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REVIEW

Harnessing Hippo in the heart: Hippo/Yap signaling and applications to heart regeneration and rejuvenation

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Abstract The adult mammalian heart exhibits limited regenerative capacity after myocardial injury, a shortcoming that is responsible for the current lack of definitive treatments for heart failure. A search for approaches that might enhance adult heart regeneration has led to interest in the Hippo/Yap signaling pathway, a recently discovered signaling pathway that regulates cell proliferation and organ growth. Here we provide a brief overview of the Hippo/Yap pathway and its known roles in the developing and adult heart. We discuss the implications of Hippo/Yap signaling for regulation of cardiomyocyte death and regeneration.

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Introduction

Myocardial infarction (MI) is a leading cause of mortality and morbidity. Although surgical and medical advances over the past several decades have greatly improved survival after acute MI (Dargie, 2005), the long term prognosis of these patients remains poor. A major reason is the heart has limited regenerative capacity. Because of the refractoriness of adult cardiomyocytes to re-enter the cell cycle, the billions of cardiomyocytes lost during acute myocardial infarction cannot be restored, being replaced instead by fibrotic myocardial tissue with little contractile function, poor diastolic compliance, and a propensity to arrhythmia. As an adaptive response, the surviving cardiomyocytes undergo pathological hypertrophic growth through activation of neurohumoral signaling pathways. Over the long term, this adverse remodeling following MI has a deleterious effect on cardiomyocyte function and survival, and ultimately leads to congestive heart failure.

Current post-MI pharmacological management suppresses neurohumoral activation that drives pathological cardiomyocyte hypertrophy. Although this strategy has been successful for heart failure management, clinical trial evidence with new neurohormonal targets did not show greater salutary effects (Mehra et al., 2003), suggesting that we are reaching a ceiling for this approach and pinpointing the need to develop new therapeutic targets.

One attractive strategy to improve outcome in heart failure is to regenerate damaged myocardium by making more functional cardiomyocytes. Extensive work over the past two decades has overturned the prevailing dogma that adult cardiomyocytes do not undergo cell division by demonstrating that adult cardiomyocytes do proliferate, albeit at a low rate (Zhou et al., 2012). Several signaling pathways have been shown to regulate cardiomyocyte proliferation, such as IGF1 (Duerr et al., 1995), perisotin (Kuhn et al., 2007), neuregulin (Baliga et al., 1999; Bersell et al., 2009), and fibroblast growth factor (Engel et al., 2005). Recently, the newly defined Hippo/Yap pathway was found to play essential roles in the regulation of heart development and postnatal cardiomyocyte proliferation. In this review, we summarize recent advances in understanding the regulation of the Hippo/Yap signaling pathway, and the role of this pathway in the developing and adult heart. In addition, we discuss opportunities for therapeutic manipulation of Hippo/Yap signaling to enhance myocardial repair and regeneration.

Drosophila growth regulation by Hippo/Yap signaling

How multicellular organisms establish and maintain proper organ size is a long-standing puzzle. Genetic screens in *Drosophila* for abnormal growth regulation phenotypes identified Merlin (Hamaratoglu et al., 2006), Warts (Xu et al., 1995), Hippo (Wu et al., 2003), Salvador (Tapon et al., 2002), Mats (Lai et al., 2005), and other mutants with similar phenotypes of tissue overgrowth, increased cell proliferation, and suppressed apoptosis. The shared phenotypes of these mutants suggested that the affected genes function in a common pathway that regulates organ growth. Further genetic and biochemical studies confirmed that these genes encode a kinase cascade, in which Merlin, a membrane-associated cytoskeletal scaffolding protein, genetically functions upstream of Hippo, a Ste-20 type kinase that phosphorylates and activates Warts. Salvador, a WW-repeat protein, serves as a scaffold to enhance Hippo activity (Wu et al., 2003), while Mats, a *Mob* superfamily protein, interacts with Warts to facilitate its kinase activity (Lai et al., 2005).

The missing link between the Hippo kinase cascade and gene expression regulation was discovered by Duojia Pan's group in 2005 (Huang et al., 2005). A yeast two hybrid screen using Warts as the bait identified the transcriptional co-activator Yorkie as a Warts binding protein. Follow-up experiments showed that Yorkie promotes cell proliferation and tissue growth, that its transcriptional activity is negatively regulated by Hippo signaling, and that it is directly phosphorylated by Warts. Moreover, Yorkie inactivation blocked the tissue overgrowth phenotype of Hippo kinase cascade mutants, indicating that phosphorylation and inactivation of Yorkie is a major output of Hippo signaling.

Yorkie is a transcriptional co-activator that lacks intrinsic DNA binding activity. The DNA binding transcription factor Scalloped was identified as a major Yorkie partner that is required for tissue overgrowth in Yorkie gain of function or Hippo pathway loss of function (Zhang et al., 2008; Wu et al., 2008; Zhao et al., 2008). Interestingly, over-expression of Yorkie but not Scalloped caused tissue overgrowth, suggesting that Yorkie, the protein modulated by Hippo signaling, is limiting for tissue growth rather than Scalloped (Wu et al., 2008).

Hippo/Yap signaling in mammals

The Hippo/Yap signaling pathway is highly conserved between *Drosophila* and mammals. The mammalian orthologs of *Drosophila* Hippo, Salvador, Warts, Mats, Yorkie and Scalloped, are Mst1/2 (mammalian sterile twenty-like), Sav1 (Salvador-like homolog 1), Lats1/2 (large tumor suppressor), Mob1, Yap (YES-associated protein), and Tead1–4 (TEA domain family member), respectively. The presence of multiple isoforms of many of the mammalian orthologs has made their genetic analysis in mammals complicated. In the remainder of this review, we will use the mammal nomenclature to describe Hippo/Yap pathway components.

Before the Hippo pathway was delineated in *Drosophila*, several crucial components of the pathway had already been cloned in mammals (Tapon et al., 2002; Xiao et al., 1991; Sudol, 1994; Creasy and Chernoff, 1995; Tao et al., 1999). The functions of these crucial Hippo pathway components have been well addressed in several reviews on Tead (Pobbati and Hong, 2013), Yap (Wang et al., 2009), and Mst (Matallanas et al., 2008). Several excellent reviews of Hippo/Yap signaling have also been published recently (Pan, 2007; Yu and Guan, 2013; Zhao et al., 2010). This section will focus on the recently defined aspects of mammalian Hippo/Yap signaling (summarized in Fig. 1).

Regulation of Yap in the submembrane compartment

In response to extracellular cues and cell–cell interactions, the Hippo kinase cascade restrains cell proliferation and

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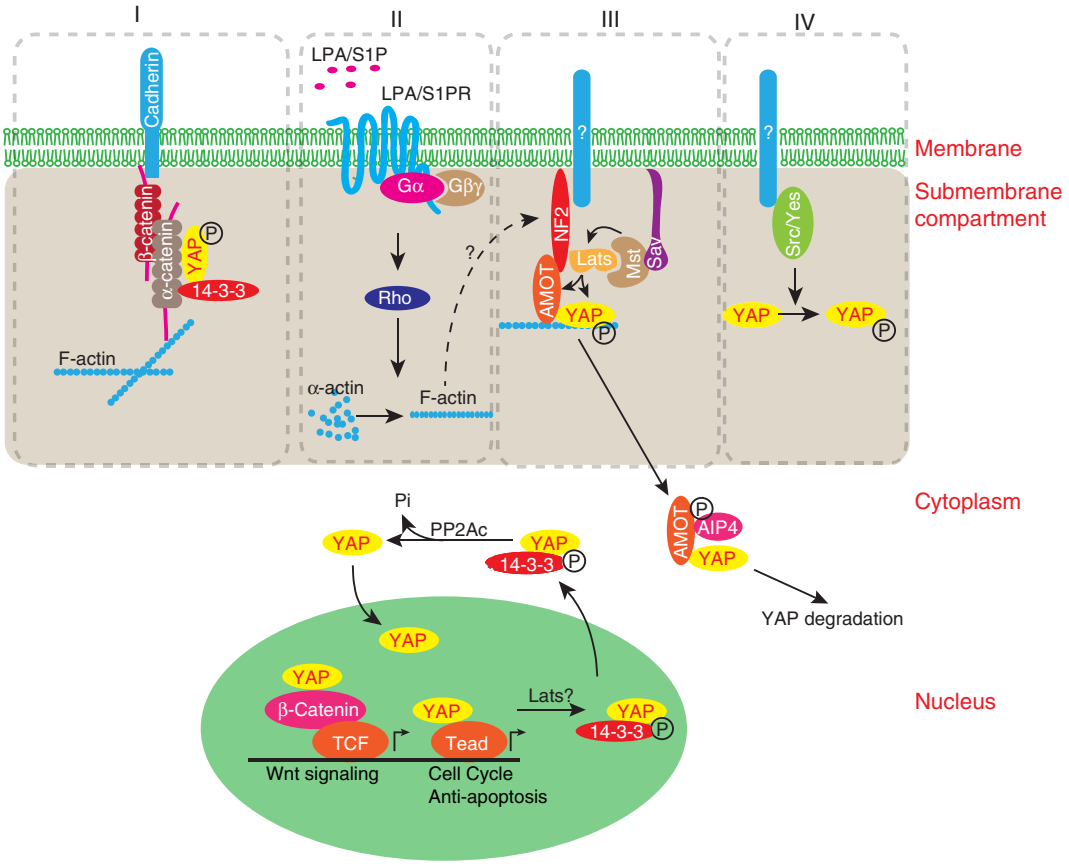

Figure 1 The mammalian Hippo/*Yap* signaling pathway. *Yap* activity is regulated by phosphorylation, subcellular localization, and degradation. Membrane receptors receive intracellular cues and relay them to the sub-membrane compartment. At adherens junctions (I), E-cadherin transduces cell–cell contact information via α/β-catenin. Under conditions of high cell density, α-catenin forms a complex with phosphorylated *Yap* and 14-3-3 proteins, thereby sequestering *Yap*. Growth factor signaling through GPCRs (II) inhibits *Lats* phosphorylation of *Yap* through a mechanism involving Rho GTPases and actin polymerization. The classic Hippos kinase cascade (III), containing the kinases *MST1/2* (mammalian Hippo ortholog) and *Lats1/2* (mammalian Warts), phosphorylates *Yap* to restrain its nuclear localization and transcriptional activity. The Hippo kinase cascade is regulated by NF2 through uncharacterized upstream receptors. *Lats* also phosphorylates AMOT, a *Yap* binding protein. Phosphorylated AMOT dissociates from F-actin and brings bound *Yap* into the cytoplasm, where the complex is degraded by the ubiquitin proteasome system via interaction with AIP4. *Yap* is also phosphorylated by YES/SRC (IV), under the control of uncharacterized upstream receptors. *Yap* is a transcriptional co-activator, and its nuclear entry is promoted by dephosphorylation by PP2A. Nuclear *Yap* promotes mitogenic and anti-apoptotic gene transcription, canonically through interaction with *Tead* transcription factors. *Yap* also enhances Wnt/β-catenin signaling through physical interactions with β-catenin. *Yap* transcriptional activity is terminated by phosphorylation by a nuclear kinase, possibly Lats1/2, leading to 14-3-3 protein binding and nuclear export.

organ growth by phosphorylating and inhibiting Yap. However, the mechanisms by which extracellular cues modulate Hippo kinase activity are incompletely understood. Recent work from Yin et al. partially filled this gap by elucidating how NF2, a protein that links cytoskeletal components with proteins in the cell membrane, controls Hippo pathway activity (Yin et al., 2013). NF2-deficient livers had decreased Lats and Yap phosphorylation. Mst was required for NF2-induced Lats activation, but unexpectedly NF2 deficiency increased Mst activity. Further biochemical studies accounted for these observations by showing that NF2 recruits Lats to the plasma membrane through direct binding. Sav1 similarly recruits Mst to the plasma membrane, where it phosphorylates and activates Lats. Liver specific single knock of either NF2 or Sav1 resulted in a mild increase of liver size, but double knock-out caused massive liver overgrowth. Based on these data, the authors raised a "parallel model" of Hippo pathway signal transduction, in which NF2 and Sav1 act as scaffolds to recruit Lats and Mst, respectively, to the membrane. In the submembrane signal transduction compartment formed by these proteins, Mst phosphorylates and activates Lats, which in turn phosphorylates and inhibits Yap.

The extracellular signals and their receptors that regulate the mammalian Hippo/Yap pathway are incompletely understood. Yu et al. found that Yap activity is regulated downstream of certain G-protein coupled receptors (GPCRs) (Yu et al., 2012). Specifically, they showed that the lipids lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) stimulate Yap activity by binding to specific GPCRs that couple to the $G\alpha_{12/13}$ subfamily of heterotrimeric G proteins. $G\alpha_{12/13}$ signaling inhibited Lats activity through a signal transduction pathway involving Rho GTPases and actin

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cytoskeleton organization, and independent of Mst. On the other hand, epinephrine stimulation of the β_2-adrenergic receptor, a GPCR coupled to $G\alpha_s$, enhanced Yap phosphorylation and thereby inhibited Yap activity.

The Hippo kinase cascade interacts with and phosphorylates Yap in the submembrane compartment, but Yap exerts its main actions in the nucleus. 14-3-3 proteins, highly conserved phosphoserine binding proteins that often bind transcription factors (Eckardt, 2001), are crucial chaperones that mediate proper Yap subcellular localization. In the absence of bound targets, 14-3-3 proteins reside in the nucleus. Target protein phosphorylation leads to 14-3-3 protein binding and export of the protein complex from the nucleus to the cytoplasm (Brunet et al., 2002). 14-3-3 proteins are required for Yap nuclear export (Dong et al., 2007), but it is not clear whether Lats is responsible for Yap phosphorylation in the nucleus.

Yap activity is regulated by cell–cell contacts, as contact inhibition leads to Yap phosphorylation, 14-3-3 binding, and exclusion from the nucleus. Yap interacts with α-catenin and angiomotin (AMOT), components of intercellular junctions which modulate Hippo/Yap signaling in response to cell–cell interactions. α-Catenin links cadherins, calcium-dependent intercellular adhesion molecules, with the actin cytoskeleton at adherens junctions. In keratinocytes, α-catenin directly interacts with 14-3-3 proteins, and the interaction is enhanced in the presence of phospho-Yap. α-Catenin negatively regulates Yap activity by recruiting it to the adherens junctions and the submembrane region (Schlegelmilch et al., 2011). Furthermore, the protein complex formed by α-catenin, 14-3-3 and phospho-Yap inhibits Yap dephosphorylation mediated by the phosphatase PP2Ac (Schlegelmilch et al., 2011). Angiomotin (AMOT) is another intercellular junction protein that regulates Yap activity (Zhao et al., 2011). AMOT binds to Yap, recruiting Yap to the sub-membrane region near intercellular junctions. AMOT also binds to filamentous actin (F-actin), and this interaction is regulated by Lats, making AMOT both a regulator and an effector of Hippo signaling. AMOT phosphorylation dissembles it from filamentous actin and releases it from junction complexes into the cytoplasm (Dai et al., 2013; Chan et al., 2013). Phosphorylated AMOT recruits the ubiquitin E3 ligase atrophin-1 interacting protein 4, leading to degradation of AMOT-bound Yap by the ubiquitin proteasome system (Adler et al., 2013).

Actin dynamics are crucial for Hippo signal transduction, as evidenced by the fact that inhibiting actin cytoskeleton dynamics initiated Hippo signaling in both *Drosophila* and mammalian cells (Dupont et al., 2011; Fernandez et al., 2011). Both actin polymerization inhibitor Latrunculin B and Rho GTPase inhibitor C3 promoted NF2 and Lats interaction (Yin et al., 2013) and increased Lats activity (Yu et al., 2012). How actin dynamics regulate Lats activity is an important gap in our understanding of Hippo signal transduction. Because the submembrane region near adherens junctions is enriched for both Hippo pathway components and actin cytoskeleton, this subcellular compartment is likely to be the arena in which actin dynamics modulate Lats.

Yap/TAZ regulation of cellular proliferation and organ size

As the crucial link between the Hippo kinase cascade and gene transcription, Yap has been intensely studied since its identification as an effector of the Hippo pathway (Huang et al., 2005). Yap has 5 HXRXXS motifs that are potential Lats1/2 phosphorylation sites. Mutation of serine 127 to alanine (S127A) largely abolished Mst2/Lats2-induced Yap phosphorylation, 14-3-3 binding, and Yap nuclear export, indicating that Yap S127 is the major Lats1/2 phosphorylation site that regulates its nuclear/cytoplasmic distribution (Zhao et al., 2007). Yap S127 phosphorylation did not directly affect Yap and Tead interaction, but rather decreased the interaction between Yap and Tead due Yap cytoplasmic localization.

In the liver, overexpression of activated Yap (Yap[S127A]) for one week doubled the liver size. This dramatic effect on organ size was due to increased proliferation and decreased apoptosis of liver cells (Dong et al., 2007; Camargo et al., 2007). In the cultured mammalian cells, Hippo/Yap signaling has been shown to be central to contact inhibition: cell–cell contacts activate Hippo to restrain Yap, thereby limiting cell proliferation. Yap overexpression overcomes contact inhibition in a *Tead*-dependent manner (Zhao et al., 2007). Yap promotes cell proliferation by stimulating the expression of *Ccna2*, *Ccnb1*, *Cdc2*, *Aurka*, *Aurkb*, and *Cdc25b* (von Gise et al., 2012). At the same time, Yap limits cellular apoptosis by stimulating expression of *Birc5* and *Birc2* (Dong et al., 2007).

As a vital transcriptional regulator, Yap is a nodal point regulated by numerous upstream signaling pathways besides the canonical Hippo kinase cascade, including the YES/SRC tyrosine kinase pathway, the Wnt/β-catenin pathway, and the JNK pathway. Yap was first identified as a YES-associated protein (Sudol, 1994). In osteoblast cells, Yap directly interacts with RUNX2 to suppress its transcriptional activity. The phosphorylation of Yap by YES/SRC tyrosine kinase is required for Yap to interact with RUNX2, as blocking YES/SRC kinase activity abolished the interaction between these two proteins (Zaidi et al., 2004). In tumor cells, Wnt signaling regulates Yap expression both transcriptionally and post-transcriptionally: in colorectal carcinoma cells, the β-catenin/TCF4 complex directly regulated Yap expression (Konsavage et al., 2012), while in liver cancer cells the Wnt/β-catenin downstream target Tribbles homolog 2 (TRIB2) stabilized Yap by preventing its degradation (Wang et al., 2013). Conversely, Yap also regulates canonical Wnt signaling by direct interaction with β-catenin. This interaction modulates β-catenin/TCF transcriptional activity (Heallen et al., 2011) and β-catenin subcellular localization (Imajo et al., 2012). In a screen for kinases that directly phosphorylate Yap, Tomlinso et al. found that JNKs are robust Yap kinases (Tomlinson et al., 2010). This study showed that PKCα and ERK2 also phosphorylate Yap. These studies indicate that Yap is a key nodal point regulated by multiple signaling pathways.

Hippo/Yap pathway in the heart

The fetal heart grows primarily by expansion of cardiomyocyte number (hyperplastic growth), while the postnatal heart grows mainly by enlargement of cardiomyocytes (hypertrophic growth). Studies of gain and loss of function mutations in Hippo/*Yap* pathway components in the heart (Table 1) have implicated this pathway in regulating both forms of heart growth.

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Table 1 Mouse models used to study cardiac Hippo/*Yap* signaling.

Mouse model	Organ phenotype	CM size	CM apoptosis	CM prolif.	Ref.
Hippo activation/Yap loss of function					
Mst1					
αMHC–*Mst1*, late fetal heart	Dilated cardiomyopathy; premature death; absence of compensatory cardiac hypertrophic growth.	↔	↑	ND	Yamamoto et al. (2003)
Lats2					
αMHC–*Lats2*, late fetal heart	Heart dysfunction, small and dilated hearts	↓	↔	ND (↑ in TAC)	Matsui et al. (2008)
Yap^{flox}					
x Nkx2.5–Cre, E7.5–	Small fetal heart, fetal demise	ND	ND	↓	von Gise et al. (2012), Xin et al. (2011), Xin et al. (2013), Del Re et al. (2013)
x Tnnt2–Cre, E7.5–	Small fetal heart, fetal demise	ND	↔	↓	
x αMHC–Cre, ~E12.5–	Dilated, lethal cardiomyopathy with normal CM number; impaired neonatal and adult heart regeneration	↓ (het; in MI)	↑ (KO or het MI)	↓ (KO or het in MI)	(het; in MI)
Tead1					
Tead1–lacZ, constitutive knockout	Small fetal heart, fetal demise	ND	ND	ND	Chen et al. (1994)
Hippo inhibition/Yap gain of function					
Sav1^{flox}					
x Nkx2.5–Cre, E7.5–x Nkx2.5–Cre, E7.5–	Large fetal heart, postnatal demise	↔	↔	↑	Heallen et al. (2011), Heallen et al. (2013)
x αMHC–MCM, adult	Normal heart function. Heart size not reported. Extended window for neonatal heart regeneration; improved outcome after MI	↓	ND	↑	
Mst					
Mst1/2^{flox} x Nkx2.5–Cre, E7.5–	Large fetal heart	ND	ND	ND	Heallen et al. (2011), Yamamoto et al. (2003), Odashima et al. (2007)
αMHC–DN–*Mst1*, late fetal heart	Normal cardiac function; preserved heart function post-MI	↔	↓ in I/R	↔ in MI	
Lats2					
Lats1/2^{flox} x Nkx2.5–Cre, E7.5–	Large fetal heart	ND	ND	ND	Heallen et al. (2011), Matsui et al. (2008), Heallen et al. (2013)
Lats1/2^{flox} x αMHC–MCM, adult	Normal heart function. Heart size not reported. Extended window for neonatal heart regeneration; improved outcome after MI.	↓	ND	↑	
αMHC–DN–*Lats*, late fetal heart	Biventricular hypertrophy, normal heart function	↑	↓ in TAC	ND	
Yap Gain of function					
cTNT–Cre; Rosa26$^{fs–rtTA}$; ColTRE–a*YAP*; Dox E8.5–	Cardiac overgrowth, embryonic lethal.	ND	↔	↑	von Gise et al. (2012), von Gise et al. (2012), Xin et al. (2011), Xin et al. (2013)

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Table 1 (*continued*)

Mouse model	Organ phenotype	CM size	CM apoptosis	CM prolif.	Ref.
βMHC–a*Yap*, E9–	Large fetal heart, viable to adulthood	ND	ND	↑	
αMHC–a*Yap*, late fetal heart	Increased heart size; Enhanced cardiac regeneration.	↔	↓ in MI	↑	
cTNT–Cre; Rosa26$^{fs-rtTA}$; ColTRE–aYAP; Dox P0–*Tead1*	Increased heart size and CM proliferation	↔	↔	↑	
MCK–*Tead1*, perinatal	Age dependent heart dysfunction and fibrosis without cardiac hypertrophy	ND	ND	ND	Tsika et al. (2010)

Table summarizes mouse models reported with gain or loss of function in the Hippo kinase cascade or in *Yap*. Approximate stage of the genetic manipulation is indicated based on the general properties of the promoter used. cTNT–Cre; Rosa26fs–rtTA; ColTRE–α*YAP* is a cardiac-specific, Dox inducible strategy to express activated *Yap* (von Gise et al., 2012).

Hippo/*Yap* pathway in heart development

The important role of the Hippo kinase cascade in suppressing cell proliferation led Heallen, Martin, and colleagues to hypothesize that Hippo signaling deficiency would cause cardiac overgrowth. This hypothesis was supported by the cardiac-restricted inactivation of *Mst1/2*, *Sav1*, and *Lats2*, which resulted in markedly enlarged hearts due to increased cardiomyocyte proliferation, trabecular expansion, and thickening of the compact myocardium (Heallen et al., 2011). However, overall cardiac structure was preserved, suggesting a selective role of Hippo signaling in cardiac growth control.

To test the hypothesis that Yap is a critical downstream effector of the Hippo kinase cascade in heart growth regulation, our group and the Olson group studied the effect of Yap gain or loss of function on heart development (von Gise et al., 2012; Xin et al., 2011). As in the Hippo-deficient mutants, overall cardiac architecture was preserved in Yap gain or loss of function. Cardiomyocyte-specific Yap inactivation early in heart development markedly depressed cardiomyocyte proliferation and caused dramatic cardiac hypoplasia. On the other hand, cardiomyocyte-specific expression of Yap[S127A] enhanced cardiomyocyte proliferation and caused profound cardiac overgrowth. The overgrowth was particularly striking in the trabecular myocardium, which is among the first cardiomyocyte populations to have withdrawn from the cell cycle. These data suggest that Hippo/Yap signaling may participate in limiting trabecular cardiomyocyte proliferation, and that the activation of Yap is sufficient to sustain cardiomyocyte cell cycle activity. Indeed, Yap activation in the neonatal heart similarly delayed cardiomyocyte cell cycle withdrawal, leading to cardiomegaly.

Tead transcription factors (Wu et al., 2008; Zhao et al., 2008), represented by Tead1–4 in mammals, are the canonical transcription factor partners of Yap. *Tead1* loss of function in the germline caused mid-gestational fetal loss due to cardiac hypoplasia (Chen et al., 1994). Yap–Tead interaction is required for Yap mitogenic activity in cardiomyocytes, as a peptide inhibitor of this interaction blocked Yap-driven cardiomyocyte cell cycle re-entry in vitro (von Gise et al., 2012). Furthermore, a Yap point mutant defective in this interaction (Yap S79A), failed to support normal fetal cardiac growth (von Gise et al., 2012). Tead transcription factors bind to the MCAT transcription factor binding motif. This motif is associated with striated muscle genes (Yoshida, 2008), suggesting that Tead, and perhaps its partner Yap, may have specialized roles in regulating muscle gene expression. Precedence for this possibility exists, as *Drosophila* Yorkie and Scalloped have specialized roles in regulating eye photoreceptor differentiation, independent of their growth-promoting roles (Jukam et al., 2013).

Ongoing studies indicate that Yap regulates heart growth and cardiomyocyte proliferation through multiple mechanisms. Hippo/Yap appears to regulate Wnt signaling. Cardiac *Sav1* inactivation caused β-catenin translocation into the nucleus and up-regulation of canonical Wnt pathway target genes (Heallen et al., 2011). Yap was shown to physically interact with β-catenin and to stimulate β-catenin/TCF transcriptional activity. Moreover, β-catenin heterozygosity suppressed the cardiac overgrowth phenotype of Hippo-deficient embryos. In agreement with these data, transgenic expression of activated Yap in fetal mouse hearts increased Wnt signaling (Xin et al., 2011). A second mechanism of Yap cardiac growth regulation involves IGF signaling. Transgenic Yap activation stimulated the IGF signal axis, a positive regulator of cardiomyocyte proliferation, hypertrophy, and survival (Xin et al., 2011). IGF pathway activation may enhance Wnt/β-catenin signaling, as Akt-mediated phosphorylation and inactivation of GSK3β enhances β-catenin stability. A third mechanism of Yap cardiac growth regulation is the transcriptional regulation of a number of cell cycle regulators. Activation of Yap in neonatal cardiomyocytes upregulated cell cycle regulators, indicating that Yap directly or indirectly stimulates expression of these cell cycle regulators (von Gise et al., 2012).

Hippo/Yap signaling pathway in cardiac hypertrophy and apoptosis

Adult mammalian cardiomyocytes have limited proliferative capacity, and thus increased size of the adult heart with

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exercise or disease is caused mainly by cardiomyocyte hypertrophy. In adult heart, the functions of the Hippo pathway kinases Mst and Lats were characterized in a series of studies carried out by the Sadoshima group, in which the effects of increasing or decreasing kinase activity were probed by cardiomyocyte-specific, transgenic overexpression of the candidate gene or a dominant negative mutant. In the case of Mst1, αMHC promoter driven expression of wild type *Mst1* increased cardiomyocyte apoptosis, decreased heart function and ventricular wall thickness, and caused death as early as 15 days after birth (Yamamoto et al., 2003). Overexpression of a dominant negative mutant form of *Mst1* (DN-Mst1) in the heart did not affect baseline heart function, but improved myocardial outcome after experimental MI. In post-MI Dn-Mst1 hearts, there was reduced cardiomyocyte apoptosis, reduced fibrosis, and preserved systolic contraction (Yamamoto et al., 2003; Odashima et al., 2007). Neither *Mst1* overexpression nor dominant negative inhibition altered cardiomyocyte size. These studies demonstrate that Mst1 activation promotes cardiomyocyte apoptosis, and that this activation adversely affects myocardial outcome after MI. Effects on Yap activity or cardiac regeneration were not directly examined, and indeed Mst1 may exert its effects through alternative pathways. For instance, cardiac Mst1 overexpression was shown to inhibit autophagy through phosphorylation of Beclin1 (Maejima et al., 2013).

Like *Mst1* overexpression, *Lats2* overexpression also caused heart dysfunction and reduced heart size (Matsui et al., 2008). However, the underlying mechanism appeared different, as *Lats2* overexpression did not increase baseline cardiomyocyte apoptosis but rather reduced cardiomyocyte size. A predisposition of *Lats2*-overexpressing cardiomyocytes to apoptosis was unmasked by pressure overload stress induced by transverse aortic constriction (TAC). Consistent with these results, dominant negative *Lats2* increased heart weight, induced cardiomyocyte hypertrophy, and suppressed TAC-induced apoptosis. The downstream targets phosphorylated by Lats2 that mediate these effects on cardiac growth and apoptosis were not established in these studies. Since Yap regulates both of these endpoints, it is likely to be essential to implement these effects of *Lats2* in the heart. However, other Lats2 substrates may also play important roles. A caveat to these experiments is the transgenic overexpression strategy, which may lead to non-physiological effects. For example, a recent study of adult stage, cardiac-specific *Lats1/2* loss of function showed that this genetic manipulation led to reduced cardiomyocyte size (Heallen et al., 2013), unlike transgenic dominant negative *Lats2*, which caused cardiomyocyte hypertrophy (Matsui et al., 2008).

The function of Yap in postnatal cardiac hypertrophy and apoptosis has been interrogated through genetic gain and loss of function approaches. Two independent studies showed that late fetal cardiac *Yap* inactivation caused dilated cardiomyopathy and premature death (Xin et al., 2013; Del Re et al., 2013). Total cardiomyocyte number was not significantly changed in Yap deficient hearts, suggesting that the major requirement of *Yap* to regulate heart size occurs early in heart development, and that myocardial hypoplasia was not a major cause of impaired heart function. *Yap* loss of function increased cardiomyocyte apoptosis rate, implicating pro-survival activity of *Yap* in postnatal cardiac homeostasis, although these measurements were made in severely failing hearts and it is not possible to discern if elevated apoptosis caused, or was an effect of, cardiac insufficiency. Del Re et al. also reported that *Yap* promotes cardiomyocyte hypertrophy after MI, based on reduced cardiomyocyte size in the remote area of post-MI hearts with cardiac *Yap* haploinsufficiency (Del Re et al., 2013). However, analysis of genetic mosaics showed that *Yap* inactivation in a small fraction of cardiomyocytes did not change the size of those cardiomyocytes or their hypertrophic response to pressure overload, indicating that *Yap* is not cell autonomously required for cardiomyocyte hypertrophy in response to pressure overload (von Gise et al., 2012). Furthermore, *Yap* activation in the postnatal heart did not drive cardiomyocyte hypertrophy (von Gise et al., 2012; Xin et al., 2013). As with *Yap* overexpression, cardiac-specific *Tead1* overexpression also did not trigger cardiac hypertrophy (Tsika et al., 2010). These *Tead1* overexpressing mice showed an age dependent heart dysfunction phenotype, and their hearts were extremely sensitive to pressure overload.

Taken together, we conclude that *Lats* may regulate adult cardiomyocyte hypertrophy independent of *Yap* or *Tead1*. Rather than controlling cardiac hypertrophic growth, the canonical Hippo/Yap pathway is crucial for regulation of cardiomyocyte survival in the adult heart. This parallels the findings in *Drosophila* post-mitotic neurons, where *Warts* and *Lats* but not Yki were required for the maintenance of dendrites (Emoto et al., 2006). The proteins and pathways through which *Lats* may regulate cardiomyocyte hypertrophic growth are currently unknown.

Hippo/Yap in heart regeneration

The finding that Hippo/Yap signaling powerfully regulates fetal heart growth and cardiomyocyte proliferation (von Gise et al., 2012; Heallen et al., 2011; Xin et al., 2011), and that Yap gain of function extends neonatal cardiomyocyte cell cycle activity in vivo (von Gise et al., 2012) raised the exciting possibility that this signaling pathway could be redeployed to promote adult heart regeneration and repair.

Initial studies on the Hippo kinase cascade and Yap suggest that this pathway remains an important regulator of cardiomyocyte cell cycle activity in the postnatal heart, although Yap's potency in driving cardiomyocyte proliferation wanes with cardiomyocyte maturation. We showed that overexpression of activated Yap beginning at E8.5 dramatically stimulated cardiomyocyte proliferation (von Gise et al., 2012). In the newborn heart, Yap1 continued to drive cardiomyocyte proliferation, although the extent of cardiomyocyte proliferation was far less than the observed in early heart development (von Gise et al., 2012; Xin et al., 2013). Consistent with the stage-dependent sensitivity of cardiomyocytes to Yap-induced proliferation, overexpression of Yap under control of the βMHC promoter, which initiates a few days after E8.5, resulted in less extensive cardiac overgrowth, and these transgenic mice were viable to adulthood (Xin et al., 2011).

The newborn mouse heart retains the ability to regenerate from myocardial injury, but this regenerative capacity is lost by postnatal day 7 (P7) (Porrello et al., 2011). Given the ability of Yap to promote neonatal cardiomyocyte proliferation, Xin, Olson, and colleagues tested the ability of Yap

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activation to prolong the regenerative window (Xin et al., 2013). P7 neonatal wild type and αMHC–aYAP transgenic mice underwent LAD ligation. Twenty-one days later, wild type mice showed extensive scar formation, loss of myocardial tissue, and ventricular dilation, while the αMHC–α*YAP* mice had regenerated the infarcted myocardium with minimal scar tissue. Consistent with these data, inactivating Yap negative regulators *Sav1* or *Lats1/2* also increased cardiomyocyte proliferation in the neonatal heart and extended the neonatal regenerative window (Heallen et al., 2013). These data suggest that neonatal inactivation of Hippo pathway kinases or activation of Yap is sufficient to maintain the proliferative competence of neonatal cardiomyocyte in a manner that enhances neonatal cardiac regeneration.

Stimulating adult cardiomyocyte cell cycle re-entry has proven to be far more challenging than increasing fetal cardiomyocyte proliferation or delaying the time of neonatal cardiomyocyte cell cycle withdrawal. Thus a crucial question is whether or not Yap activation can promote adult heart regeneration and stimulate adult cardiomyocyte proliferation. When 28-day old αMHC–αYap mice or wild type controls were challenged with myocardial infarction (MI), *Yap* transgenic mice showed better preservation of heart function and smaller scar size at 21 day post-MI (Xin et al., 2013). αMHC–α*YAP* mice had 2.5-fold higher cardiomyocyte proliferation compared to wild-type control post-MI. However, the extent of adult cardiomyocyte proliferation was limited – cardiomyocyte proliferation stimulated by expression of activated Yap in adult cardiomyocytes was twenty-fold lower than the rate observed in neonatal wild-type mice. These data suggest that Yap activation in the adult heart does promote cardiomyocyte proliferation, although the level of proliferation may be insufficient to support meaningful cardiac regeneration. Yap-dependent modulation of other processes, such as reduction of apoptosis (Xin et al., 2013), likely contributes to the salutary effect of Yap after MI.

The effect of *Sav1* or *Lats1/2* inactivation on adult cardiomyocyte proliferation has also been examined. Inactivation of these Hippo pathway components in the adult heart increased cardiomyocyte DNA synthesis and karyokinesis (Heallen et al., 2013). Qualitatively, the result was similar to what was observed in Yap gain of function (Xin et al., 2013). However, inactivation of *Sav1* or *Lats1/2* caused quantitatively much higher levels of cardiomyocyte cell cycle activity (~2.5% EdU^+ cardiomyocytes after 4 days of labeling). This discrepancy may be due to the different experimental methods used by the two groups, or it might suggest that the Hippo pathway regulates proliferation through additional targets other than Yap.

The contribution of resident cardiac progenitors in adult heart homeostasis, and their potential recruitment for therapeutic heart regeneration, has been widely debated (Kajstura et al., 2008; Bergmann et al., 2009; Senyo et al., 2013; Hsieh et al., 2007). The Hippo–Yap pathway plays a critical role in the multipotency and differentiation of embryonic stem cells, neuronal progenitors, and intestinal stem cells (Lian et al., 2010; Cao et al., 2008; Cai et al., 2010; Barry et al., 2013). These data suggest that Hippo–Yap may modulate cardiac progenitor activity. However, since most animal models used to study the cardiac function of this pathway (Table 1), they have primarily used cardiomyocyte-specific drivers that are presumed to be inactive in cardiac progenitors, experimental data on Hippo–Yap signaling in cardiac progenitors is currently lacking. Clarifying the contribution of resident cardiac progenitors to adult heart homeostasis and their potential redeployment for therapeutic heart regeneration, and the function of Hippo/Yap in this processes, are clearly priorities for future studies.

Concluding comments: harnessing Hippo/Yap for heart disease therapy

The fetal heart enlarges through hyperplastic growth. At this developmental stage, the heart is similar with other organs, such as liver or skin, and its size is controlled by Hippo/Yap signaling. Shortly after birth, cardiomyocytes largely exit the cell cycle, and the heart increases its size by hypertrophic rather than hyperplastic growth. The Hippo/Yap pathway continues to influence heart size by regulating cardiomyocyte proliferation in the newborn mice, but it has less of an effect on adult cardiomyocyte proliferation. In addition to cardiomyocyte proliferation, Hippo kinases also regulate cardiac hypertrophic growth. Hippo/Yap signaling also influences cardiomyocyte autophagy and apoptosis.

The studies summarized in this review demonstrate the importance of the Hippo/Yap pathway in the regulation of heart growth during fetal development. Modulation of this pathway in the neonatal heart prolongs the neonatal regenerative window, highlighting the potential for enhancing cardiac regeneration. Yap activation in the injured adult heart is also beneficial at least in the short-term, although stimulation of cardiomyocyte proliferation was modest and additional mechanisms are also likely to contribute. Over the past two decades, many paracrine factors and their pathways have been found to stimulate cardiomyocyte proliferation, such as IGF1 (Duerr et al., 1995), perisotin (Kuhn et al., 2007), neuregulin (Baliga et al., 1999; Bersell et al., 2009), and fibroblast growth factor (Engel et al., 2005). However, these agents also cause cardiomyocyte hypertrophy (Baliga et al., 1999; McMullen et al., 2004; House et al., 2010; Oka et al., 2007), which may be deleterious to heart function in the long run. When compared with the other signaling pathways involved in cardiac repair, the Hippo/Yap pathway stands out by promoting cardiomyocyte proliferative growth and enhancing myocardial recovery after MI without stimulating cardiomyocyte hypertrophy.

A number of major questions remain, and the answers have important implications for our understanding of adult cardiomyocyte homeostasis and the therapeutic potential of Hippo/Yap pathway modulation for cardiac repair and regeneration.

Why does Yap stimulation of cardiomyocyte proliferation wane from fetal to neonatal to adult heart? One possibility is that Yap activation alone is insufficient to overcome the multiple barriers to postnatal cardiomyocyte cell cycle activity. Another possibility is that unknown factors block Yap activity in the adult heart. Future work addressing this question may further our understanding of Hippo/Yap pathway regulation and allow us to develop strategies to augment Yap-driven cardiac regeneration regulation.

Why does inactivation of Yap in the late fetal heart cause progressive, lethal dilated cardiomyopathy? Does this reflect a developmental defect that becomes expressed postnatally,

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or an ongoing requirement for Yap in the maintenance of adult heart function? Current data from the failing adult heart suggests that Yap is required to suppress cardiomyocyte apoptosis. However, cardiomyocyte number in these failing hearts was not detectably reduced. Perhaps Yap has additional roles in the postnatal cardiomyocyte, as it does in the *Drosophila* eye (Jukam et al., 2013).

Finally, a prerequisite to considering modulation of the Hippo/Yap pathway for therapeutic cardiac regeneration is a demonstration that this can be done without induction of tumors, since activation of Yap is highly oncogenic in proliferative tissues such as skin or liver. Presently, the long term safety of Yap activation in cardiomyocytes has not been reported. Furthermore, approaches need to be developed to stimulate Yap in cardiomyocytes and not in other tissues or organs. This might be achieved by the identification of beneficial downstream genes regulated by Hippo/Yap, which may yield more cardiac-selective drug targets. Alternatively, localized or cell-type specific drug or gene delivery technologies may be used to achieve cardiomyocyte-specific Yap activation.

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