fold change 1.4–1.5). These data indicate that YAP1 stimulates cardiomyocyte proliferation through the coordinate activation of a number of cell-cycle genes.

YAP1 Stimulation of Cardiomyocyte Proliferation Requires TEAD Interaction. In many contexts, coactivator YAP1 binds to TEAD family factors to stimulate cell proliferation (2). However, in some cases YAP1 regulation of cell growth is independent of TEAD factors (8). To assess the role of YAP1–TEAD interactions in promoting cardiomyocyte proliferation, we expressed GFP fused to a region of YAP1 sufficient to bind TEAD (25). We reasoned that this peptide, corresponding to amino acid residues 47–155 of YAP1, would interfere with YAP1–TEAD interaction (Fig. 7A) and named the peptide YTIP (YAP1–TEAD Interfering Peptide). In coimmunoprecipitation experiments, we confirmed that GFP–YTIP impaired YAP1–TEAD1 interaction (Fig. 7B).

We next measured the effect of GFP–YTIP on activated YAP1 stimulation of cardiomyocyte proliferation. GFP–YTIP strongly attenuated the stimulatory effect of activated YAP1 on proliferation of P4 neonatal cardiomyocytes, as measured by BrdU uptake and pH3 immunoreactivity (Fig. 7C). The antiproliferative effect of GFP–YTIP was paralleled by decreased activation of cell-cycle genes Cdk1 and Cyclin A2 by aYAP1 (Fig. 7D). These data indicate that YAP1 mitogenic activity in cardiomyocytes requires YAP1–TEAD interaction.

Substitution of YAP1 serine 79 by alanine selectively abolished TEAD interaction, and a YAP1S79A allele was unable to restore proliferation defects caused by ablation of wild-type Yap1 (11). We generated Yap1S79A;Tnn2T–Cre embryos, in which only the mutant, and not the wild-type, YAP1 protein would be expressed in the heart. These mutant mice exhibited cardiomyocyte hypoplasia at least comparable in severity to that caused by cardiomyocyte Yap1 ablation (Fig. 7E). These data indicate that YAP1 stimulation of cardiomyocyte proliferation requires TEAD interaction, both in vitro and in vivo.

Discussion

Growth of the mammalian heart occurs through both cardiomyocyte proliferation and hypertrophy, with proliferation and hypertrophy primarily driving fetal and postnatal heart growth, respectively. YAP1 has been shown to promote cellular proliferation and organ growth in multiple systems in flies and mammals (2). Although YAP1 regulation of organ growth through control of cellular proliferation has been studied in depth, little has been reported about YAP1 regulation of hypertrophic organ growth. Our results indicate that YAP1 is both necessary and sufficient for fetal cardiomyocyte proliferation. However, YAP1 is neither necessary nor sufficient for postnatal hypertrophic growth of the heart. Thus, our data indicate that YAP1 regulates organ size predominantly by controlling cell number.

Normal heart development requires precisely regulated regional organ growth. Selective hypoplasia of heart structures are seen in congenital heart disease such as hypoplastic left heart syndrome. Our study showed that proper regulation of YAP1 activity is critical for normal growth of the fetal heart. Interestingly, overall size of the left ventricular chamber was more severely affected in some YAP1TNT loss-of-function mutants (e.g., Fig. 1C and Fig. 3D). Moreover, it is likely that YAP1 activity is regionally controlled, so that localized disruption of YAP1 regulation could lead to selective chamber hypoplasia. Thus, mutation of genes in the YAP pathway may participate in pathogenesis of congenital heart disease involving abnormalities of myocardial growth.

Myocardial trabeculation is essential for fetal heart growth and function. Cardiomyocytes in trabeculae exit the cell cycle through unknown mechanisms, suggesting that growth of trabeculae occurs from addition of cardiomyocytes from compact myocardium. Neuregulin signaling to ErbB2/ErbB4 is essential for myocardial trabeculation (26, 27), and recent studies suggest that ErbB2 acts by promoting compact myocardial proliferation and directional cardiomyocyte migration into trabeculae (28). Our data indicate that

![Fig. 6. YAP1 promotes expression of cell-cycle genes through interaction with TEAD1. (A) Heat map displaying two-way hierarchical clustering of cell-cycle genes in P4 neonatal cardiomyocytes expressing either aYAP1 or LacZ (control). Expression of cell-cycle genes segregated samples into treatment groups. (B) Validation of differential expression of cell-cycle genes by qRT-PCR. **P < 0.001. n = 4.

![Fig. 7. YAP1 stimulation of cardiomyocyte proliferation requires TEAD interaction. (A) YAP1–TEAD inhibitory peptide (YTIP) strategy. (B) Endogenous TEAD1 and YAP1 interaction was blocked by Ad:GFP–YTIP in M513 cells. (C) aYAP1 stimulation of P4 neonatal cardiomyocyte proliferation was attenuated by GFP–YTIP, as measured by BrdU and pH3 immunostaining. *P < 0.01. n = 3. (D) Inhibition of TEAD interaction reduced expression of YAP1-activated cell-cycle genes, as measured by qRT-PCR. **P < 0.05. n = 3. (E) Cardiomyocyte YAP1TNT, deficient in TEAD interaction, did not support normal fetal myocardial growth. Yap1Tnnt2T–Cre indicates the genotype Yap1TNT::Tnn2T–Cre. H&E-stained sections of E12.5 heart are shown. (Right) Boxed areas are enlarged. Brackets indicate compact myocardial thickness. (Scale bars: 100 μm.)

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YAP1 is essential for regulating myocardial trabeculation, because trabeculation was reduced in YAP1knockout (1) mutants. This reduction of trabeculation may be a consequence of reduced proliferation of cardiomyocytes in compact myocardium. YAP1 activation in cardiomyocytes prevented trabecular cardiomyocyte cell-cycle exit, suggesting that active regulation of the YAP1 pathway is essential for limiting trabecular myocardial expansion. Interestingly, YAP1 activation also prevented expression of the trabecular cardiomyocyte marker Nppa, suggesting that YAP1-induced trabecular proliferation is linked to impaired differentiation. Abnormalities of YAP1 regulation may contribute to myocardial noncompaction, which is seen in some forms of human cardiomyopathy.

The Hippo pathway is the best-known regulator of YAP1 activity. However, these kinases have multiple downstream targets and influence diverse processes such as apoptosis and autophagy (29). Indeed, the roles of Hippo pathway kinases LATS and MST in regulating these later processes have been explored by cardiomyocyte-restricted transgenic gain and inhibition of function (30, 31). Recently, cardiac-restricted inactivation of the Hippo pathway components Sav1, Lats2, or Mst1/2 was shown to cause cardiac overgrowth (32). Thus, the findings of that study combined with those of the present study indicate that Hippo kinases restrain the proliferative activity of YAP1 on fetal cardiomyocytes.

Gene expression profiling showed that activated YAP1 potentially and coordinately activated gene programs that promote cell-cycle activity. YAP1 activates transcription by binding to DNA-binding transcription factors, with TEAD1-4, orthologs of Yorkie, being the best known (2). However, not all proliferative activities of YAP1 or its Drosophila ortholog Yorkie are mediated by TEAD/Scalloped. For example, Yorkie but not Scalloped, is required for growth of Drosophila imaginal discs (8). Using a dominant negative peptide that blocks YAP1–TEAD and a YAP1 point mutation-defective TEAD interaction, we showed that YAP1 stimulation of cardiomyocyte proliferation requires its interaction with TEAD.

Activated YAP1 potently promoted proliferation of fetal trabecular and neonatal ventricular cardiomyocytes that normally exit the cell cycle. These data show that activation of YAP1 can overcome signals that normally lead cardiomyocytes to exit the cell cycle. However, a limitation of the current study is that we cannot distinguish whether YAP1 is preventing cell-cycle withdrawal or reinitiating cell-cycle reentry. This limitation will need to be addressed further to test the hypothesis that activated YAP1 can induce cell-cycle reentry in adult, fully mature cardiomyocytes, with important potential ramifications for regenerative approaches to heart injury.

Materials and Methods
Please see SI Materials and Methods for details.

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Supporting Information

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SI Materials and Methods

Mice. All animal procedures were approved by the Institutional Animal Care and Use Committee. Col-TetO-YAP1 (1), Yap1Box (2), Yap1S79A (2), Rosa26mTmG (3), Rosa26hTmG (4), and Tnnt2-Cre (5) alleles were described. Dox was administered to pregnant dams at 1 mg/mL in drinking water. For delivery to lactating pups, Dox in maternal drinking water was supplemented with IP injection of 100 μg per g of body weight daily. Gestational age was determined by checking for vaginal plugs, with noon of the day of the plug defined as E0.5. Ascending aortic constriction was performed as described (6).

Adenoviruses. Tnnt2-Cre adenovirus was constructed using the rat Tnnt2 promoter (7) and the AdEasy system (Stratagene). 3xFlag–YAPI adenovirus was generated by cloning S127A-mutated human YAPI cDNA with an N-terminal triple FLAG epitope tag into pENTR3C (Invitrogen). These expression cassettes were then transferred to pAd/CMV/V5-DEST by using LR clonase (Invitrogen). Viruses were purified on cesium chloride gradients and titered by using the AdEasy adenoviral titer kit (Stratagene). Retro-orbital adenoviral injection to neonatal pups was performed as described (8).

Cardiomyocyte Isolation and Culture. Fetal and neonatal rat cardiomyocyte culture was performed by using the Neonys cardiomyocyte dissociation kit (Cellutron). Cardiomyocytes were enriched by preplating on tissue culture plastic to remove nonmyocytes. P4 neonatal cardiomyocytes were initially cultured for 48 h in the presence of 20 μM cytosine B-d-arabinofuranoside (araC; Sigma) and 5% (vol/vol) horse serum to prevent proliferation of nonmyocytes. Then cardiomyocytes were transduced with virus (multiplicity of infection, 25) in serum-free medium and cultured for an additional 48 h. For BrdU labeling, we treated cells for 24 h with 10 μM BrdU. Cells were fixed with 4% (wt/vol) PFA and immunostained. For quantitation of cardiomyocyte number, cells were cultured on labeled, gridded dishes (MatTek) and sequentially imaged. Adult cardiomyocytes were isolated by antegrade collagenase perfusion and purified by differential centrifugation.

Histology. H&E staining was performed on paraffin-embedded sections. Immunostaining was performed on cryosections and detected with Alexa-labeled secondary antibodies (Invitrogen). Antibody sources are listed in Table S1. EdU was administered intraperitoneally at 200 μg (pregnant dams) or 5 μg per g of body weight (pups), 2 or 24 h before tissue collection, respectively. EdU was detected with Click-it chemistry (Invitrogen). Imaging was performed on a Nikon TE2000 epifluorescent microscope with deconvolution (Volocity; Perkin-Elmer) or on an Olympus FV1000 confocal.

Gene Expression. Western blotting was performed by using specific antibodies (Table S1). Total RNA was isolated by using the RNeasy kit (Qiagen) and hybridized to Affymetrix microarrays (Rat Gene 1.0 ST). Array data were deposited at the Gene Expression Omnibus (GEO) database (accession no. GSE33019). For qRT-PCR, RNA was reverse transcribed (Superscript III) and specific transcripts were measured by using Sybr Green chemistry and normalized to GAPDH. Primer sequences are provided in Table S2.

Fig. S1. Growth of the mammalian heart. Fetal growth occurs through cardiomyocyte proliferation, and postnatal growth occurs through cardiomyocyte hypertrophy. The fold increase in heart weight between E10.5 and birth and birth to adulthood is comparable. The rate of change of heart weight normalized to the size of the heart, a measure of the growth velocity at the cellular level, decreases rapidly with increasing age.

Fig. S2. Cardiac YAP1 expression. (A) Postnatal YAP1 expression in myocardium. Postnatal age is indicated. d, days; w, weeks. (B) YAP1 expression in cardiomyocytes (CM) and nonmyocytes (NM) at indicated developmental stages. Myocardium was dissociated by collagenase digestion, and the cardiomyocyte fraction was isolated by differential plating (fetal and neonatal) or differential centrifugation (adult). E, embryonic.
Fig. S3. Histological and FACS analysis of Yap1-deficient fetal hearts. (A) Whole-mount view of backlit embryo showing translucent mutant chest (arrowheads) due to pericardial effusion. (B) Example of a mutant heart with severe global myocardial hypoplasia. Overall cardiac patterning was preserved. Lungs shape was normal despite cardiac hypoplasia. (C) Membranous VSD in a rare Yap1\textsuperscript{Tm2\textsuperscript{m}TmG/\textsuperscript{m}} mutant that survived to E16.5. (D) Expression of chamber-specific markers MYL2 and MYL7 was unchanged in Yap1-deficient hearts. (Scale bars: 100 μm.) (E) TUNEL staining indicated no significant change in apoptosis rate in Yap1\textsuperscript{Tm2\textsuperscript{m}TmG/\textsuperscript{m}} heart. Arrowheads indicate apoptotic nuclei in skin. (Scale bar: 10 μm.) (F) FACS analysis of fetal cardiomyocytes, isolated by sorting for Tnnt2-Cre\textsuperscript{activated} expression of mGFP from the Rosa26\textsuperscript{mTmG} allele. (G) Total cardiomyocyte number was reduced in Yap1\textsuperscript{Tm2\textsuperscript{m}TmG/\textsuperscript{m}} mutant heart compared with control. \(n = 9–11\). (H) Cell size, as estimated by forward scatter, was unchanged between control and Yap1-deficient cardiomyocytes. \(n = 9–11\).
Fig. S4. YAP1 is not required for pathological cardiac hypertrophy. Mice with mosaic cardiac YAP1 inactivation were treated with ascending aortic constriction (AAC) or no operation at 6 wk. One week later, cardiomyocyte cross-sectional areas were measured. GFP⁺ (YAP1-depleted) and RFP⁺ (control) cardiomyocyte cross-sectional areas were not statistically different (NS). More than 280 cardiomyocytes were measured per group. ***P < 0.001. (Scale bars: 10 µm.)
Activated YAP1 stimulated proliferation of cultured fetal and postnatal cardiomyocytes. (A) Adenoviral expression of activated YAP1 (aYAP1) in neonatal rat cardiomyocytes. YAP1 was detected with specific antibody. (B) aYAP1 stimulated proliferation of E16.5 fetal rat cardiomyocytes. \( n = 3 \). *\( P < 0.05 \). (C) Different phases of mitosis induced by expression of aYAP1. Red, TNNI3. (Scale bar: 50 \( \mu \)m.) (D) Serial observation of cardiomyocyte division. P4 NRVMs were plated on gridded tissue culture dishes and observed sequentially for cytokinesis. Times indicate hours after virus treatment. White arrows indicate cardiomyocytes undergoing cytokinesis. Yellow arrowheads indicate cardiomyocytes just after cytokinesis. TNNI3 staining was performed on fixed cells after the culture period to confirm lineage identity. Cardiomyocyte cytokinesis was not observed in untreated (control) or LacZ adenovirus-treated groups. (Scale bars: 50 \( \mu \)m.) (E) A subset of P4 NRVMs underwent apoptosis during the culture period, as indicated by TUNEL staining. (Scale bar: 20 \( \mu \)m.) (F) aYAP1 reduced apoptosis of cultured cardiomyocytes. (G) Quantitation of absolute cell number by sequential imaging indicated that cardiomyocyte number declined during the culture period. Cardiomyocyte number declined least in the aYAP1 group. *\( P < 0.05 \) vs. LacZ.
Fig. S6. Fetal YAP1 gain of function stimulated cardiomyocyte proliferation in vivo. (A) Genetic strategy for cardiomyocyte-restricted YAP1 gain of function. Tnnt2-Cre recombination of Rosa26<sup> fs-rtTA</sup> resulted in cardiomyocyte-restricted expression of the rtTA, which stimulated activated human YAP1 expression from a Dox-regulated promoter positioned distal to the collagen locus. (B) Induction of the human YAP1 transgene by Dox. qRT-PCR was performed with primers specific for human YAP1. (C) Peripheral hemorrhage and growth retardation of mutant embryo at E12.5. Hearts of mutant embryos were enlarged and exhibited pockets of pooled blood. (Left bars: 1 mm; right bars: 250 μm.) (D) Nppa in situ hybridization of E12.5 left ventricle. Trabecular myocardium (red arrowheads) expressed markedly lower Nppa transcript levels in TetO-aYap1<sup>Tnnt2</sup> compared with control. (Scale bars: 100 μm.)
Fig. S7. YAP1 promotes expression of cell-cycle genes. (A and B) Gene Set Enrichment Analysis was performed by using gene expression profiles of P4 NRVMs expressing activated YAP1 or control (LacZ) on curated canonical pathway gene sets. (A) Gene sets significantly enriched (FDR < 0.05) in activated YAP1 expressing NRVMs. (B) Enrichment graphs showing overrepresentation of indicated gene set members among genes more highly expressed in activated YAP1 compared with control. Red numbers correspond to ranks indicated in A. (C) Heat map displaying two-way hierarchical clustering of cell cycle genes in P4 NRVMs expressing either aYAP1 or LacZ (control). Expression of cell-cycle genes clustered samples into treatment groups. This is the same heat map as in Fig. 6A but in expanded form with gene symbols.
Table S1. Antibodies and other staining reagents used in this study

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Table S2. qRT-PCR primers used in this study

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Other Supporting Information Files

Dataset S1 (XLS)