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Activation of Yap1/Taz signaling in ischemic heart disease and dilated cardiomyopathy

Ning Hou^{a,b}, Ying Wen^e, Xun Yuan^e, Haodong Xu^c, Xuejun Wang^d, Faqian Li^{b,e,f,g,**}, and Bo Ye^{e,*}

^aDepartment of Pharmacology, School of Pharmaceutical Sciences, and The Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou 511436, PR China

^bDepartment of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY 14642, USA

^cDepartment of Pathology, Center for Cardiovascular Biology and Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195, USA

^dDivision of Basic Biomedical Sciences, University of South Dakota Sanford School of Medicine, Vermillion, SD 57069, USA

^eDepartment of Laboratory Medicine and Pathology, University of Minnesota, 420 Delaware St SE, Minneapolis, MN 55455, USA

^fMasonic Cancer Center, University of Minnesota, 420 Delaware Street SE, Minneapolis, MN 55455, USA

^gLillehei Heart Institute, Cancer & Cardiovascular Research Center, 2231 6th Street SE, Minneapolis, MN 55455, USA

Abstract

Genetic manipulation of key components of the evolutionally conserved Hippo pathway has shown that the precise control of these signaling molecules is critical to cardiac development and response to stresses. However, how this pathway is involved in the progression of cardiac dysfunction in different heart diseases remains unclear. We investigated the expressional levels and subcellular localization of Yap1, Taz, and Tead1 and determined Hippo target gene expression in failing human hearts with ischemic heart disease (IHD) and idiopathic dilated cardiomyopathy (IDC) and mouse desmin-related cardiomyopathy (DES). Our results demonstrated that Yap1, Taz, and Tead1 were significantly increased in failing human and DES hearts compared with the non-failing controls (NFH) or wild type (WT) mouse hearts at both mRNA and protein levels. Interestingly, adult human and mouse hearts had more Taz than Yap1 by mRNA and protein expression and their increases in diseased hearts were proportional and did not change Yap1/Taz ratio. Yap1, Taz, and Tead1 were accumulated in the nuclear fraction and cardiomyocyte nuclei of

*Correspondence to: Bo Ye, MMC 76, Department of Laboratory Medicine and Pathology, Room 293, Dwan Variety Club Cardiovascular Research Center, 425 E River Pkwy, Minneapolis, MN 55455, USA. bye@umn.edu (B. Ye). **Correspondence to: Faqian Li, MMC 76, Department of Laboratory Medicine and Pathology, C420 Mayo Bldg, 420 Delaware St SE, Minneapolis, MN 55455, USA. lif@umn.edu (F. Li).

Conflict of interest

None.

diseased hearts. The ratio of Yap1 phosphorylated at serine 127 (human) or serine 112 (mouse) to the total Yap1 (pYap1/Yap1) was significantly lower in the nuclear fraction of diseased hearts than that in normal controls. More importantly, Hippo downstream targets Ankrd1, Ctgf, and Cyr61 were transcriptionally elevated in the diseased hearts. These results suggest that Yap1/Taz signaling is activated in human and mouse dysfunctional hearts. Further investigation with relevant animal models will determine whether this pathway is a potential target for preventing and reversing abnormal remodeling during the progression of different cardiac disorders.

Keywords

Hippo; Yap1; Taz; Heart failure

Subject code and head

Cardiomyopathy; Cell signaling/signal transduction

1. Introduction

The Hippo pathway originally identified in *Drosophila* is a highly conserved kinase cascade and plays an important role in cell proliferation and organ size determination (Harvey et al., 2003; Hayashi et al., 2015; Pan, 2007). Genetic mouse models targeting this evolutionally conserved Hippo signaling have proven its critical roles in cardiac development, proliferation, and hypertrophy. However, inconsistent and even contradictory results of this pathway in cardiac regeneration and stress response were reported. Furthermore, it is not clear how this pathway regulates cardiac remodeling in different heart diseases when its component levels are not artificially elevated. Therefore, understanding how key effectors of this pathway change during natural progress of different heart diseases can help to explain the phenotypic difference of the animal models missing or gaining function of its key components.

Yap1 (yes-associated protein 1), a transcriptional coactivator first identified as a binding partner of the SH3 domain of c-Yes (Sudol, 1994), and its paralog PDZ-binding motif (Taz), are two essential downstream effectors of the Hippo signaling. The function and stability of Yap1/Taz are regulated by posttranslational modifications, especially phosphorylation. Akt or Hippo signaling kinases such as Lats can phosphorylate mouse Yap1 at Ser¹¹², which corresponds to Ser¹²⁷ in human Yap1 (Taz serine 89), and inhibits its activity by retaining it in the cytoplasm through binding to protein 14-3-3 (Basu et al., 2003; Lei et al., 2008; Zhao et al., 2007). Therefore, deletion of Lats increases Yap1 nuclear translocation and consequently promotes its activity via enhanced interaction with its binding partners such as Tead family members (Heallen et al., 2011). The substitution of human Ser¹²⁷ or mouse Ser¹¹² with Ala (S127A or S112A) generates a Yap1 protein that is constitutively active and localized to the nucleus.

The function of Yap1/Taz in development and tumorigenesis has been extensively investigated (Yu and Guan, 2013). In many cancerous cells, Yap1/Taz are activated and are essential for their growth and survival (Zanconato et al., 2016). Recently, Yap1/Taz were

shown to play critical roles in cardiac development, regeneration and response to stresses. Mice with Yap1 deletion mediated by Nkx2.5-cre die by embryonic day (E) 10.5 with compromised cardiomyocyte proliferation (Xin et al., 2011) while mice with cardiac specific knockout of Yap1 mediated by α -MHC-cre develop progressive dilated cardiomyopathy after birth (Xin et al., 2013). The gain-of-function by cardiac expression of activated Yap1 promotes cardiomyocyte division and builds bigger hearts (von Gise et al., 2012; Xin et al., 2011). More importantly, Yap1 activation enhances cardiac regeneration without affecting hypertrophy in adult mice (Lin et al., 2014; Xin et al., 2013). On the other hand, cardiac overexpression of wild type Yap1 promotes cardiac hypertrophy, but not cardiomyocyte proliferation in adult mice (Xu et al., 2014). Moreover, Yap1 haplodeficiency attenuates cardiac hypertrophy and worsens cardiac dysfunction after myocardial infarction (Del Re et al., 2013). These findings point to important roles of Yap1 in the maintenance of normal cardiac homeostasis and cardiac response to stresses, but also emphasize its divergent effects in different context.

Although Yap1/Taz can potentially activate gene transcription, they do not contain any DNA binding domain for directly binding to regulatory DNA motifs. They have been shown to interact with many DNA binding proteins, especially TEA-domain-containing (Tead, also named transcriptional enhancer factor, TEF) family transcription factors. The consensus DNA binding M-CAT (5'-CATTCCT-3') motif for Teads is present in several hypertrophy-induced genes and Tead activity is up-regulated during cardiac hypertrophy (Molkentin and Markham, 1994). Among 4 Tead family members, Tead1 is the most abundant in the heart (Kaneko et al., 1997) and developmentally regulated (Molkentin and Markham, 1994). Its expression is detected in fetal hearts at embryonic day 12, continues to increase during development with the highest expression level reached at neonatal day 1 and 2 and thereafter declines gradually to approximately 60% of the peak value in adult hearts. Tead1 is required for normal cardiac development and its global deletion causes ventricular hypoplasia without significant effects on myofibrillogenesis (Chen et al., 1994). Moreover, cardiac Tead1 overexpression causes heart failure and promotes reactivation of the fetal gene program in the adult heart (Tsika et al., 2010). However, the role of Tead1 in human and animal models of heart diseases remains unclear.

Although the inhibition and activation of the Hippo pathway have been shown to affect the maintenance of normal cardiac homeostasis and cardiac stress response in animal models, little is known about its involvement in the pathogenesis of many heart diseases, especially in dysfunctional human and animal hearts. Yap1 protein is increased in human hypertrophic cardiomyopathy and mouse pressure overload hypertrophy induced by transverse aortic constriction but its phosphorylation at human serine 127 and mouse serine 112 is reduced, indicative of Hippo signaling inhibition in these settings (Xu et al., 2014). A decreased expression of Yap1 has been reported in the ventricular tissue obtained from children with ventricular septal defects, the most common type of congenital heart disease (Ye et al., 2015). Dilated and ischemic cardiomyopathy are two main causes of congestive heart failure. Whether Yap1/Taz signaling is also altered in the heart with systolic dysfunction have not been explored. In the present study, we systemically investigated the changes of main Hippo signaling effectors: Yap1/Taz, Yap1/Taz binding partner Tead1, and their downstream targets in failing human hearts with ischemic heart disease (IHD) and idiopathic

dilated cardiomyopathy (IDC) and mouse desmin-related cardiomyopathy (DES) (Wang et al., 2001).

2. Materials and methods

2.1. Human hearts

All failing human hearts were from patients who were receiving an orthotopic cardiac transplantation. Informed consent was obtained at the time each subject was listed for transplant. Non-failing hearts were acquired from brain-dead organ donors whose hearts were deemed unacceptable for transplantation. Informed consent for research use of unused donor hearts was obtained from the appropriate next of kin by the organ procurement organization (Gift of Life, Inc., Philadelphia, PA). The research protocol was approved by the Institutional Review Boards of the University of Rochester and University of Minnesota. All research was conducted in compliance with Good Clinical Practice standards and all applicable NIH research requirements. Tissues from the left ventricular free wall of 15 human hearts, including 5 failing hearts with ischemic heart disease (IHD), 5 failing hearts with idiopathic dilated cardiomyopathy (IDC) and 5 non-failing controls (NFH) without a history of cardiac diseases, were procured using a standardized protocol. All hearts were arrested in situ with ice-cold cardioplegia solution (Viaspan®, DuPont, Wilmington, DE), transported to the laboratory on wet ice, snap frozen in liquid nitrogen within 2 h, and stored at -80 °C until protein extraction or mRNA isolation was performed. Additional left ventricular tissues from the same hearts were fixed in 10% buffered formalin and embedded in paraffin.

2.2. Mouse model with desmin mutation

The 7-amino-acid (R172 through E178) deletion mutant of desmin (DES) was expressed under the control of the *MYH6* promoter as previously reported (Wang et al., 2001). All animal procedures were performed in conformity with the guidelines from the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or *the Guide for the Care and Use of Laboratory Animals* (US Department of Health, Education, and Welfare, Department of Health and Human Services, NIH Publication 85-23). Left ventricular tissues were collected for immunohistochemical stains, protein and mRNA isolation. These animal protocols were approved by the Institutional Animal Care and Use Committee at the University of South Dakota.

2.3. RNA isolation, semi-quantitative and quantitative RT-PCR

Total RNA was prepared using TRIzol (Life Technologies, Grand Island, NY). Total RNA (1 µg) from each sample was reverse transcribed using 1 µmol/l oligo (dT) primers and 4 units of Ominiscript reverse transcriptase (Qiagen, Valencia, CA) in a total volume of 20 µl. PCR reaction was performed with 1 µl cDNA in a total volume of 20 µl containing 2 µl 10× Taq buffer with KCl and 15 mM MgCl₂ (Thermo Fisher Scientific, Rockford, IL), 0.2 mM dNTPs, 0.5 µl Taq DNA Polymerase and 0.5 µM of specific sense and antisense primers for each gene (Table 1). For semi-quantitative RT-PCR, the PCR products were loaded onto a 2% gel and the intensity of the specific product was measured with Quantity One (Bio-Rad Laboratories, Hercules, CA) normalized to that of the internal control of glyceraldehyde

phosphate dehydrogenase (GAPDH). Real-time quantitative RT-PCR (qRT-PCR) was performed in a 20 µl reaction with iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA) on an iCycler MyiQ single color real-time qRT-PCR detection system from BioRad Laboratories. GAPDH was used as normalization for qPCR.

2.4. Protein extraction and Western blot

Nuclear and cytoplasmic proteins were prepared using the NE-PER® nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacture's protocol. RIPA lysis and extraction buffer (Thermo Fisher Scientific, Rockford, IL) with protease and phosphatase inhibitor (Thermo Fisher Scientific, Rockford, IL) were used for whole tissue protein extraction. Protein concentrations were determined by the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Equal protein amounts were resolved on 4–20% SDS-Tris-glycine-polyacrylamide gels (BioRad Laboratories, Hercules, CA) and transferred to a PVDF membrane. The membrane was blocked with 5% wt/vol powdered non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 60 min at room temperature. The blot was incubated with primary antibodies for overnight at 4°. Antibodies specific for YAP1, YAP1 phosphorylated at human Ser127 (mouse Ser112), TEAD1, β tubulin and an antibody recognizing both YAP1 and TAZ (YAZ/TAZ) (Loforese et al., 2017) were purchased from Cell Signaling Technology Inc. (Danvers, MA). After 3 washes in TBST, the membrane was incubated with a peroxidase-conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL) at 1:10,000 dilution for 45 min at room temperature. After being washed in TBST, the blot was developed with Pico Western blotting detection reagents (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's instruction.

2.5. Antigen retrieval and immunohistochemical staining

Four µm tissue sections were cut from paraffin-embedded formalin-fixed heart tissues for immunohistochemical staining as previously described (Yi et al., 2002). After 3 cycles of deparaffinization in xylene and tissue rehydration, antigen retrieval was performed in pH 9 Tris buffer (Dako North America, Inc., Carpinteria, CA) by heating to 99 °C for 20 min. The endogenous peroxidase activity was quenched with 3% H₂O₂ and non-specific binding was blocked with 10% non-immune goat serum (Life Technologies, Grand Island, NY) and then incubated with primary antibodies at 4 °C for overnight. YAP1 specific antibody and YAP/TAZ antibody recognizing both YAP1 and TAZ from Cell Signaling Technology Inc. (Danvers, MA) were used at 1:200 dilution. The signal was amplified with the Histostain-SP Kit (Life Technologies, Grand Island, NY) and detected with DAB substrate (Dako North America, Inc., Carpinteria, CA). Color development of DAB for diseased and control hearts was microscopically monitored in parallel with the same reaction time. Hematoxylin was used as a counterstain. Negative controls were performed with the omission of primary antibodies.

2.6. Immunofluorescent labeling and confocal microscopy

Cardiac Troponin T (cTnT) was employed as a specific cardiac marker. Paraffin-embedded formalin-fixed heart tissues were double labeled with pYAP1 or YAP1 and cTnT antibodies for overnight at 4 °C. After washing, secondary antibody conjugated with DyLight-555 and

488 was added. Nuclear counterstain was performed with 4', 6-Diamidino-2-Phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO). Confocal images were collected with Olympus FV1000 confocal microscope (Olympus America Inc., Melville, NY) under uniform settings. For each antibody, staining was performed on at least three samples of each group. Ten high-power fields (60×) were randomly selected from each sample and percentage of YAP1 positive cardiomyocytes was compared among groups.

2.7. Statistics

All data are shown as mean $\pm$ standard error of the means (SEM). Statistical analysis was performed with SPSS 18.0 software (IBM Corporation, Armonk, NY). The equality of variance before all *t*-test and one-way ANOVA was conducted with Levene's test. Student *t*-test was performed for two-group analysis with equal variances. If equal variance was not assumed, the Satterthwaite's approximate *t*-test was used for two-group analysis. One-way ANOVA followed by Tukey post hoc testing was used for multiple group comparison when equal variances were present. Otherwise, Tamhane's T2 test was used for multiple group comparison. A *P* value smaller than 0.05 was considered statistically significant and all tests were two-tailed.

3. Results

3.1. The expression and cellular localization of YAP1, TAZ and TEAD1 in adult human and mouse hearts

Consistent with previous study which has shown Yap1 decreases during postnatal development (von Gise et al., 2012), we detected low levels of Yap1 expression by Western blots, RT-PCR and immunohistochemical stains in normal adult human (Figs. 1 and 2, Supplement Fig. S1A) and mouse hearts (Figs. 3 and 4, Supplement Fig. S1B). Similarly, Tead1 was also low in adult hearts (Figs. 1 and 3). In contrast, there was much more Taz than Yap1 in adult human (Figs. 1 and 2) and mouse hearts (Figs. 3 and 4, Supplement Fig. S2). Using YAP/TAZ antibody which can recognize both YAP1 and TAZ (Loforese et al., 2017), we found that TAZ was 5.09 (human) or 2.25 (mouse) folds over YAP1 in whole lysates of normal adult hearts by relative density in the same membrane for Western blotting (Figs. 1E and 3E). Quantitative RT-PCR also showed that *TAZ* mRNA was 1.97 or 2.31 folds of *YAP1* in non-failing human hearts and wild-type mouse hearts respectively by log₂ CT values (Figs. 2E and 4D). These results indicate that Taz is the main mediator of Hippo signaling with higher expression level than Yap1 in adult mammalian hearts.

3.2. Upregulation of YAP1 and TAZ in failing human hearts

Western blots were conducted with specific antibodies only reacting to YAP1 or pSer127-YAP1 and YAP/TAZ antibody recognizing both YAP1 and TAZ to examine their protein levels in the lysates purified from left ventricles of the patients with end stage heart failure resulting from IHD or IDC and of non-failing controls. The pSer127-YAP1 was slightly increased, while the total YAP1 and TAZ levels determined by both YAP1 and YAP/TAZ antibodies in the whole lysates were significantly increased in the failing samples compared with that in the non-failing controls (Fig. 1A, left panel, and Fig. 1B–E). In addition, TEAD1, one of the major binding partners of YAP and TAZ, was also upregulated in the

diseased hearts relative to normal controls (Fig. 1A, left panel, and Fig. 1G). Next, we evaluated these protein levels in nuclear and cytoplasmic fractions. As shown in Fig. 1A (middle and right panels), 1C and 1E, the nuclear level of YAP1 and TAZ was higher, but the ratio of pSer127-YAP1 over YAP1 was lower in the nuclear fraction of the diseased hearts than that in the control hearts, while no significant differences between these protein levels was observed in the cytoplasmic fractions of diseased and control hearts (Fig. 1D). Similar findings were observed for TEAD1 (Fig. 1A, G). The increased nuclear levels of pSer127-YAP1, YAP1 and TAZ were further confirmed by the immunostaining on the sections of cardiac tissues prepared from NFH, IHD and IDC using specific antibodies against pSer127-YAP1, YAP1 and YAP/TAZ recognizing both YAP1 and TAZ (Fig. 2A, B, Supplement Fig. S1A and S1C). pSer127-YAP1 staining was also observed in non-cardiomyocytes of NFH, IHD and IDC, but mainly located in the cytoplasm. Moreover, nuclear and cytoplasmic YAP1 was increased in non-cardiomyocytes of IHD and IDC, partially contributing to the elevation of nuclear YAP1 level in human diseased hearts by Western blots. Real-time RT-PCR also revealed that *YAP1* and *TAZ* mRNA levels were increased in failing human hearts (Fig. 2C, D). Thus, we conclude that the Yap1/Taz signaling is activated in failing human hearts with IHD and IDC.

3.3. Activation of Yap1/Taz signaling in mouse hearts with desmin mutation

Mouse hearts with desmin mutation develop cardiomyopathy (Wang et al., 2001) and show similar Wnt signaling activation as failing human hearts (Hou et al., 2016). The Hippo transducers YAP/TAZ have been shown to play positive as well as negative roles in Wnt signaling. In the heart, YAP overexpression promotes Wnt signaling and enhances cardiomyocyte proliferation (Xin et al., 2011). On the other hand, YAP suppresses Wnt signaling to restrict intestinal stem cell expansion (Barry et al., 2013). We employed the same approaches to determine the expression and localization of pYAP1, YAP1, TAZ and TEAD1 in mouse hearts. YAP1 and TAZ levels detected by both YAP1 and YAP/TAZ antibodies were significantly elevated (Fig. 3A, C and E), but the ratio of pSer112-YAP1 over total YAP1 was significantly decreased in both whole tissue lysates and nuclear fraction of DES relative to WT hearts, while no significant differences in the cytoplasmic fraction were observed between DES and WT hearts (Fig. 3B, D). Whole heart lysates and nuclear fractions extracted from DES hearts also had higher levels of TEAD1 than that of WT hearts (Fig. 3A, left and middle panels, Fig. 3G). Immunostains demonstrated significant nuclear accumulation of pYAP1, YAP1 and TAZ in cardiomyocyte nuclei of DES hearts (Fig. 4A, Supplement Fig. S1B and S1D). Moreover, real-time RT-PCR further showed that *Yap1* and *Taz* mRNA levels were increased in DES hearts (Fig. 4B, C). Hence, Yap1/Taz signaling is activated in DES hearts like failing human hearts.

3.4. Transcriptional upregulation of YAP1 and TAZ targets in diseased human and mouse hearts

To determine if Yap1 and Taz regulate target gene expression in adult hearts, we first performed semi-quantitative and quantitative RT-PCR on RNA purified from human IHD, IDC and NFH as well as WT and DES mouse hearts. Ankyrin repeat domain 1 (*ANKRD1*), cysteine-rich angiogenic inducer 61 (*CYR61*, also known as *CCN1*) and connective tissue growth factor (*CTGF*, also known as *CCN2*) are well-known Hippo pathway target genes

(Yu et al., 2012). As shown in Fig. 5A and B, the transcriptional levels of *ANKRD1*, *CYR61*, and *CTGF* were significantly increased in diseased human and mouse hearts compared with NFH and WT hearts, respectively; and these changes were further confirmed by real-time RT-PCR (Fig. 5C and D). Thus, Hippo pathway target genes are transcriptionally activated in failing human hearts with IHD or IDC and in mouse DES hearts.

4. Discussion

Recently, the Hippo signaling has gained much attention as it mediates cardiomyocyte proliferation and is a potentially therapeutic target in cardiac regeneration. In the present study, we have found that nuclear mediators of the Hippo pathway: Yap1, Taz and Tead1, are significantly increased at both mRNA and protein levels in human hearts with IHD and IDC and mouse DES hearts. More importantly, Yap1, Taz, and Tead1 are accumulated in the nucleus of cardiomyocytes of diseased human and mouse hearts. Moreover, Hippo pathway target genes: *ANKRD1*, *CYR61*, and *CTGF*, are dramatically upregulated in these diseased hearts. These results indicate that transcriptional activity of Yap1 and Taz is increased in ischemic and cardiomyopathic hearts, resulting in the upregulation of their target genes.

4.1. The Hippo pathway during cardiac development

The Hippo pathway and its nuclear effectors: Yap1/Taz and Teads play important and obligatory roles in normal heart development. Fetal, neonatal and adolescent hearts have abundant Yap1 proteins while adult hearts have barely detectable Yap1 protein by 12 weeks of age (von Gise et al., 2012). There is more Yap1 protein in cardiomyocytes than non-cardiomyocytes in fetal hearts, but its level becomes comparable between cardiomyocytes and non-cardiomyocytes in neonatal and adolescent hearts. At mRNA levels, there is more Yap1 than Taz mRNA copy numbers by quantitative RT-PCR in the heart at 6 weeks of age (Xin et al., 2013). The developmental expression pattern of Taz, especially at the protein level is less clear. Furthermore, it is not well defined whether there is differential expression of Yap1 and Taz in adult hearts. Here we report that Taz is more abundant than Yap1 in adult human and mouse hearts. Yap1 deletion in embryonic and neonatal mouse hearts significantly reduces cardiomyocyte proliferation and impairs regeneration (von Gise et al., 2012; Xin et al., 2013, 2011) while cardiac Taz deletion has no detectable effect on cardiac structure and function. Compound deletion of Yap and Taz indicates gene dosage dependent effect of the Hippo pathway on cardiac function and a predominant role of Yap in fetal and preadolescent hearts (Xin et al., 2013). As Taz is more abundant than Yap1 in adult hearts, it will be important to determine whether Taz has a predominant role in adult hearts and if it is required for cardiac hypertrophic response to stresses.

Tead1 is the most abundant among 4 Tead family members in the heart (Kaneko et al., 1997) and its expression changes during development (Molkentin and Markham, 1994). Tead1 DNA binding activity is detected in fetal hearts at embryonic day 12, reaches the highest level at neonatal day 1 and 2, and starts to decline by neonatal day 13 to a steady-state level of approximately 60% the peak value in adult mouse hearts. Our results show that Tead1 is present in adult human and mouse hearts and its protein level is relatively low. Tead1 is

required for embryonic heart development and its global deletion causes severe cardiac defects with thin ventricular wall and trabeculae and embryonic lethality between E11 and 12 (Chen et al., 1994).

4.2. Yap1/Taz in cardiac hypertrophy and failure

Yap1 regulates cardiomyocyte proliferation in fetal hearts (von Gise et al., 2012; Xin et al., 2013, 2011), but its role in adult hearts remains to be clarified. Yap1 overexpression has been shown to promote either cardiomyocyte hypertrophy or proliferation in adult hearts and this appears dependent on the phosphorylation status of Yap1. Constitutively active mouse or human Yap1 mutants with S112A or S127A promote cardiomyocyte proliferation without inducing cardiomyocyte hypertrophy in vivo and in vitro (Lin et al., 2014; Xin et al., 2013). On the other hand, overexpression of wild type Yap1 enhances cardiomyocyte hypertrophy in vivo and in vitro (Del Re et al., 2013; Xu et al., 2014). These findings indicate that the phosphorylation status of Yap1 may be critical to its regulation on hypertrophic or proliferative growth of cardiomyocytes. However, Yap1 levels may also control these two processes as mutant Yap1 is more stable than its wild type counterpart. It is also possible that these two forms of Yap1 target to different cellular compartments and thus have different cellular partners. Conducting parallel experiments with these 2 forms of Yap1 will be essential to determine which of these explanations stand. Interestingly, Yap1 protein is increased in human hypertrophic cardiomyopathy and mouse pressure overload hypertrophy by transverse aortic constriction (Xu et al., 2014). Additionally, nuclear Yap1 is detected in the border zone of mouse myocardial infarction (Del Re et al., 2013). Furthermore, overexpression of wild type Yap1 promotes cardiomyocyte hypertrophy in cultured cardiomyocytes and in vivo mouse hearts (Del Re et al., 2013). These findings suggest that Yap1 can cause abnormal cardiac remodeling in addition to its beneficial effect on cardiac regeneration. Our current study has revealed that Yap1 is upregulated at both mRNA and protein levels and accumulates in cardiomyocyte nuclei in IDC and IHD. Moreover, the pS127-Yap1/Yap1 ratio is decreased in IDC and IHD, indicating Hippo signaling inhibition. We have also showed that similar Yap1 upregulation in DES mouse hearts. As the proliferative activity of cardiomyocytes in adult mouse and human hearts is negligible, the increased Yap1 in these diseased hearts is mainly contributing to maladaptive cardiac hypertrophy. Interestingly, adult human and mouse hearts have more Taz than Yap1. Their upregulation in diseased human and mouse hearts is proportional and thus their ratio does not change. Although cardiac Taz deletion has no detectable effect on cardiac morphology and function, its predominance and inducibility in diseased human and mouse hearts suggest that Taz may have important roles in cardiac stress response. A recent study has revealed that Hippo signaling is elevated and Yap/Taz activity is suppressed in mouse and human arrhythmogenic right ventricular dysplasia (ARVD) (Chen et al., 2014). It is highly likely that different cardiac disorders may exhibit different functional outcomes of the Hippo signaling pathway. Another possibility is that diminished Yap/Taz activity in ARVD reflects reduced Hippo signaling in fibrofatty tissue which characteristically replaces myocardial tissue in this disease, rather than in cardiomyocytes.

How Yap1/Taz signaling is activated in the heart is controversial. During cardiac hypertrophy, the PI3k/Akt/Foxo3 pathway transcriptionally suppresses Mst1 expression and

thus reduces its activity leading to a decrease in Yap1 phosphorylation and thus its transactivation (Wang et al., 2014). In contrast, Mst1/Lats2 are activated after myocardial infarction to increase Yap1 phosphorylation (Del Re et al., 2013). However, Yap1 is not retained in the cytoplasm, but stayed in the nucleus of cardiomyocytes in the border zone. Our findings in failing hearts also indicate Yap1 is stabilized and accumulated in the nucleus despite an increase in its phosphorylation. Therefore, Hippo signaling activation does not necessarily cause Yap1/Taz suppression as it can interact with other pathways to keep phosphorylated Yap1 in the nucleus.

4.3. Tead1 and cardiomyopathy

Tead1 regulates cardiac gene expression during cardiac stresses. Pressure overload enhances the binding of Tead1 to many cardiac specific gene promoters and activates their transcription (Maeda et al., 2002; Molkentin and Markham, 1994). Moreover, overexpression of Tead1 driven by the muscle creatine kinase (MCK) promoter induces age-dependent dilated cardiomyopathy with impaired cardiac function including diminished ejection fraction and fractional shortening as well as the activation of the fetal gene program (Tsika et al., 2010). Our results demonstrate that Tead1 is upregulated and accumulates in the nucleus of human and mouse cardiomyocytes with ischemia and cardiomyopathy. These findings indicate that Tead1 is involved in cardiac gene expression and maladaptive remodeling during the progression of heart diseases with different etiologies.

4.4. Yap1/Taz target genes in heart diseases

Ankrd1, Cyr61 and Ctgf are three well-known Yap1/Taz transcriptional targets and play important roles in the maintenance of normal cardiac function. Their dysregulation can cause cardiac dysfunction. Naturally occurring mutations in Ankrd1 are associated with hypertrophic cardiomyopathy and IDC (Arimura et al., 2009; Moulik et al., 2009). Furthermore, expression of Ankrd1 is increased in the patients with IDC and its levels correlate with heart failure severity; hence, Ankrd1 has been proposed to act as a potential biomarker for the progression of IDC. Cyr61 has been shown to be induced in the human arteriosclerotic lesions (Hilfiker et al., 2002). Ctgf mediates expressions of extracellular matrix genes in hypertrophic cardiomyopathy and promotes cardiac remodeling and fibrosis in a murine dilated cardiomyopathy model (Koshman et al., 2015). In the agreement with the above observations, our data show that the expression of these three genes is elevated in human and mouse diseased hearts with and without ischemia in parallel to Yap1/Taz signaling activation. Therefore, the developmentally important Hippo pathway is operational in adult human and mouse hearts and likely contributes significantly to cardiac gene regulation and structural remodeling in different heart disorders.

In conclusion, we have demonstrated in the present investigation that Yap1/Taz signaling is activated as evidenced by the elevation of Yap1, Taz and Tead1 levels and their nuclear accumulation in 3 types of human and mouse heart failure. This activation coincides with increased Hippo downstream target gene upregulation. Whether the activation of Yap1/Taz is protective or deleterious requires further investigation with relevant animal models. Several studies overexpressing non-phosphorylatable Yap1 at the key consensus site have revealed protective effect by promoting cardiomyocyte proliferation (von Gise et al., 2012; Xin et al.,

2013, 2011). However, overexpression of wild type Yap1 has shown deleterious effect by promoting cardiomyocyte hypertrophy (Del Re et al., 2013; Xu et al., 2014). These findings suggest the phosphorylation status of Yap1 may determine its effect on the heart. Dissecting the differential roles of Yap1 and Taz as well as different forms of Yap1 may provide better strategy to attenuate pathologic hypertrophy without compromising the beneficial effect of this pathway on cardiac regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexmp.2017.11.006>.

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Fig. 1.

YAP1 and TAZ were expressed in adult human hearts and increased in the nuclear fraction of failing human hearts indicating Hippo signaling inhibition. A: Representative Western blots with p-Ser127-YAP1 (pYAP1), YAP1, YAP/TAZ and TEAD1 antibodies in whole (12.5 µg), cytoplasmic (25 µg), and nuclear (25 µg) lysates extracted from human non-failing heart (NFH), ischemic heart disease (IHD), and idiopathic dilated cardiomyopathy (IDC). Histone H3 and tubulin were used as loading controls for whole, nuclear, and cytoplasmic proteins. B–D: Fold increases of protein levels for pYAP1(B) and total YAP1(C) in whole lysates, nuclear fractions and cytoplasmic fractions were determined by phosphor-specific YAP1 and total YAP1 antibodies respectively. The ratio of pYAP1/YAP1 (D) was also compared within different groups. E and F, Increased YAP1 and TAZ expressions in the whole lysates and nuclear fractions from IHD and IDC hearts were also confirmed by YAP/TAZ antibody which can recognize both 65KD YAP1 and 50KD TAZ (E). The ratio of

YAP1/TAZ (F) was unchanged in IHD, IDC and NFH. G, TEAD1 was elevated in the whole lysates and nuclear fractions of IHD and IDC hearts compared to NFHs. Cytoplasmic TEAD1 was undetectable in human heart tissues by Western blot. N = 5 in each group. *Error bars* represent mean $\pm$ SEM. *N.S.*, not significant. * $P < 0.05$ and † $P < 0.01$ vs. NFH (ANOVA).

Fig. 2.

YAP1 and TAZ accumulated in the cardiomyocyte nuclei and their mRNA levels were up-regulated in failing human hearts. A: Representative confocal images of triple staining of p-Ser127-YAP1 (pYAP1, red), cTnT (green), and 4',6-diamidino-2-phenylindole (DAPI, blue) revealed that both cardiomyocytes and non-cardiomyocytes form IDC, IHD and NFH showed pYAP1 positivity in the cytoplasm. Nuclear pYAP1 was strong in cardiomyocytes of IDC and IHD, but not detected in cardiomyocytes of NFH (arrows). Cytoplasmic pYAP1 was observed in non-cardiomyocytes of NFH, IDC and IHD (triangles). N = 3 in each group.

B: Representative images of immunohistochemical staining revealed unchanged expression level of YAP1 and YAP/TAZ in cardiomyocyte cytoplasm of IDC, IHD and NFH, but a dramatic increase in the cardiomyocyte nucleus of IDC and IHD compared to NFH. N = 3 in each group. Cardiomyocyte nuclei are indicated by arrows while non-cardiomyocyte nuclei are pointed by triangles. Asterisk indicates small vessels with endothelial cells. C: Semi-quantitative RT-PCR of *YAP1* and *TAZ* in human hearts. D: Log₂ CT values of *YAP1* and *TAZ* in human failing hearts by real-time RT-PCR. E: The ratio of *YAP1* and *TAZ* expression levels in the same sample was analyzed by real-time RT-PCR in human hearts. Five human hearts were used for each group. *Error bars* represent mean ± SEM. *N.S.*, not significant. **P* < 0.05 and †*P* < 0.01 vs. control groups. NFH, non-failing heart; IHD, ischemic heart disease; IDC, idiopathic dilated cardiomyopathy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

YAP1 and TAZ were expressed in adult mouse hearts and activated in hearts with desmin mutation (DES). A: Representative Western blots with p-Ser112-YAP1 (pYAP1), YAP1, YAP/TAZ, and TEAD1 antibodies in whole (12.5 µg), cytoplasmic (25 µg), nuclear (25 µg) lysates from wild-type (WT) and DES hearts. B–D: Fold increases of protein levels for pYAP1(B) and total YAP1(C) in whole lysates, nuclear and cytoplasmic fractions from wild type (WT) and desmin mutation (DES) hearts were determined by phosphor-specific YAP1 and total YAP1 antibodies respectively. The ratio of pYAP1/YAP1 (D) was also compared between WT and DES animals. E and F, Increased YAP1 and TAZ expressions in the whole lysates and nuclear fractions from DES hearts were further confirmed by YAP/TAZ antibody which can recognize both 65KD YAP1 and 50KD TAZ. The ratio of YAP1/TAZ (F) was

decreased in nuclear fractions, but unchanged in whole lysates and cytoplasmic fractions of DES hearts. G, TEAD1 was upregulated in the whole lysates and nuclear fractions of DES hearts compared to WT hearts. Cytoplasmic TEAD1 was undetectable in adult mouse heart tissues by Western blot. N = 6 in each group. *Error bars* represent mean $\pm$ SEM. *N.S.*, not significant. * $P < 0.05$ and † $P < 0.01$ vs. WT (*t*-test).

Fig. 4.

YAP1 and TAZ accumulated in the cardiomyocyte nuclei and their mRNA levels were up-regulated in mouse hearts with DES. A: Representative images of immunohistochemical staining with pYAP1, YAP1 and YAP/TAZ antibodies from wild type (WT) and DES mice showed no difference in the cytoplasm, but a significant increase in the nucleus of cardiomyocytes of DES hearts relative to WT controls. N = 3 in each group. Cardiomyocytes are indicated by arrows while non-cardiomyocytes are pointed by triangles. B: Semi-quantitative RT-PCR of *Yap1* and *Taz* in mouse hearts. C: Log₂ CT values of *Yap1* and *Taz* in mouse DES hearts by real-time RT-PCR. D: *Yap1/Taz* expression ratio in the same sample was analyzed by real-time RT-PCR in mouse hearts (F). Six mouse hearts were used for each group. Error bars represent mean ± SEM. N.S., not significant. **P* < 0.05 and †*P* < 0.01 vs. control groups (*t*-test). WT, wild type; DES, mouse with desmin mutation.

Fig. 5.

YAP1 target genes were up-regulated in failing human hearts and mouse DES hearts. A and B: Representative images of semi-quantitative RT-PCR for *ANKRD1*, *CYR61* and *CTGF* in human (A) and mouse hearts (B). C and D: Fold increases evaluated by real-time RT-PCR of *ANKRD1*, *CYR61* and *CTGF* in human (C) and mouse hearts (D). Five human hearts and six mouse hearts per group were used for every analysis. Error bars represent mean $\pm$ SEM. N.S., not significant. * $P < 0.05$ vs. NFH or WT; † $P < 0.01$ vs. NFH or WT. IDC, idiopathic dilated cardiomyopathy; IHD, ischemic heart disease; NFH, non-failing heart. WT, wild type; DES, mouse with desmin mutation.

Table 1

Sequences of primers used in this study.

Species	Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')
Human	<i>YAP1</i>	GCAACTCCAACCAGCAGCAACA	CGCAGCCTCTCCTTCTCCATCTG
	<i>TAZ</i>	ACCCACCCACGATGACCCCA	GCACCCTAACCCAGGCCAC
	<i>ANKRD1</i>	CACTTCTAGCCCACCCTGTGA	CCACAGGTTCCGTAATGATTT
	<i>CYR61</i>	AGCCTCGCATCCTATACAACC	TTCTTTCACAAGGCGGCACTC
	<i>CTGF</i>	CCAATGACAACGCCTCCTG	TGGTGCAGCCAGAAAGCTC
	<i>GAPDH</i>	CTCCTCCACCTTTGACGCTG	CATACCAGGAAATGAGCTTGACA
Mouse	<i>Yap1</i>	TACATAAACCATAAGAACAAGACCACA	GCTTCACTGGAGCACTCTGA
	<i>Taz</i>	GAAGGTGATGAATCAGCCTCTG	GTTCTGAGTCGGGTGGTTCTG
	<i>Ankrd1</i>	GGAGGAACAACGGAAGCG	GGCACATCCACAGGTTCACT
	<i>Cyr61</i>	GCTCAGTCAGAAGGCAGACC	GTTCTTGGGACACAGAGGA
	<i>Ctgf</i>	CTGCCTACCGACTGGAAGAC	CATTGGTAACTCGGGTGGAG
	<i>Gapdh</i>	AGGTCGGTGTGAACGGATTG	GGGGTCGTTGATGGCAACAA