Published in final edited form as:

Sci Signal. ; 4(196): ra70. doi:10.1126/scisignal.2002278.

Regulation of Insulin-Like Growth Factor Signaling by Yap Governs Cardiomyocyte Proliferation and Embryonic Heart Size

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Abstract

The Hippo signaling pathway regulates growth of the heart and other tissues. Hippo pathway kinases influence the activity of various targets, including the transcriptional coactivator Yap, but the specific role of Yap in heart growth has not been investigated. We show that Yap is necessary and sufficient for embryonic cardiac growth in mice. Deletion of Yap in the embryonic mouse heart impeded cardiomyocyte proliferation, causing myocardial hypoplasia and lethality at embryonic stage 10.5. Conversely, forced expression of a constitutively active form of Yap in the embryonic heart increased cardiomyocyte number and heart size. Yap activated the insulin-like growth factor (IGF) signaling pathway in cardiomyocytes, resulting in inactivation of glycogen synthase kinase 3 β , which led to increased abundance of β -catenin, a positive regulator of cardiac growth. Our results point to Yap as a critical downstream effector of the Hippo pathway in the control of cardiomyocyte proliferation and a nexus for coupling the IGF, Wnt, and Hippo signaling pathways with the developmental program for heart growth.

INTRODUCTION

Embryonic development depends on the ability of the heart to supply oxygen and nutrients to developing organs. Growth of the embryonic heart involves cardiomyocyte proliferation, especially along the outer surface, referred to as the compact zone, which provides the muscular layer required for contraction. Growth factors, including insulin-like growth factor (IGF), bone morphogenetic proteins (BMPs), Wnts, and neuregulins, regulate embryonic cardiac growth (1), but the transcriptional effectors of and inputs to these pathways are poorly understood.

The Hippo signaling pathway, which was discovered in *Drosophila*, is a pivotal regulator of cell proliferation and organ size (2). This pathway consists of a series of kinases and

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Author contributions: M.X., R.B.-D., and E.N.O. designed the study and wrote the paper; M.X., R.B.-D., E.N.O., and J.A.R. interpreted the data; M.X., Y.K., L.B.S., X.Q., and J.M. performed the experiments; and R.J.S. provided the Nkx-KI-Cre mouse line.

Competing interests: The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

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adaptors that phosphorylate and inhibit the transcriptional coactivator Yorkie (Yki). Hippo interacts with Salvador to phosphorylate and activate the complex of Warts and Mats, which in turn phosphorylates and inactivates Yki (3). Hippo, Salvador, Warts, Mats, and Yki are structurally and functionally homologous to mammalian MST1 or 2 (MST1/2), WW45, LATS1/2, Mob1, and Yap, respectively (2). Yap interacts with various transcription factors and has been implicated in liver growth, tumorigenesis, and proliferation of neural, skin, and intestinal progenitor cells (2, 4, 5). Genetic deletion of the upstream kinases in the Hippo pathway—WW45, MST1/2, and LATS—results in excessive cardiomyocyte proliferation (6). In addition to Yap, these kinases have other targets, such as the Yap-related protein Taz, histones H2AX and H2B, Foxo3, and Foxo1, that may mediate the actions of this pathway (7). Furthermore, Yap can also be regulated in a Hippo-independent manner (8, 9). These complexities raise uncertainties about the precise role of Yap and its potential downstream effectors in cardiac growth in vivo.

To investigate the possible function of Yap in cardiomyocyte proliferation, we used loss- and gain-of-function approaches in mice. We show that genetic deletion of Yap leads to lethality at E10.5 (embryonic day 10.5), accompanied by reduced cardiomyocyte proliferation and consequent myocardial hypoplasia and contractile insufficiency. Conversely, cardiac-specific overexpression of a constitutively active form of Yap promotes cardiomyocyte proliferation, leading to an increase in cardiomyocyte number and heart size. Yap increases the abundance of IGF1 receptor (IGF1R), resulting in activation of downstream effectors that inactivate glycogen synthase kinase 3 β (GSK-3 β), leading to stabilization and increased abundance of β -catenin, a positive regulator of proliferation. Our findings substantiate a role of Yap as an essential regulator of embryonic cardiac growth and development and identify Yap as a nexus that connects the Hippo, Wnt, and IGF pathways to control embryonic heart size.

RESULTS

Deletion of *Yap* during cardiogenesis impairs cardiomyocyte proliferation

To assess the potential role of Yap in heart development and circumvent the early embryonic lethality resulting from global deletion of *Yap* (10), we generated mice with a conditional *Yap* mutant allele (*Yap^{loxP}*) (fig. S1, A to D). This *Yap* allele was deleted specifically in cardiac progenitor cells with the knock-in Nkx2.5-Cre mouse line (11), which allows for deletion at E7.5 in the cardiac crescent. Wild-type and mutant *Yap^{loxP/loxP}; Nkx2.5-Cre* (*Yap* nKO) embryos were indistinguishable at E8.5, whereas by E9.5, mutant embryos had a slower heartbeat compared to that of wild-type embryos. By E10.5, the mutant embryos died and had an abnormally thin myocardium as measured by a ~50% decrease in the number of cardiac troponin T–positive cardiomyocytes (Fig. 1, A and B). Deletion of *Yap* with Nkx2.5-Cre (fig. S1E) did not affect cardiac looping or chamber formation. However, mutant embryos displayed a statistically significant reduction in ventricular myocyte number compared to control littermates (Fig. 1, A and B). Phosphorylation of H3 occurs in mitotic cells, and decreased immunostaining for phosphorylated H3 (PH3) confirmed the diminished proliferation of cardiomyocytes in *Yap* nKO embryonic hearts at E9.5 compared to control embryos (Fig. 1, A and B). These findings revealed an essential role of Yap in promoting cardiomyocyte proliferation.

Yap promotes cardiomyocyte proliferation in vivo

We investigated whether expression of Yap was sufficient to induce cardiomyocyte proliferation by generating transgenic mice expressing an active form of Yap under control of the β MHC (β -myosin heavy-chain) promoter, which is transcriptionally activated in the heart beginning at E9. The Hippo kinase cascade phosphorylates mouse Yap at Ser¹¹²,

which corresponds to Ser¹²⁷ in human Yap. Substitution of Ser¹¹² with Ala (S112A) generates a Yap protein that is constitutively active and localized to the nucleus (12). Transgenic mice with twofold overexpression of YapS112A (fig. S2A) were viable, but displayed an abnormally thickened myocardium and expanded trabecular layer (Fig. 1, C and D, and fig. S2B). Increased PH3 immunostaining confirmed the enhanced proliferation of cardiomyocytes in hearts of transgenic embryos (Fig. 1, B and D). When transgenic mice reached adulthood, heart size was normal (fig. S2C). However, the average cross-sectional area of cardiomyocytes in the adult transgenic hearts was reduced relative to that of littermate control hearts (fig. S2D), despite a reproducible increase in the number of cardiomyocytes in the transgenic hearts (fig. S2E). These findings suggest a compensatory mechanism to normalize cardiac mass by reduction of cardiomyocyte size.

Yap promotes neonatal cardiomyocyte proliferation in vitro

To further determine the effect of Yap on cardiomyocyte proliferation, we infected primary neonatal cardiomyocyte cultures with adenovirus expressing YapS112A (Ad-YapS112A) (fig. S3A). Cardiomyocytes expressing YapS112A showed enhanced DNA synthesis, as demonstrated by a threefold increase in 5-bromo-2'-deoxyuridine (BrdU) incorporation (Fig. 2, A and B) and an about threefold increase in karyokinesis, as demonstrated by PH3 staining (Fig. 2C and fig. S3B).

Cardiomyocytes proliferate during fetal life but exit the cell cycle soon after birth. Before terminal withdrawal from the cell cycle, cardiomyocytes can undergo a final round of incomplete cell division, during which karyokinesis becomes uncoupled from cytokinesis, resulting in binucleated cardiomyocytes (13). Cardiomyocytes expressing YapS112A showed a fourfold increase in Aurora B kinase staining (Fig. 2, D and E), a marker of cytokinesis. An increase of ~50% in the number of cardiomyocytes was also seen with YapS112A expression compared to green fluorescent protein (GFP) expression 3 days after adenovirus infection (Fig. 2F). Thus, the active form of Yap is capable of promoting cardiomyocyte proliferation by inducing DNA synthesis, karyokinesis, and cytokinesis.

Yap regulates genes critical for pro-growth signaling pathways

Microarray analysis of wild-type and β MHC-YapS112A transgenic hearts at E10.5 revealed increased transcription of genes involved in IGF signaling, such as *IGF1*, *IGF binding proteins* (*Igfbp2* and *Igfbp3*), β -catenin (*Ctnnb1*), and β -catenin downstream target genes in β MHC-YapS112A transgenic hearts (fig. S4A). *Kcne3*, *Ndr1*, and *Ier3*, which are regulated by β -catenin, are among the genes with the largest increase in transcription upon stabilization of β -catenin in cardiomyocytes in vivo (14), suggesting that YapS112A might promote stabilization of β -catenin protein. The increased abundance of IGF1R and β -catenin in cardiomyocytes expressing YapS112A was confirmed by Western blot analysis (Fig. 3, A and B). The nonphosphorylated active form of β -catenin was also increased in abundance by YapS112A expression (Fig. 3A). Consistent with the microarray data, the abundance of *Igfbp2* was increased twofold in β MHC-YapS112A transgenic hearts at E10.5 compared to control mice (fig. S4B).

Expression of YapS112A also induced the expression of genes encoding proteins involved in mitosis and cytokinesis, including *cyclin A2* and *B*, *Cdc2*, and *Ect2* (fig. S4C), whereas genes encoding proapoptotic factors, such as *Cabc1* and *Dapk*, showed decreased expression. Thus, Yap may promote cardiomyocyte proliferation by inducing DNA synthesis, karyokinesis, and cytokinesis through activation of genes encoding factors that support growth.

Yap increases the activity of the IGF signaling pathway

Stimulation of the IGF signaling pathway activates the kinases phosphoinositide 3-kinase (PI3K) and Akt (15). Indeed, we found that the abundance of PI3K and phosphorylation of Akt were increased in YapS112A-expressing cardiomyocytes, whereas total Akt abundance was unchanged (Fig. 3, A and B). These findings further indicate that the IGF signaling pathway is activated in cardiomyocytes expressing YapS112A. Phosphorylated Akt inhibits GSK-3 β activity by phosphorylating GSK-3 β , and phosphorylated GSK-3 β , the inactive form of GSK-3 β , was increased in YapS112A-expressing cells (Fig. 3, A and B).

β -Catenin expression is tightly regulated by the GSK-3 β -Axin-APC (adenomatous polyposis coli) degradation complex (16). Upon phosphorylation by GSK-3 β , phosphorylated β -catenin is ubiquitinated and degraded by proteasome-mediated proteolysis. Consistent with the increased abundance of inactive phosphorylated GSK-3 β , the abundance of β -catenin protein and the active nonphosphorylated form of β -catenin was increased in YapS112A-expressing cardiomyocytes (Fig. 3, A and B). These findings suggest that activated Yap increases IGF signaling, and the consequent decrease in phosphorylated GSK-3 β accounts for the stabilization of β -catenin. Accordingly, cardiomyocytes expressing YapS112A showed increased activation of a TOPflash luciferase reporter, a transcriptional readout of β -catenin signaling. YapS112A activated the TOPflash luciferase reporter more effectively than wild-type Yap, and deletion of the activation domain of Yap (Yap- Δ AD) abolished transcriptional activity (Fig. 3C). Thus, Yap increases β -catenin signaling in cardiomyocytes, and the transcriptional activity of Yap is required for this process.

Wnt signaling promotes proliferation of cardiac progenitors, an effect that is antagonized by GSK-3 β (17). Given that Yap inhibits GSK-3 β signaling by increasing the phosphorylation of GSK-3 β , we investigated whether increased β -catenin is necessary for Yap-dependent stimulation of cardiomyocyte proliferation. To explore this possibility, we treated cardiomyocytes with the β -catenin inhibitor IWR1 (18) and observed that IWR1 partially inhibited YapS112A-induced cytokinesis (Fig. 3D).

To examine whether Yap could augment IGF signaling in cardiomyocytes, we infected cardiomyocytes with adenoviruses expressing GFP or YapS112A, serum-starved the cells, and then treated the cells with IGF. Basal phosphorylation of Akt in YapS112A-expressing cells not treated with IGF was similar to that of IGF-treated control cells. However, after 20 min of IGF stimulation, phosphorylation of Akt was enhanced in Ad-YapS112A infected cells, indicating that Yap sensitized cardiomyocytes to IGF signaling (Fig. 3E).

To further investigate whether up-regulation of IGF signaling by Yap affects β -catenin and its activity, we inhibited the IGF pathway in cardiomyocytes with the PI3K inhibitor LY294002. This compound inhibited the Yap-mediated increase in TOPflash luciferase reporter activity (Fig. 3F). Consistent with this observation, the amount of active β -catenin was decreased in the presence of LY294002 (Fig. 3G). The blockade in β -catenin activity suggests that IGF mediates the regulation of β -catenin and cardiomyocyte proliferation by Yap.

To circumvent possible off-target effects of the inhibitors and directly determine whether β -catenin and IGF1R are essential mediators of cardiomyocyte proliferation in response to YapS112A, we knocked down β -catenin and IGF1R by RNA interference (RNAi) (Fig. 3H). The number of dividing cells was significantly reduced by RNAi knockdown compared with that of non-RNAi-treated cells, suggesting that β -catenin and IGF1R are necessary for cardiomyocyte proliferation induced by Yap (Fig. 3I).

DISCUSSION

Our findings define Yap as a nodal integrator that links pro-growth signaling pathways to coordinate heart growth during embryogenesis. Although the IGF and PI3K-Akt signaling pathways promote cardiomyocyte proliferation (19, 20), the downstream effectors of these pathways are incompletely understood. Our results identify Yap as a key transcriptional mediator of the growth-stimulatory effects of IGF on cardiomyocyte proliferation and indicate that GSK-3 β and its downstream substrate β -catenin are important mediators of the proliferative effects of IGF on cardiomyocytes. Thus, Yap appears to function as a nexus that connects the Hippo, IGF, and Wnt signaling pathways to govern embryonic heart growth (Fig. 4). The results of this study extend previous reports showing that deletion of upstream kinases in the Hippo pathway promote cardiomyocyte proliferation (6). Although one interpretation of those previous findings is that Yap mediates the antiproliferative effects of these kinases, note that Hippo pathway kinases have numerous substrates, both transcriptional and non-transcriptional, in addition to Yap. Thus, the current work unequivocally shows that deletion of Yap in the embryonic heart impairs cardiomyocyte proliferation, whereas expression of constitutively active Yap promotes cardiomyocyte proliferation. We conclude that Yap, a terminal transcriptional effector of the Hippo pathway, is both necessary and sufficient for embryonic heart growth.

The IGF signaling pathway regulates cardiac growth and development

IGF signaling plays essential roles in regulating cell proliferation during development, such that dysregulation of IGF signaling results in aberrant growth phenotypes. Lack of IGF1, IGF2, and IGF1R in mice leads to growth deficiency (21, 22). Both IGF1- and IGF2-null mice display reduction in body weight and partial perinatal lethality. IGF1R-null mice exhibit a more severe phenotype with complete lethality at birth and hypoplasia of multiple organs. Moreover, fibroblasts from IGF1R-null mice are resistant to tumor formation. Conversely, increased IGF signaling has been implicated in promoting cardiomyocyte proliferation (23, 24).

Several studies have shown that IGF signaling not only stimulates neonatal cardiomyocyte division in vitro (25, 26) but also enhances cardiomyocyte proliferation during embryogenesis (20). Relatively little is known of the mechanisms that control IGF signaling or the transcriptional effectors of this pathway. Our data show that GSK-3 β and its downstream substrate β -catenin are important mediators of the growth-stimulatory actions of IGF on cardiomyocytes. This conclusion is supported by several observations. First, IGF1 action is primarily mediated by the cell surface IGF receptor. YapS112A increases the abundance of Igfbp2 and IGF1R and activates the downstream mediators PI3K and Akt. Inactivation of GSK-3 β by activated Akt leads to an increase in β -catenin abundance. Second, Yap augments IGF-dependent phosphorylation of Akt. Inhibition of PI3K activity blocks the IGF-mediated Yap-stimulated increase in active β -catenin. Third, suppression of β -catenin and IGF1R abrogates the proliferative effects of YapS112A in cardiomyocytes. Our data show that IGF signaling and β -catenin are important mediators of Yap-induced growth-stimulatory actions on cardiomyocytes, but we cannot totally rule out the possibility that IGF1 and β -catenin may independently mediate Yap-induced proliferation.

GSK-3 β signaling plays important roles in regulating cardiomyocyte proliferation during development. Mice lacking GSK-3 β exhibit a cardiomyocyte hyperproliferation phenotype (27). Furthermore, the GSK-3 β inhibitor BIO promotes cardiomyocyte proliferation in vitro (28). PI3K-Akt signaling also regulates cardiomyocyte proliferation (19, 29, 30). Our study identifies Yap as a previously unrecognized transcriptional cofactor that promotes embryonic and neonatal cardiomyocyte proliferation by activating IGF-dependent PI3K-Akt signaling.

Yap is a nexus of multiple signaling pathways

Regulation of tissue growth and the control of organ size require precise coordination of multiple developmental signaling pathways. Yap has been reported to mediate the actions of various growth factors, including BMP, Wnt, epidermal growth factor (EGF), and Hedgehog (16). Yap activates Smad1-dependent transcription downstream of the BMP pathway and is required for BMP suppression of neural differentiation of mouse embryonic stem (ES) cells (31). Yap also provides an integration point in Hedgehog signaling, acting as a transcriptional coactivator for Gli transcription factors (32). Yap has non-cell-autonomous functions through its interaction with the EGF receptor (EGFR) in *Drosophila* (33), and activation of Yap promotes proliferation of neighboring cells in an EGFR-dependent manner (33). There is also crosstalk between Hippo and Wnt signaling (6, 34). The Yap homolog, WW domain-containing transcription regulator 1 (Wwtr1; also referred to as Taz), interacts with Disheveled in the cytoplasm, thereby inhibiting Wnt- β -catenin signaling, revealing a cytoplasmic function of Taz that is independent of its effects on transcription (34).

It will be interesting to investigate the potential of Yap to promote myocardial regeneration after injury. The neonatal mouse heart can regenerate in response to surgical resection of the ventricular apex (35), but this regenerative response is lost after the first week of postnatal life. Whether Yap contributes to the regenerative response of the neonatal heart such that its sustained expression in later life can extend the regenerative response is currently under investigation.

MATERIALS AND METHODS

Creation of a conditional *Yap* mutant allele

The *Yap* targeting vector was constructed with pGKneoF2L2DTA harboring two loxP sites encompassing a neomycin resistance cassette flanked by two FRT sites (36). Targeting arm sequences were isolated from 129SvEv genomic DNA by polymerase chain reaction (PCR). The *Yap* 5' targeting arm (5 kb), *Yap* 3' targeting arm (4 kb), and *Yap* knockout arm (2 kb) were cloned into the vector with Not I, Xma I, and Eco RV, respectively. The sequence-verified targeting vector was linearized and electroporated into 129SvEv-derived ES cells. Targeting of the mutant allele was screened through Southern blot analysis. *Yap* 5' probe detects a 6-kb DNA fragment in addition to the wild-type 9-kb fragment after Eco RI digestion and *Yap* 3' probe detects a 10-kb DNA fragment plus the wild-type 8-kb fragment after Pst I digestion in the presence of *Yap* targeted allele. Targeted ES cells were injected into blastocysts to generate chimeric mice. High-percentage chimeric males were bred with mice expressing FLPe recombinase to remove the neomycin resistance cassette, producing the *Yap*^{loxP} allele (37). PCR genotyping with a forward primer upstream of 5' loxP site, a first reverse primer located in the knockout arm, and a second reverse primer downstream of 3' loxP site produces 600-, 457-, and 338-bp (base pair) bands for *Yap*^{loxP}, *Yap*^{WT}, and *Yap*^{flxed} alleles, respectively. Primer sequences for PCR genotyping are as follows: YF, 5'-ACATGTAGGTCTGCATGC-CAGAGGAGG-3'; YR1, 5'-AGGCTGAGACAGGAGGATCTCTGT-GAG-3'; and YR2, 5'-TGTTGAGACAGCGTCACTATGGAGC-3'. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center.

Transgenic mice

Transgenic mice were generated by pronuclear injection according to standard procedures. A full-length mouse complementary DNA (cDNA) encoding YapS112A with a FLAG epitope tag was cloned 3' to a 5.5-kb segment of the β MHC promoter and 5' to a 0.6-kb polyadenylation signal from the human growth hormone gene. The transgene was linearized

with Not I to remove vector sequence, injected into fertilized oocytes from B6C3F1 female mice, and implanted into pseudopregnant ICR mice.

Histology and immunohistochemistry

Mouse embryos and adult hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned at 5- μ m intervals. Hematoxylin and eosin (H&E) staining was performed using standard procedures. Neonatal cardiomyocytes were fixed in 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100 in PBS. Immunohistochemistry was carried out on paraffin-embedded sections and fixed cardiomyocytes as described previously (38). Antibodies used in this study were anti- α -actinin antibody (Sigma), anti-phospho-H3 antibody (Cell Signaling), anti-BrdU antibody (Roche), and anti-Aurora B kinase antibody (Cell Signaling). Conjugated secondary antibodies Alexa Fluor 555 and 488 were purchased from Invitrogen. Slides were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were obtained with a Leica Microsystems DMRXE compound microscope.

Western blot analysis

Total heart lysate was prepared by homogenizing frozen tissue in radio-immunoprecipitation assay (RIPA) buffer [50 mM tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, and protease inhibitor cocktail (Roche)]. Total cell lysates from neonatal rat cardiomyocytes were prepared by lysing the cells with RIPA buffer. Lysates were then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed to detect Akt, phospho-Akt, GSK-3 β , phospho-GSK-3 β , and β -catenin with rabbit anti-Akt (Cell Signaling) at a dilution of 1:1000, anti-phospho-Akt (Cell Signaling) at a dilution of 1:1000, anti-GSK-3 β (Cell Signaling) at a dilution of 1:1000, anti-phospho-GSK-3 β (Cell Signaling) at a dilution of 1:1000, anti- β -catenin (Cell Signaling) at a dilution of 1:1000, and anti-active β -catenin (Millipore) at a dilution of 1:1000. Anti-rabbit horse-radish peroxidase (HRP) and anti-mouse HRP (Bio-Rad) at a dilution of 1:5000 were used as secondary antibodies, followed by detection with ECL detection kit (Amersham).

Generation of adenoviruses

For adenovirus production, cDNAs encoding FLAG-tagged wild-type Yap, Yap-S112A, or Yap- Δ AD were subcloned into the p-AdTrack-CMV (cytomegalovirus) vector and transformed in bacteria BJ5183-AD-1 (Stratagene) for homologous recombination. Adenoviruses were generated by transfecting linearized recombinant adenoviral plasmids into a mammalian packaging cell line Ad-293. Primary lysates were used to reinfect Ad-293 cells to generate higher-titer viruses.

Cardiomyocyte cultures and stimulation studies

Neonatal rat ventricular myocytes (NRVMs) were prepared by dissociation of 1- to 2-day-old Sprague-Dawley rats with the Isolation System for Neonatal Rat/Mouse Cardiomyocytes (Cellutron) according to the manufacturer's instructions. After isolation, NRVMs were maintained in Dulbecco's modified Eagle's medium (DMEM)/199 medium (4:1) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. For assessing cardiomyocyte proliferation, NRVMs were infected with recombinant adenovirus 24 hours after plating in plating medium. NRVMs were grown in serum-free medium for 24 hours and incubated with 10 μ M BrdU for another 24 hours. Cardiomyocytes were stained by anti- α -actinin antibody and assessed by indirect immunofluorescence. For TOPflash luciferase assay, 24 hours after virus infection, cardiomyocytes were transfected with TOPflash luciferase reporter [TOPflash reporter contains three TCF response elements

(CCTTTGATC) upstream of a basal *c-fos* promoter] and incubated with or without LY294002 (Sigma) at 50 μ M for 36 hours, and luciferase activity was measured. NRVMs were infected with recombinant adenovirus, incubated with or without 50 μ M LY294002, or with or without 100 μ M IWR1 (provided by L. Lum) for 2 days followed by Western blot analysis or immunostaining with anti-Aurora B kinase antibody, respectively. For IGF stimulation studies, cardiomyocytes were infected with recombinant adenovirus and incubated in serum-free medium for 48 hours before IGF was added to cardiomyocytes at 50 nM for 20 min.

RNAi knockdown studies

RNAi double-stranded oligos were purchased from Sigma. Cardiomyocytes were transfected with RNAi oligos with Lipofectamine 2000 (Invitrogen) 1 day after the cardiomyocytes were infected with adenoviruses. Two days after transfection, cells were either fixed with 4% paraformaldehyde for immunostaining or lysed for Western blot analysis.

RNA analysis

Total RNA was purified from isolated heart ventricles or cardiomyocytes with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of RNA was used as template to synthesize cDNA with random hexamers.

Statistics

Data are presented as means $\pm$ SEM. Differences between groups were tested for statistical significance with the unpaired two-tailed Student's *t* test, analysis of variance (ANOVA) with the Bonferroni post-test, or the Mann-Whitney test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Differences at $P = 0.05$ and lower were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to L. Lum for providing IWR1 small-molecule inhibitor. We thank T. Carroll, B. Johnson, D. Patrick, C. Grueter, E. Small, R. Frost, and M. Murakami for sharing insightful discussions. We thank C. Nolen and G. Vitug for technical support. We also thank J. Cabrera for graphics.

Funding: M.X. was supported by a Beginning Grant-in-Aid from the American Heart Association. Work in the laboratory of E.N.O. was supported by grants from the NIH, the Robert A. Welch Foundation (grant I-0025), the American Heart Association: Jon Holden DeHaan Foundation, the Donald W. Reynolds Center for Clinical Cardiovascular Research, and the Fondation Leducq TransAtlantic Network of Excellence in Cardiovascular Research Program.

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Fig. 1.

Yap is necessary and sufficient for embryonic heart growth. (A) Whole-mount, H&E, and phosphorylated H3 (PH3) immunostaining of *Yap^{loxP/+}* (Control) and *Yap^{loxP/-}; Nkx2.5-Cre* (*Yap* nKO) embryos at E9.5. PH3 (red)–, *Nkx2.5* (blue)–, and cardiac troponin T (green)–stained section of *Yap* nKO. lv, left ventricle; la, left atrium; v, common ventricle; a, atrium. $n = 3$ embryos per phenotype. Arrowheads, PH3-positive cells. Asterisks, blood cells. (B) Quantification of number of cardiomyocytes per section in control (Ctrl) and *Yap* nKO at E9.5, PH3 immunostaining in Ctrl and *Yap* nKO at E9.5, and of PH3-positive cells in wild-type (WT) and β MHC-YapS112A transgenic (Tg) mice at E10.5. $n = 3$ embryos per genotype. Data are presented as means $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, by t test. (C) H&E-stained sections of embryonic hearts from β MHC-YapS112A Tg and WT mice at E14.5. The top panels show the entire heart, and the lower panels show higher magnification of the ventricles of the hearts. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. Scale bars, 100 μ m. (D) H&E staining of transverse section of WT and β MHC-YapS112A Tg embryos at E10.5 (left column). PH3-stained sections of hearts from WT and β MHC-YapS112A Tg embryos at E10.5 (middle column). Higher magnification of H&E staining of the left ventricles (right column). la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. Scale bars, 100 μ m.

Fig. 2.

Yap promotes neonatal cardiomyocyte proliferation in vitro. **(A)** BrdU (red) and α -actinin (green) staining of neonatal rat cardiomyocytes infected with adenovirus expressing GFP or YapS112A. Arrowheads, BrdU-positive cells. Scale bar, 20 μ m. **(B)** Quantification of BrdU incorporation assays. Cells were counted in six randomly selected fields for each condition. Number of cells counted per field was >100. Data are presented as means $\pm$ SEM. *** P < 0.001 by t test. **(C)** Quantification of PH3-positive cardiomyocytes. Cells were counted in eight randomly selected fields for each condition. Number of cells counted per field was >100. Data are presented as means $\pm$ SEM. *** P < 0.001 by t test. **(D)** Representative image of cardiomyocytes infected with Ad-YapS112A, immunostained for Aurora B kinase (red) and α -actinin (green). Arrows, cells undergoing cytokinesis. Scale bar, 20 μ m. **(E)** Quantification of Aurora B kinase immunostaining. Cells were counted in six randomly selected fields for each condition. Number of cells counted per field was >100. Data are presented as means $\pm$ SEM. ** P < 0.01 by t test. **(F)** Quantification of cardiomyocyte number by cardiac α -actinin staining. Cells were counted in nine randomly selected fields for each condition. Number of cells counted per field was >100. Data are presented as means $\pm$ SEM. *** P < 0.001 by ANOVA with the Bonferroni post test.

Fig. 3.

Yap increases the activity of the IGF signaling pathway. **(A)** Neonatal rat cardiomyocytes were infected with adenovirus expressing GFP or YapS112A and immunoblotted with antibodies against the indicated proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. **(B)** Quantification of abundance of IGF, Akt, and β -catenin signaling proteins in neonatal rat cardiomyocytes infected with adenoviruses expressing GFP or YapS112A. Values were determined with data from four Western blots. Protein quantification was normalized to Ad-GFP-infected cells. $*P < 0.05$, Mann-Whitney test. **(C)** Neonatal rat cardiomyocytes were infected with adenovirus expressing the indicated genes followed by transfection with a TOPflash luciferase reporter. Luciferase activity was normalized to β -galactosidase activity. Data are presented as means $\pm$ SEM. $*P < 0.05$, Mann-Whitney test. **(D)** Aurora B kinase immunostaining was quantified after cardiomyocytes were infected with the indicated recombinant adenovirus and incubated with

the β -catenin inhibitor IWR1. Data are presented as means $\pm$ SEM. *** $P < 0.001$, Mann-Whitney test. **(E)** Neonatal rat cardiomyocytes were infected with adenovirus expressing GFP or YapS112A, serum-starved, stimulated with IGF, and immunoblotted for active Akt. GAPDH was used as loading control. Protein quantification was normalized to Ad-GFP without IGF treatment. Quantification of three Western blots is shown. * $P < 0.05$, *** $P < 0.001$ by ANOVA with the Bonferroni post test. **(F)** Neonatal rat cardiomyocytes were infected with recombinant adeno-virus expressing GFP or YapS112A, transfected with TOPflash luciferase reporter, and incubated with the PI3K inhibitor LY294002. Luciferase activity was normalized to β -galactosidase activity. Data are presented as means $\pm$ SEM. ** $P < 0.01$, Mann-Whitney test. **(G)** Neonatal rat cardiomyocytes were infected with adenovirus expressing GFP or YapS112A, incubated with LY294002, and immunoblotted for active β -catenin. Quantification of protein expression from three Western blots is shown in the bar graph, with a representative Western blot shown below. ** $P < 0.01$, ANOVA with the Bonferroni post test. **(H)** Neonatal rat cardiomyocytes were infected with adenovirus expressing GFP or YapS112A, transfected with small interfering RNAs (siRNAs) against β -catenin or IGF1R, and immunoblotted for β -catenin or IGF1R antibodies to confirm knockdown. **(I)** Quantification of Aurora B-positive cells. Cells were counted in eight randomly selected fields for each condition. Number of cells counted per field was >100 . Data are presented as means $\pm$ SEM. *** $P < 0.001$, ANOVA with the Bonferroni post test.

Fig. 4.

Model showing a role for Yap in governing cardiac growth and survival by interlinking the Hippo, IGF, and Wnt pathways. Yap promotes cardiomyocyte proliferation through activation of IGF signaling and stabilization of the active form of β -catenin. Inhibition of IGF signaling or β -catenin blocks Yap-induced cardiomyocyte proliferation. Yap interlinks the Hippo pathway (MST1/2 and LATS1/2), the Wnt pathway [Wnt, Disheveled (Dvl), Axin, APC, and GSK-3 β], and the IGF pathway (IGF, IGF1R, PI3K, Akt, and GSK-3 β) to control β -catenin signaling and regulate cardiac growth and development. Each of the three pathways influenced by Yap is demarcated by a box.