Endocardial Hippo signaling regulates myocardial growth and cardiogenesis

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Summary
The Hippo signaling pathway has been implicated in control of cell and organ size, proliferation, and endothelial-mesenchymal transformation. This pathway impacts upon two partially redundant transcription cofactors, Yap and Taz, that interact with other factors, including members of the Tead family, to affect expression of downstream genes. Yap and Taz have been shown to regulate, in a cell-autonomous manner, myocardial proliferation, myocardial hypertrophy, regenerative potential, and overall size of the heart. Here, we show that Yap and Taz also play an instructive, non-cell-autonomous role in the endocardium of the developing heart to regulate myocardial growth through release of the paracrine factor, neuregulin. Without endocardial Yap and Taz, myocardial growth is impaired causing early post-natal lethality. Thus, the Hippo signaling pathway regulates cell size via both cell-autonomous and non-cell-autonomous mechanisms. Furthermore, these data suggest that Hippo may regulate organ size via a sensing and paracrine function in endothelial cells.

Introduction
The mechanisms by which organ size is regulated during development and in the setting of regeneration after injury remain fundamental questions in biology. How is organ size sensed by the organism and what are the cellular effectors that mediate homeostasis? In the embryonic heart, cardiomyocyte proliferation continues through gestation and into the early post-natal period, but subsequent growth in the size of the mammalian postnatal heart is achieved largely via myocardial hypertrophy. In the setting of neonatal cardiac injury in the mouse and zebrafish, myocardial cells re-enter the cell cycle and proliferate until normal heart size is reestablished, while in the adult mouse and human, myocardial injury leads to compensatory myocardial hypertrophy in the non-injured regions of the heart (Eschenhagen

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et al., 2017; Laflamme and Murry, 2011; Xin et al., 2013b). The pathways and molecular signals that regulate homeostasis of heart organ size, however, remain largely unknown.

The Hippo signaling pathway has been implicated in the regulation of cardiomyocyte proliferation in the embryo and in the setting of neonatal cardiac regeneration (Xin et al., 2013a). Hippo was first elucidated in Drosophila (Huang et al., 2005) where genetic screens to identify effectors of an overgrowth phenotype identified the Hippo serine/threonine kinase (Mst1/2 in mammals) and the downstream kinase Warts (Lats1/2 in mammals) that phosphorylates the transcription co-factor Yorkie (Yap/Taz in mammals) (Justice et al., 1995; Xu et al., 1995). Upstream activators of the Hippo kinase cascade remain only partially elucidated. Mechanical stress (Benham-Pyle et al., 2015; Codelia et al., 2014; Dupont et al., 2011; Kim et al., 2014; Porazinski et al., 2015) and cell contact can activate Hippo, as can G-protein coupled receptors signaling through Gα12/13 (Yu et al., 2012). In mice, the Hippo kinase cascade is required in cardiomyocytes for normal heart development (Del Re et al., 2013; Heallen et al., 2011; Lin et al., 2014; von Gise et al., 2012; Xin et al., 2013a; Xin et al., 2011). Lats phosphorylation of Yap at serine 127 sequesters Yap in the cytoplasm. When a constitutively active form of Yap (YapS127A) is forced into the nucleus and overexpressed in myocardial cells, this results in hyperproliferation of cardiomyocytes and improved regeneration after neonatal myocardial injury (Lin et al., 2014). Embryonic inactivation of Mst1/2 or Lats2 also produces cardiomyocyte hyperproliferation, increased heart size and lethality. Thus, Hippo has been implicated as a therapeutic target to modulate the regenerative response of the heart to injury and as a fundamental regulator of heart size.

Myocardial growth and maturation is regulated in part by signals from the underlying endocardium (Brutsaert, 2003; de la Pompa and Epstein, 2012; Rentschler et al., 2010). More generally, endothelial cells play an increasingly appreciated role in instructing organogenesis by providing inductive signals and by secreting growth factors and cell surface ligands in addition to their important role in blood vessel formation, and thus in delivering oxygen and nutrients to developing tissues. In endodermal tissues, endothelial cells play an instructive function in the formation of liver and pancreas (Matsumoto et al., 2001; Yoshitomi and Zaret, 2004). In the developing heart, endocardial cells secrete neuregulin 1 (Nrg1) and express the cell surface ligand ephrin B2 (Efnb2), both of which signal to neighboring myocardial cells to regulate growth and maturation of the myocardium. Notch signaling between endocardium and myocardium is also required for myocardial growth and trabeculation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). Active Notch signaling in the endocardium up-regulates Efnb2, which subsequently enhances Nrg1 expression (Grego-Bessa et al., 2007). Nrg1 is a ligand for the erb-b2 receptor tyrosine kinases 2 and 4 (ErbB2 and ErbB4) on the adjacent myocardium and engagement of ligand stimulates ErbB2-ErbB4 dimerization and activation of ErbB2 tyrosine kinase activity to promote differentiation of trabecular myocytes (Grego-Bessa et al., 2007).

In this report, we show that Hippo signaling in endocardium regulates myocardial growth during embryonic development. In the absence of endocardial Yap and Taz, Nrg1 expression is deficient and myocardial ErbB2/4 signaling is impaired. This culminates in thin myocardial walls and early post-natal lethality. Interestingly, activation of Notch in
endocardium is intact, suggesting that both Notch and Hippo converge on Nrg1 expression. These results indicate that the Hippo pathway can regulate cell, and possibly organ size via both cell autonomous and non-cell autonomous mechanisms.

**Results**

_Yap_ and _Taz_ are each expressed in the developing heart by both myocardial cells and eNOS-expressing endocardial cells at embryonic day 13.5 (E13.5) (Figure 1A,C). We used the _Nfatc1<sup>IRES-Cre/+</sup>_ allele (Wu et al., 2012) to delete _Yap_ and _Taz_ in endocardial cells, but not other extra-cardiac vascular endothelial cells, to produce _Yap<sup>flox/flox</sup>_; _Taz<sup>flox/flox</sup>_; _Nfatc1<sup>IRES-Cre/+</sup>_ embryos (hereinafter referred to as mutants). We show by immunohistochemistry that endocardial Yap and Taz are efficiently deleted, while myocardial Yap and Taz expression remain intact (Figure 1B,D). By examining E13.5 embryos that harbor a Td/tomato Cre-reporter allele (recognized by an RFP antibody), we confirmed that _Nfatc1<sup>IRES-Cre/+</sup>_ activity was restricted to the heart in both control and mutant embryos (Figure 1E–G). Endothelial cells outside of the heart were not labeled. At this stage of development, endocardial cells also exhibit active Notch signaling as evidenced by co-expression of the cleaved Notch intracellular domain (NICD, Supplemental Figure 1).

Genetic deletion of _Yap_ and _Taz_ from endocardial cells produced mutant embryos that appear grossly normal at E13.5 (Figure 2A,E), and mutant animals were present at normal Mendelian ratios at that time point; however, mutants die between postnatal day P1 and P7 (Table 1). In the mutants, we observe abnormal interventricular septae, suggestive of serpiginous ventricular septation defects; no overt valve mesenchyme or coronary vasculature defects were appreciated. Histological sections of hearts from E13.5 mutant embryos also reveal thin right and left ventricular walls as compared to controls (Figure 2B–D,F–H). The thickness of the ventricular myocardium in E13.5 mutant hearts was significantly decreased in both the right and left ventricles compared with controls (Figure 2I,J), and this thin ventricular myocardial phenotype remained evident at perinatal time points (E18.5 and P0) (Supplemental Figure 2).

To interrogate the molecular basis for the myocardial phenotype in mutant hearts, we performed quantitative real-time PCR (qRT-PCR) expression analysis of genes encoding critical endocardium-derived molecules including _Nrg1_ and _Efnb2_. _Nrg1_ expression was reduced in E13.5 mutant hearts as compared to controls, while _Efnb2_ expression levels did not notably differ (Figure 3A). We also measured the expression of other endocardium-enriched genes, _Vegfr2_, _Nfatc1_ and _Pecam1_, and found these transcripts to be present at similar levels in control and mutant hearts (Figure 3A). These observations are consistent with a specific Yap/Taz-dependent regulation of _Nrg1_ in endocardium.

Consistent with the qRT-PCR results, antibody staining revealed reduced Nrg1 expression in the Pecam1-positive endocardial cells of _Yap/Taz_ mutants compared to control hearts (Figure 3B–G). Consistent with reduced endocardial Nrg1 ligand expression, we also noted reduced phosphorylation of the Nrg1 co-receptor, ErbB2 (phospho-ErbB2, p-ErbB2), on adjacent cardiac troponin T (cTnT)-positive myocardial cells in the mutants compared to controls (Figure 3H–M). Collectively, these data are consistent with the Hippo effector...
molecules Yap and Taz acting in endocardium upstream of Nrg1/ErbB2 signaling in the developing heart.

Given the reduced levels of Nrg1 expression observed in the Yap/Taz mutants, we sought to examine the possibility that Nrg1 expression requires Yap and Taz. To do so, we examined murine embryonic fibroblasts (MEFs) isolated from Yap^flox/flox;Taz^flox/flox embryos. Treatment with an adenovirus-expressing Cre recombinase (AAV1-Cre) significantly reduced Yap and Taz expression in these cells (Supplemental Figure 3A). We observed a reduction of Nrg1 expression in response to Cre-mediated deletion of Yap and Taz similar to that found in mutant mouse hearts (Supplemental Figure 3A). Transient overexpression of Yap and Taz, individually or in combination, in the AAV1-Cre-treated Yap^flox/flox;Taz^flox/flox MEFs rescued Nrg1 expression (Supplemental Figure 3B), confirming the Yap/Taz-dependent expression of Nrg1.

Further, we asked whether Yap binds Nrg1 regulatory elements in endothelial cells. We used published chromatin profiles of human umbilical vein endothelial cells (HUVECs) to identify relevant Nrg1 regulatory elements (Ernst et al., 2011) that control the most highly expressed Nrg1 transcript (Figure 4A). We identified two well conserved putative TEAD binding sites (one in the promoter and one in a strong enhancer) and demonstrated Yap occupancy in HUVECs by chromatin immunoprecipitation followed by qPCR (Figure 4B). These chromatin regions showed Yap enrichment of 2.5- to 4-fold over IgG controls. As expected, Yap was not enriched at either of two nearby control genomic regions (NC1, NC2) upstream of these putative TEAD binding sites, or at another more distant region (DC1) on the same chromosome (Figure 4B and see methods for genome coordinates). This suggests that Yap/Taz may directly regulate Nrg1 expression in endothelial cells.

To determine whether Nrg1 acts as the primary molecular effector underlying the Yap/Taz mutant heart phenotype, we cultured explanted E12.5 embryonic hearts in serum-free medium supplemented with insulin/transferrin/selemin formula (Sigma) with or without recombinant Nrg1. Following 24 hours of culture, with or without Nrg1, both control and mutant hearts were beating, suggesting myocardial viability. Vehicle-treated mutant hearts, however, had thin compact myocardium compared with control hearts, which is consistent with our in vivo analysis (Figure 5A,C). Nrg1-treated mutant hearts had a significantly thickened compact myocardial wall compared to vehicle-treated mutant hearts (Figure 5C,D) and had enlarged to a degree similar to Nrg1-treated control hearts (Figure 5). We assessed phenotypic rescue of the thin compact myocardium by comparing the number of cells in the compact myocardium of vehicle- and Nrg1-treated control and mutant hearts. Upon treatment with exogenous Nrg1, the thin compact myocardium of mutant hearts was rescued and the number of cells in this region increased to similar levels as control hearts (Figure 5E). This is consistent with the reported role of Nrg1 as a growth factor essential for ventricular trabeculation (Meyer and Birchmeier, 1995; Rentschler et al., 2002). Since Nrg1 has also been shown to affect the cell size of myocardial cells (Zhao et al., 1998), we assessed the size of the compact myocardial cells by staining heart sections with wheat germ agglutinin to demarcate the cell boundary, and measuring the distance between nearby nuclei. Nrg1-treated control and mutant hearts had larger compact myocardial cells compared to vehicle-treated control and mutant hearts (Figure 6). Thus, exogenous Nrg1
treatment can rescue the thin myocardium of mutant hearts by increasing myocardial cell number as well as cell size. Taken together, these data suggest that endocardial Yap and Taz are required for proper Nrg1 expression and for normal myocardial development.

**Discussion**

In this study, we show that conditional loss of Yap/Taz in the endocardium results in a thin compact myocardium and early postnatal lethality. We also report that loss of Yap/Taz in the endocardium leads to diminished expression of Nrg1, which is a crucial endocardial-derived factor that shapes the myocardium (Brutsaert, 2003; Ford et al., 1999; Lai et al., 2010; Meyer and Birchmeier, 1995; Rentschler et al., 2002). The myocardial phenotype of hearts with Yap/Taz-deficient endocardium is partially rescued by addition of recombinant Nrg1. Moreover, we demonstrate that Yap can occupy the promoter/enhancer regions of Nrg1, suggesting that Yap may directly regulate Nrg1 expression. This study reveals a novel Yap/Taz-dependent regulation of Nrg1 in the endocardium that is required for proper myocardial development.

Both the Hippo pathway and the ErbB pathway have been implicated in cell-autonomous control of cardiomyocyte proliferation during development and after injury, and both are attractive therapeutic targets to enhance cardiac regeneration. Our results highlight an unexpected convergence of these pathways in endocardial cells where Yap/Taz function to regulate Nrg1 expression. Nrg1 secretion from endocardium activates ErbB2/4 receptors on adjacent cardiac myocytes, and it is attractive to hypothesize that active ErbB signaling in cardiac myocytes once again converges with Hippo signaling to regulate proliferation. Indeed, recent studies in mammary cells indicate that Nrg1 binding to ErbB receptors induces cleavage of ErbB4 and subsequent liberation of an intracellular domain (ICD) that binds Yap via the WW domain (Haskins et al., 2014). The Yap:ErbB4-ICD complex enters the nucleus, binds TEAD factors, and induces the expression of Yap-dependent proliferation-associated genes. Assuming a similar capacity for ErbB4/Yap interactions in cardiac myocytes, we speculate that Hippo activation in endocardium results, indirectly, in activation of Yap/Taz target genes in cardiac myocytes in a Nrg1/ErbB-dependent manner. Thus, cell-autonomous and non-cell-autonomous Hippo signaling may be linked via Nrg1/ErbB signaling to regulate cardiogenesis.

A growing body of evidence identifies endothelium as an important source of paracrine signaling during organogenesis. For example, endothelial cells promote liver organogenesis during development even before formation of the regional vasculature (Freedman et al., 2007; Matsumoto et al., 2001). In the heart, disruption of multiple pathways, including PlexinD1 (Gitler et al., 2004), Neurofibromin (Gitler et al., 2003), Notch (de la Pompa and Epstein, 2012; High and Epstein, 2008) and Shp2 (Araki et al., 2009; Lauriol et al., 2016), can result in structural or functional heart disease despite the presence of apparently intact blood vessels and oxygen-carrying capacity. In neural crest cells, we have previously shown that Notch and Hippo signaling can interact since Yap/Taz functionally bind to Rbp-J to regulate Notch target genes (Manderfield et al., 2015). Others have provided evidence that ErbB signaling is disrupted when the Notch pathway components Numb and Numb-like are deleted (Hirai et al., 2017). It will be of interest to determine how these and additional
pathways intersect to form the network of endocardial signaling that impacts upon cardiac myocyte homeostasis.

In the setting of a major ischemic episode, the mammalian heart fails to mount a robust regenerative response sufficient to replace the loss of cardiomyocytes. The Nrg1-ErbB signaling axis has been a focus of translational efforts to ameliorate ischemia-reperfusion injury and to enhance regeneration. Nrg1 stimulates proliferation of mono-nucleated mouse cardiomyocytes and enhances cardiac repair after injury in both mice and zebrafish (Bersell et al., 2009; Gemberling et al., 2015). Gain- and loss-of-function studies in mice involving both ErbB2 and ErbB4 support the rationale for further studies focused on the therapeutic potential of the Nrg1-ErbB signaling pathway (Bersell et al., 2009; D’Uva et al., 2015; D’Uva and Tzahor, 2015; Polizzotti et al., 2015). Indeed, clinical trials using recombinant Nrg1 have been initiated (Gao et al., 2010; Jabbour et al., 2011). Our results suggest that endothelial Yap/Taz activity may be a relevant therapeutic target for modulation of Nrg1-ErbB signaling. Further studies to determine if endothelial Hippo signaling could act as a sensor to regulate myocardial regeneration in neonatal or adult life will be of great interest.

In summary, we provide evidence to support non-cell autonomous Hippo signaling as a regulator of myocardial growth, in addition to the known role of Hippo within myocardium. Our results demonstrating Hippo regulation of Nrg1 in endocardium provide a link between two signaling pathways strongly implicated in control of myocardial proliferation and regeneration.

Methods

Mice

All mice were maintained on a mixed genetic background. Yap\textsuperscript{flox/+} (Xin et al., 2011), Taz\textsuperscript{flox/+} (Xin et al., 2013a) and Nfatc1\textsuperscript{IRES-Cre/+} (Wu et al., 2012) alleles were genotyped as previously described. R26\textsuperscript{tdTomato} mice (B6.Cg-Gt(ROSA)26Sor\textsuperscript{tm14(CAG-tdTomato)Hze/J}) were obtained from Jackson Labs (strain #007914) and genotyped as previously described (Madisen et al., 2010). All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Mouse embryo fibroblast preparation

Mouse Embryo Fibroblasts (MEFs) were isolated from E14.5 Taz\textsuperscript{flox/flox};Yap\textsuperscript{flox/flox} embryos as described (Manderfield et al., 2014). To delete the floxed Taz and Yap alleles, MEFs were treated for 48 hours with an adeno-associated virus expressing a constitutively active Cre-recombinase (AAV1-CMV-Cre, Penn Vector Core, University of Pennsylvania, Philadelphia, PA) at a dosage of 1000 genome copies per cell.

RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from untreated or AAV1-CMV-Cre treated Taz\textsuperscript{flox/flox};Yap\textsuperscript{flox/flox} MEFs or from isolated embryonic hearts using the Qiagen RNeasy kit following manufacturer’s instructions (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using the Superscript III system (Invitrogen, Grand Island, NY). qRT-PCR was performed in
triplicate with SYBR Green reagents (Applied Biosystems, Grand Island, NY). Relative gene expression was normalized to Gapdh. qRT-PCR primer sequences are:

- **Gapdh Forward**: 5′ CGTCCCGTAGACAAAATGGT 3′
- **Gapdh Reverse**: 5′ GAATTGCGCTGAGTGAGT 3′
- **Taz Forward**: 5′ GAGAGGATTAGGATCGTCAAG 3′
- **Taz Reverse**: 5′ GGATCTGAGCTACTGGTG 3′
- **Yap Forward**: 5′ ACCATAAGAACAAGACACATCC 3′
- **Yap Reverse**: 5′ CTTCACCTGGAGCACTCGAG 3′
- **Efnb2 Forward**: 5′ ATTATTGCCCCAAAGTGAGACTC 3′
- **Efnb2 Reverse**: 5′ GCACCGCCGTTATTCTCCTTC 3′
- **Nrg1 Forward**: 5′ TCTGAGCGCAAAGAAGGCAG 3′
- **Nrg1 Reverse**: 5′ CTGGTTTCACACCGAAGCA 3′
- **ErbB2 Forward**: 5′ TGTGCTAGACAAACGAGACC 3′
- **ErbB2 Reverse**: 5′ CGGATCAAACCTCCTCCTTA 3′
- **ErbB4 Forward**: 5′ CCTTCTGCCTGCTATCCGA 3′
- **ErbB4 Reverse**: 5′ CCAAAGTGGCCATCTTCTGTA 3′

**Histology and immunofluorescence**

Samples were harvested, fixed overnight in 2% paraformaldehyde and dehydrated through an ethanol series. All samples were paraffin-embedded and sectioned. All primary antibodies were incubated overnight at 4°C at the specified dilutions. Antibodies used for immunofluorescence were anti-Nrg1 (1:50, Abcam, Cambridge, MA, ab2369), anti-Yap (1:200, Cell Signaling, Danvers, MA, #4912S and #12395S), anti-Taz (1:200, Cell Signaling, Danvers, MA, #4883), anti-PECAM1 (1:20, HistoBioTech, Miami Beach, FL, DIA 310), anti-eNOS (1:250, BD Biosciences, San Jose, CA, #610296), anti-NICD (1:25, Cell Signaling, Danvers, MA, #2421) and phospho-ErbB2 (1:25, Cell Signaling, Danvers, MA, #2243).

**ChIP-qPCR**

HUVEC (ATCC® CRL-1730™) in 15cm² dishes were fixed in 1% formaldehyde for 10 minutes and the fixation quenched by adding glycine to 125 mM for 5 minutes. Cells were washed twice in 1× PBS and harvested by scraping cells from the plate. ChIP was performed as described previously (Lee et al., 2006), with the exception that cellular extracts were sonicated in miliTUBE AFA Fiber tubes (Covaris, Woburn, MA, #520130) for 10 minutes in a Covaris S220 (Covaris, Woburn, MA) using the following conditions: Temperature: 5–9°C, Peak Power: 140 W, Duty Cycle: 5%, Cycles per Burst: 200. All ChIPs were performed using 500 μg of total extract and 2 μg of antibody per sample. Antibodies used were anti-Yap (Novus Biologicals, Littleton, CO, #NB110-58358) and normal rabbit IgG (Cell Signaling, Danvers, MA, #2729). Thirty microliters of Protein G Dynabeads (Invitrogen, Grand Island,
NY. #100.02D) were used per ChIP. IgG and no antibody controls were routinely performed in parallel. After elution, ChIP DNA was analyzed by standard qPCR methods using a Step One Plus Real-Time PCR System (Applied Biosystems, Grand Island, NY).

ChIP qPCR primers:

| Primer    | Sequence (5’ – 3’) | Genome Coordinates (GRCh38/hg38) of Fwd/Rev Amplicon |
|-----------|--------------------|------------------------------------------------------|
| TEAD1 Forward | GATTCAGTCTCTGCTACGG | chr8: 32,549,393-32,549,492 |
| TEAD1 Reverse | GCAGAGCTGATTCCTGACAC |                                           |
| TEAD2 Forward | TGGCTTAACAGTTCTATTTTCAAG | chr8: 32,552,594-32,552,646 |
| TEAD2 Reverse | CAGGTTGAAACAGATCACTGG |                                           |
| NC1 Forward | CTGTGGGACCAAGTGAACATC | chr8: 31,262,787-31,262,839 |
| NC1 Reverse | AACAATTAGGCCATTCTGCTG |                                           |
| NC2 Forward | GCAGCAATTCTGCAATCAGT | chr8: 31,258,368-31,258,485 |
| NC2 Reverse | AAATGCTTTGCAAGAGGTC |                                           |
| DC1 Forward | AATGCTAGGACCAAGGAGTG | chr8: 77,958,614-77,958,743 |
| DC1 Reverse | TCCAAATGTGTGTTAGCATTC |                                           |

Quantification of ventricular myocardium thickness

Paraffin sections from control (Taz\textsuperscript{flox/flox}; Yap\textsuperscript{flox/flox} or Nfatc\textsuperscript{IRES-Cre/+}; Taz\textsuperscript{flox/+}; Yap\textsuperscript{flox/+}) or mutant (Nfatc\textsuperscript{IRES-Cre/+}; Taz\textsuperscript{flox/flox}; Yap\textsuperscript{flox/flox}) hearts were stained with haematoxylin and eosin (H&E). Left and right ventricles from 4 controls and 4 mutants were imaged and quantified using ImageJ software. Statistical differences between conditions were analyzed using a Student’s t-test.

Embryonic cardiac explants

E12.5 embryonic hearts were dissected from the embryo in cold PBS. Beating embryonic hearts were then cultured in DMEM/F-12 media (Gibco, Waltham, MA, #11039-021) supplemented with 1000U/mL Penicillin-Streptomycin (Gibco, Waltham, MA, #15140-122), 1× ITS liquid media (Gibco, Waltham, MA, #41400-045) and either 12.5nM of recombinant Nrg1 (Cell Signaling, Danvers, MA, #5218) or vehicle control (20 mM citrate, pH 3.0, 100 mM NaCl) for 24 hours at 37°C. Hearts were then fixed in 4% paraformaldehyde overnight at 4°C and processed for paraffin embedding and sectioning.

Quantification of cell number in the compact myocardium

Transverse sections from cardiac explants were co-stained with cTnT (Thermo MA5-12960) (demarcating myocardial cells) and Hoechst nuclear counterstain. ImageJ was used to calculate the total area of the compact myocardium and the number of cells within each of these regions was counted. Data are reported as an average of 2 to 4 sections from each condition ± standard error of the mean (SEM) measured in three independent replicates. Statistical differences between conditions were analyzed using Student’s t-test.
Quantification of cell size in the compact myocardium

To quantify the size of myocardial cells in the compact zone, cross sections of vehicle- or Nrg1-treated embryonic cardiac explants were imaged using a Leica STED 3X super resolution microscope. Three-dimensional renderings of cardiac tissues were created using Imaris image analysis and visualization software (Bitplane, Zurich, Switzerland). As a proxy for cell size, the average of the distances between the center of the cell nucleus in question and the nuclei of its three nearest neighbors in the compact zone were measured.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Eric Olson for providing Yap/Taz floxed mice, and to members of the Epstein lab for helpful comments and advice. This work was supported by NIH R01 HL118768, R35 HL140018, the Cotswold Foundation and the WW Smith Endowed Chair to J.A.E.

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Highlights

- Non-cell autonomous Hippo signaling regulates myocardial growth and organ size.
- Myocardial development requires Yap/Taz-dependent regulation of endocardial Nrg1.
- Yap/Taz regulate myocardial growth through release of the paracrine factor, Nrg1.
Figure 1. Mutant embryos display loss of both Yap and Taz in endocardial cells, but not other vascular endothelial cells
(A,B) Transverse sections of hearts from E13.5 (A) control (Yap$^{\text{flox/+}}$; Taz$^{\text{flox/+}}$; Nfatc1$^{\text{IRES-Cre/+}}$) and (B) mutant (Yap$^{\text{flox/flox}}$; Taz$^{\text{flox/flox}}$; Nfatc1$^{\text{IRES-Cre/+}}$) embryos stained for Yap and eNOS (endothelial marker) with nuclear counter-stain (Hoechst). Throughout, boxed areas are shown at higher magnification in images to the right. White arrowheads indicate cells co-expressing Yap and eNOS. Yellow arrowheads denote eNOS-positive/Yap-negative cells. Transverse sections of hearts from E13.5 control (C) and mutant (D) embryos stained for Taz and eNOS with nuclear counter-stain (Hoechst). White arrowheads indicate cells co-expressing Taz and eNOS. Yellow arrowheads denote eNOS-positive/Taz-negative cells. RV=right ventricle, LV=left ventricle. Scale bars: 100 μm. (E) Bright field and corresponding fluorescence image of whole-mount E13.5 control mouse embryo on the background of the R26$^{\text{td-Tomato}}$ reporter (Nfatc1$^{\text{IRES-Cre/+}}$; Taz$^{\text{flox/+}}$; Yap$^{\text{flox/+}}$; R26$^{\text{td-Tomato}}$) shows specificity of Cre activity to the heart. Cross sections of hearts from control (F) (Nfatc1$^{\text{IRES-Cre/+}}$; Taz$^{\text{flox/+}}$; Yap$^{\text{flox/+}}$; R26$^{\text{td-Tomato}}$) and mutant (G) (Nfatc1$^{\text{IRES-Cre/+}}$; Taz$^{\text{flox/flox}}$; Yap$^{\text{flox/flox}}$; R26$^{\text{td-Tomato}}$) E13.5 mouse embryos stained with Pecam1 and RFP antibodies. RFP expression is restricted to endocardial/endothelial cells and the mesenchyme of the endocardial cushions derived from endocardium. Scale bars: 100 μm, inset: 10 μm.
Figure 2. Loss of endocardial Yap and Taz results in thin myocardium

(A) Control (Nfatc1\textsuperscript{IRES-Cre/+}; Taz\textsubscript{floxed}/+; Yap\textsubscript{floxed}/+) E13.5 embryo imaged in bright field. (B–D) H&E stained transverse sections from the heart of a control E13.5 embryo. Blue lines indicate representative region used in ventricular wall thickness calculations. (E) Mutant (Nfatc1\textsuperscript{IRES-Cre/+}; Taz\textsubscript{floxed}/+; Yap\textsubscript{floxed}/+) E13.5 embryo imaged in bright field. (F–H) H&E stained transverse sections from the heart of a mutant E13.5 embryo. Blue lines indicate representative region used in ventricular wall thickness calculations. (I,J) Quantification of RV wall thickness (I) or LV wall thickness (J) in control and mutant embryos. Data represent the mean ± standard deviation. Statistics were completed using a Student’s t-test, *** p<0.001. RA=right atrium, LA=left atrium, RV=right ventricle, LV=left ventricle, Scale bars: 100 μm.
Figure 3. Endocardial deletion of Yap/Taz causes reduction of Nrg1 expression and Nrg1-ErbB signaling

(A) qRT-PCR of RNA isolated from E13.5 hearts for Nrg1, Efnb2, Vegfr2, Nfatc1 and Pecam1. Data depicted are the mean ± standard error of the mean (SEM) and the statistics were completed using a Student’s t-test * p<0.05; n=3. (B–D) Transverse sections from control E13.5 embryo hearts stained for (B) Nrg1 or (C,D) Nrg1 and Pecam1. White arrowheads indicate Pecam1/Nrg1 co-positive endocardial cells. (E–G) Transverse sections from mutant E13.5 embryo hearts stained for (E) Nrg1 or (F,G) Nrg1 and Pecam1. Yellow arrowheads denote Pecam1-positive/Nrg1-negative endocardial cells. (H–J) Transverse sections from control E13.5 embryo hearts stained for (H) phosphorylated ErbB2 (p-ErbB2) or (I,J) p-ErbB2 co-stained with cardiac troponin T (cTnT) and DAPI nuclear counterstain. White arrowheads indicate p-ErbB2/cTnT co-positive myocardial cells. (K–M) Transverse sections from mutant E13.5 embryo hearts stained for (K) p-ErbB2 or (L,M) p-ErbB2 co-stained with cTnT and nuclear counterstain. Yellow arrowheads denote cTnT-positive/p-ErbB2-negative myocardial cells. LV=left ventricle, Scale bars: 100 μm, inset: 10 μm.
Figure 4. Yap occupies promoter and enhancer regions of NRG1

(A) Schematic diagram of the human NRG1 locus showing active promoter (red) and enhancer (orange) regulatory regions in HUVECs, including two putative TEAD binding sites (purple) within evolutionary conserved regions of NRG1 promoter and enhancer regions. (B) ChIP-qPCR signal for Yap occupancy and IgG control at putative TEAD binding sites (TEAD1 and TEAD2), nearby experimental control regions (NC1, NC2), or distal experimental control region (DC1). See methods for genomic coordinates of control regions. Yap occupancy expressed as percent enrichment of Yap ChIP signal normalized to input. Data depicted are the mean + standard error of the mean (SEM) and the statistics were completed using a Student’s t-test * p<0.05; n=3.
Figure 5. Exogenous Nrg1 rescues the thin myocardium phenotype of Yap/Taz mutant hearts

Transverse sections of control (Yap\textsuperscript{flx/+}, Taz\textsuperscript{flx/+}, Nfatc\textsuperscript{IRES-Cre/+}) (A,B) and mutant (Yap\textsuperscript{flx/flx}, Taz\textsuperscript{flx/flx}, Nfatc\textsuperscript{IRES-Cre/+}) (C,D) E12.5 cardiac explants cultured for 24 hours with either vehicle (A,C) or recombinant Nrg1 (B,D). Sections were stained for cTnT to mark myocardial cells and with nuclear counter-stain (Hoechst). Merged images were generated by combining respective green and blue channels using ImageJ software. Dotted white lines demarcate the compact myocardium. (E) Quantification of cell number in the compact myocardium in vehicle- or Nrg1-treated control and mutant hearts. Box and whiskers plot with median, minimum, and maximum indicated depict cell number per section. Statistics were completed using a One-way ANOVA, P < 0.01 (Tukey’s multiple comparisons, ***P < 0.001, *P < 0.05). Scale bars: 100 μm.
Figure 6. Exogenous Nrg1 increases the size of compact myocardial cells

(A) Representative cross section of an E12.5 cardiac explant that was stained for wheat germ agglutinin (WGA), cTnT, and nuclear counter-stain (Hoechst) (left) and rendered in 3D (right) for quantification of cell size. Nuclei within the cTnT-low compact myocardium are dark blue. (B–E) Three-dimensional renderings of cross sections of control (Yap\textsuperscript{flox/+}; Taz\textsuperscript{flox/+}; Nfatc\textsuperscript{IRES-Cre/+}) (B,D) and mutant (Yap\textsuperscript{flox/+}; Taz\textsuperscript{flox/+}; Nfatc\textsuperscript{IRES-Cre/+}) (C,E) E12.5 cardiac explants that were treated with vehicle or recombinant Nrg1. For each representative explant cross section, only the nuclei of the compact myocardial cells are shown (blue spheres) with and without WGA-marked cell outlines (red). (F) Quantification of cell size as measured by average distance (in μm) between nuclei of the compact myocardial cells for each treatment. Statistics were completed using a Student’s t-test; * p < 0.001; N > 180 for each condition.
Table 1

Genotype of offspring from \textit{Nfatc}^{IRES-Cre/+}; \textit{Taz}^{flox/+}; \textit{Yap}^{flox/+} x \textit{Taz}^{flox/flox}; \textit{Yap}^{flox/flox} matings.

| Age       | Mutant (n) | Total (n) | Observed Mutants (%) | Expected Mutants (%) | \(\chi^2\) Test (p-value) |
|-----------|------------|-----------|----------------------|----------------------|--------------------------|
| E13.5–16.5| 8          | 79        | 10.1%                | 12.5%                | 0.524                    |
| E18.5     | 3          | 37        | 8.1%                 | 12.5%                | 0.419                    |
| P0        | 4          | 66        | 6.1%                 | 12.5%                | 0.114                    |
| P7        | 0          | 41        | 0.0%                 | 12.5%                | 0.016                    |

Ratios of genotypes were tested for goodness of fit to the expected Mendelian segregation (1:7) by chi-square analysis, calculated with 1 degree of freedom. P denotes postnatal day; postnatal day 0 is the day of birth; E denotes embryonic day.