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A growing role for the Hippo signaling pathway in the heart:

Hippo pathway function in the heart

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Abstract

Heart disease is a major cause of clinical morbidity and mortality, and a significant health and economic burden worldwide. The loss of functional cardiomyocytes, often a result of myocardial infarction, leads to impaired cardiac output and ultimately heart failure. Therefore, efforts to improve cardiomyocyte viability and stimulate cardiomyocyte proliferation remain attractive therapeutic goals. Originally identified in *Drosophila*, the Hippo signaling pathway is highly conserved from flies to humans and regulates organ size through modulation of both cell survival and proliferation. This is particularly relevant to the heart, an organ with limited regenerative ability. Recent work has demonstrated a critical role for this signaling cascade in determining heart development, homeostasis, injury and the potential for regeneration. Here we review the function of canonical and non-canonical Hippo signaling in cardiomyocytes, with a particular focus on proliferation and survival, and how this impacts the stressed adult heart.

Introduction

First identified and defined in *Drosophila melanogaster*, the signaling cascade now recognized as the Hippo pathway is a critical molecular mechanism regulating fundamental cell processes that determine organ size. Genetic screens for overgrowth phenotypes in the fly revealed key components of the pathway including the serine/threonine kinases hippo (MST1/2 in mammals) and warts (LATS1/2), and the scaffold proteins salvador (SAV1/WW45) and mob as tumor suppressor (MOB1A)(Table)[1–8]. Importantly, the Hippo pathway is highly conserved from flies to humans, and activation of these core constituents leads to phosphorylation and inhibition of the downstream transcriptional co-factor yorkie (YAP/TAZ), thereby restricting cell proliferation and survival[9, 10]. YAP/TAZ lack a DNA binding domain and instead associate with multiple transcription factors. The best studied to date is the TEA domain (TEAD) family, which has been shown to mediate many of the biological functions of YAP/TAZ (Figure)[11].

Regulation of Hippo signaling

Since its discovery, regulation of the Hippo pathway has been of great interest and much effort has been devoted to elucidating upstream signals that feed into the pathway. Altered

mechanical stress, resulting from changes in substrate rigidity or cell-cell contacts, influences many cell processes and has been shown to regulate the subcellular localization and activity of YAP/TAZ[12, 13]. When a cell experiences increased force, actin polymerization is upregulated and active YAP/TAZ localize to the nucleus[14]. Conversely, cells grown on soft substrates promote cytosolic retention of inactive YAP/TAZ. The ability of extracellular matrix stiffness to modulate YAP/TAZ is dependent upon the actin cytoskeleton, and disruption of F-actin, or inhibition of the small GTPase RhoA, which is known to promote actin polymerization, block YAP/TAZ activation in this context[15]. Potential proximal mediators of mechanical strain-induced regulation of YAP/TAZ include cell surface integrins, angiomotins and α -catenins, which have been shown to associate with YAP in the cytosol and inhibit YAP function[16–19]. Whether or not this mechanism is LATS-dependent remains somewhat unclear.

The Hippo pathway is also regulated by soluble factors via cognate cell surface receptors. G-protein coupled receptors (GPCRs), the largest class of receptors in the human genome and highly relevant to heart disease, can directly modulate the Hippo pathway through engagement of specific heterotrimeric G-proteins and initiation of downstream signaling. Two phospholipid signaling molecules with demonstrated cardioprotective effects, sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), both signal through GPCRs that couple strongly to $G_{\alpha 12/13}$. These factors were shown to trigger inhibition of Hippo signaling and subsequent activation of YAP/TAZ[20]. On the other hand, hormones such as epinephrine and glucagon, which signal through GPCRs that couple primarily to $G_{\alpha s}$, activate the Hippo pathway and inhibit YAP/TAZ. These findings demonstrate specificity of GPCR signaling toward Hippo pathway regulation. Activation of $G_{\alpha 12/13}$ is known to stimulate RhoA and cytoskeletal rearrangement, and YAP/TAZ activation was blocked by inhibition of RhoA or F-actin[21, 22]. This mechanism also appears dependent on LATS function, which may be regulated by PKA.

YAP/TAZ nuclear signaling

Hippo inactivation triggers the nuclear accumulation of YAP/TAZ. Lacking the ability to bind directly to DNA, nuclear YAP/TAZ associate with numerous transcription factors to modulate gene expression. Partners of YAP/TAZ include MRTF-A[23], FoxO1[24], Tbx5[25, 26], Runx[27, 28], p73[29], Smads[30, 31], and TEADs among others[32]. YAP/TAZ have been shown to regulate expression of genes that promote cell proliferation, survival and metabolic function. Established YAP/TAZ targets include connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (CYR61 or CCN1), survivin (also known as baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5), amphiregulin and fibroblast growth factor 2 (FGF2)[10, 33, 34]. In cardiomyocytes, the catalytic subunit of phosphoinositide-3 kinase (PI3K) p110 β was identified as an important transcriptional target of YAP-TEAD that mediates enhanced proliferation[35]. Additional work has identified a subset of antioxidant genes as critical mediators of YAP-dependent cardioprotection, indicating that suppression of reactive oxygen species is an important function of YAP/TAZ signaling in the heart[24, 36]. YAP was also found to promote expression of genes that influence cytoskeletal dynamics and promote cell protrusions, which may be a critical step in the proliferative process of cardiomyocytes[37]. Interestingly,

nuclear YAP/TAZ can also repress gene expression through recruitment of the NuRD complex and subsequent histone modification and transcriptional inhibition[38]. This mechanism mediated YAP/TAZ attenuation of TNF-related apoptosis inducing ligand (TRAIL) and DNA damage-inducible transcript 4 (DDIT4) expression, which was necessary for enhanced cell survival.

Hippo and heart development

An important distinction between the embryonic/early postnatal and the adult heart is the underlying mechanism of organ growth. During development, the heart enlarges through the addition of cardiomyocytes (proliferation), whereas this process almost entirely ceases shortly after birth and the heart continues its growth via enlargement of individual myocytes (hypertrophy). Indeed, cardiomyocytes within the adult mammalian heart have little capacity for proliferation, although low-level turnover has been observed[39, 40], and therapies attempting to enhance this ability, and thereby enable cardiac regeneration, remain attractive.

In the embryonic mouse heart, the Hippo pathway plays an important role in determining cardiomyocyte number and, subsequently, overall organ size. Cardiomyocyte-targeted inducible expression of a constitutively active YAP mutant (S127A) in the developing heart caused increased myocyte proliferation, thickening of the myocardium, and embryonic lethality[41]. Genetic inactivation of Hippo pathway components through targeted deletion of SAV1, MST1/2 or LATS2 during heart development resulted in augmented YAP activation and was sufficient to drive increased cardiomyocyte proliferation resulting in cardiomegaly and postnatal death[42]. Conversely, cardiomyocyte-restricted deletion of YAP in the fetal mouse heart resulted in reduced cardiomyocyte proliferation, myocardial hypoplasia yet also resulted in embryonic death[41]. In sum, these studies clearly demonstrate the involvement and ability of Hippo signaling through YAP to regulate embryonic cardiomyocyte proliferation and heart size during development. Mechanistic inquiry revealed Hippo crosstalk with Wnt/ β -catenin signaling, which was demonstrated to play a critical role in mediating the positive effect of YAP on cell cycle-related gene expression and cardiac overgrowth[42, 43].

Interference with YAP-TEAD interaction can also modify cardiomyocyte proliferation and heart growth. Recent findings from Pu and colleagues identified vestigial-like 4 (VGLL4) as a critical modulator of cardiomyocyte proliferation and survival in postnatal mice[44]. VGLL4 is highly expressed in the heart and VGLL4 protein levels are upregulated with age. VGLL4 contains a Tondu domain that allows for its binding to TEAD transcription factors in the cardiomyocyte nucleus. This association competes with YAP for TEAD binding and also promotes TEAD degradation, thereby negatively regulating YAP-TEAD transcriptional output. Acetylation of VGLL4 prevents its association with TEAD, and overexpression of an acetylation-resistant VGLL4 mutant in neonatal mouse hearts resulted in the dissociation of YAP-TEAD complexes, attenuation of cardiomyocyte proliferation and increased cell death. These results signal an important role for VGLL4 as an age-dependent mechanism that represses YAP function in the heart.

Hippo signaling in adult heart

Hypertension is a major risk factor for coronary artery disease and heart failure. In mice, transverse aortic constriction (TAC) is commonly used to generate pressure overload-induced cardiac hypertrophy and heart failure[45]. In this model of increased hemodynamic afterload, the heart initially responds through compensatory hypertrophy to maintain output despite increased resistance. However, with time, the response becomes maladaptive leading to remodeling of the myocardium, dilatation and decreased cardiac function. Examination of human failing hearts revealed activation of several key Hippo pathway components[46–48]. Additionally, at the cellular level, Hippo signaling regulates responses that contribute to a failure phenotype, including aberrant cardiomyocyte growth and death, suggesting a role for this pathway during chronic stress[49]. For example, endogenous LATS2 is upregulated in the heart during pressure overload in mice, and inhibition of LATS2 via expression of a kinase-inactive dominant-negative LATS2 mutant suppresses cardiomyocyte death in response to TAC[50]. Conversely, overexpression of LATS2 is sufficient to cause cardiomyocyte apoptosis in cell culture. Increased activation of LATS2 also attenuates cardiomyocyte hypertrophy in response to phenylephrine stimulation in vitro and TAC-induced hypertrophy in vivo, while inhibition of LATS2 increased ventricle growth both at baseline and in response to TAC. While the potential role of YAP downstream of LATS2 was not investigated, these results indicate that LATS2 is an important endogenous regulator of cardiomyocyte hypertrophy and remodeling during pressure overload and may also contribute to cardiomyocyte death initiated by TAC.

The protein scaffold and tumor suppressor, RASSF1A (Ras association domain family member 1A), has been shown to engage the Hippo pathway kinase MST1 via the SARAH domains present in each, and promote MST1 activation[51]. Studies in mice demonstrated that RASSF1A is an important regulator of Hippo signaling in the pressure-overloaded heart[52, 53]. Endogenous RASSF1A expression was increased by TAC, and RASSF1A association with MST1 was observed in myocardial samples that were subjected to pressure overload. Importantly, both systemic and cardiomyocyte-restricted deletion of RASSF1A attenuated TAC-induced activation of MST1 and cardiomyocyte apoptosis. On the other hand, increased cardiac expression of RASSF1A, but not a SARAH domain mutant RASSF1A that is deficient for MST1 binding, augmented MST1 activation and cardiomyocyte apoptosis[52]. Somewhat unexpectedly, systemic deletion of RASSF1A did not confer cardioprotection against TAC-induced cardiac remodeling and dysfunction. In fact, these RASSF1A KO mice had worsened hypertrophy and fibrosis, and heart function similar to WT mice after TAC. This was in contrast to cardiomyocyte-restricted deletion of RASSF1A, which showed attenuated hypertrophy, reduced fibrosis and better heart function. Further analysis revealed a protective function of RASSF1A in cardiac fibroblasts that restricted secretion of TNF- α and prevented paracrine-mediated fibrosis and hypertrophy during pressure overload[52]. These studies demonstrated a role for RASSF1A in activating the Hippo pathway, and delineated cell type specific functions for RASSF1A, in the chronically stressed heart.

The function of MST2 has also been investigated in the adult mouse heart[54]. MST2 systemic KO and WT mice were subjected to pressure overload stress and characterized.

Although baseline cardiac phenotypes were similar, MST2 KO mice showed attenuation of TAC-induced cardiac hypertrophy, fibrosis and cardiomyocyte apoptosis. Interestingly, despite these differences, cardiac function was decreased comparably to WT counterparts. Results from cultured cardiomyocytes indicated that MST2 promotes activation of the MAP kinase ERK as well as cardiomyocyte growth, and may explain why hypertrophy is blunted in MST2 KO mice. These findings also suggest that the function of MST1 and MST2 in the heart may not be redundant, as each isoform appears to mediate opposing outcomes with regard to cardiomyocyte growth and survival.

Hippo and myocardial infarction

Myocardial infarction (MI) contributes significantly to the development of chronic heart failure and is a major source of morbidity and mortality worldwide [55, 56]. MI triggers large-scale death of cardiomyocytes that increases the workload of surviving cardiomyocytes and initiates a maladaptive remodeling process, further impairing cardiac output. Treatment of MI by primary percutaneous coronary intervention (PCI) restores blood flow to salvage heart muscle, but also paradoxically contributes to heart damage by promoting reperfusion injury, which exacerbates cardiomyocyte death following ischemia[57].

MST1 is activated during hypoxia/reoxygenation in cultured cardiomyocytes and in the heart following ischemia/reperfusion (I/R) [58, 59]. Activation of MST1, either by transgenesis or I/R, induces cardiomyocyte apoptosis. MST1-Tg (α MHC) mice had robust increases in TUNEL-positive cardiomyocytes and developed a lethal dilated cardiomyopathy as early as postnatal day 15 [58]. Conversely, inhibition of MST1 during I/R by cardiac expression of dominant negative (DN) MST1(K59R) reduced myocyte apoptosis and attenuated cardiac dysfunction [58, 59], indicating that endogenous MST1 is an important mediator of cardiomyocyte death caused by MI and a potential therapeutic target.

Investigation into the underlying molecular mechanisms responsible for MST1 activation in cardiomyocytes revealed the involvement of Neurofibromin 2 (NF2) and RASSF1A, both previously identified Hippo pathway members. NF2 is a scaffold, whose active conformation is regulated by phosphorylation, that activates Hippo signaling in the mammalian brain and liver[60, 61]. Recent work demonstrated that oxidative stress promotes the active conformation of NF2 in cardiomyocytes, a response localized to the nucleus and mediated by the targeting subunit of myosin light chain phosphatase (MYPT-1)[62]. This acute activation of NF2 promotes complex formation with MST1 and LATS2 and facilitates MST1 activation during I/R. NF2 cardiac-specific knockout mice (cKO) had significantly smaller infarcts with attenuated cardiomyocyte apoptosis and improved heart function after I/R. Mechanistically, NF2 cKO hearts showed upregulation of YAP activity and this cardioprotective effect was abolished in NF2-YAP double mutant mice, suggesting that NF2-mediated MST1 activation signals through a nuclear canonical MST1-LATS2-YAP pathway [62].

MST1 activation can also occur through a mitochondrial mechanism involving the small GTPase K-Ras and RASSF1A. Here, oxidative stress activates K-Ras, which promotes its association with RASSF1A, and the recruitment and activation of MST1 to mitochondria.

Interestingly, RASSF1A-activated MST1 does not engage canonical LATS2-YAP signaling in cardiomyocytes, but instead phosphorylates Bcl-xL at Serine 14. This post-translational modification antagonizes the ability of Bcl-xL to associate with Bax thereby leading to increased Bax activation and the mitochondrial cell death pathway [47]. Bcl-xL Serine 14 phosphorylation appears to be physiologically relevant as it is increased in failing human hearts and mouse hearts subjected to I/R. Furthermore, Bcl-xL S14A knock in (KI) mice, which are resistant to phosphorylation by MST1, have increased Bcl-xL-Bax interaction, reduced cardiomyocyte apoptosis and smaller infarcts after I/R compared to control mice [47, 63].

MST1 is also activated during chronic MI (permanent ligation model) in the mouse heart, although the mechanism remains unclear[46]. In this condition, MST1 inhibits cardiomyocyte autophagy and promotes the accumulation of aggresomes. MST1 loss-of-function mice (MST1^{-/-} and DN-MST1-Tg) had smaller infarcts and better heart function after MI; however, this was abolished in Beclin1^{+/-} background further implicating regulation of autophagy as mechanistically important. MST1 was shown to directly phosphorylate Beclin1 at Threonine 108, and endogenous Beclin1 phosphorylation was observed in human and mouse MI hearts. Phosphorylation at this site enhances the interaction between Beclin1 and Bcl-2, thereby inhibiting the formation of Beclin1-Vps34-Atg14L complex that is critical for activation of autophagy. Additionally, the enhanced binding of Beclin1 with Bcl-2 frees Bax to be activated and stimulates apoptosis[46]. Taken together, these results reinforce MST1 as a central regulator of cardiomyocyte death and suggest that compartmentalization of MST1 dictates downstream signaling during I/R and chronic MI.

LATS2 is also activated by I/R and chronic MI in the heart[24, 64]. Inhibition of endogenous LATS2 through cardiac expression of DN-LATS2 afforded cardioprotection against I/R injury. DN-LATS2-Tg mice showed attenuated cardiomyocyte apoptosis, reduced infarcts and increased activation of YAP[24]. Interestingly, YAP is globally inactivated in the MI heart, yet shows selective activation in cardiomyocytes at the infarct border region[64]. Activation of YAP is likely cardioprotective as adenoviral gene transfer of YAP into the adult mouse heart elicited protection against I/R[24]. Similarly, AAV9-mediated expression, or transgenic expression, of constitutively active YAP both resulted in significantly enhanced cardiomyocyte proliferation at the border zone and preservation of heart function after MI[65, 66]. Conversely, YAP heterozygous cKO mice have increased cardiomyocyte death, augmented chamber dilatation and worsened cardiac function compared to control mice after 4 weeks MI[64].

Several mechanisms may underlie the beneficial effect of YAP activation in the heart. In the setting of I/R, YAP interacts with FoxO1 and promotes expression of the FoxO1 target genes catalase and MnSOD, which attenuate oxidative stress, DNA damage and apoptosis[24]. YAP also promotes the expression of microRNA (miR)-206[67]. During I/R, YAP activity is inhibited and miR-206 levels decline. Increased miR-206 expression promoted cardiomyocyte survival and protected the heart against I/R injury, which may be mediated by downregulation of Forkhead box protein P1 (FoxP1). Studies have also demonstrated that YAP enhances PI3K-AKT signaling[35, 43, 64]. As mentioned above, YAP was shown to

promote expression of p110 β , a catalytic subunit of PI3K, and demonstrated a critical role for p110 β and enhanced AKT activity in YAP-induced cardiomyocyte proliferation and survival[35]. AKT phosphorylation and p110 β expression were decreased in YAP cKO hearts. On the other hand, YAP gain-of-function promoted AKT activation, and PI3K inhibition prevented the anti-apoptotic and proliferative effects of YAP in cardiomyocytes[35, 64]. As a regulator of transcription, further studies are likely to reveal additional gene targets through which YAP affords protective effects; however, it may also be of interest to explore potential non-transcriptional YAP functions[68].

Hippo pathway in cardiomyocyte proliferation and heart regeneration

In addition to protecting cardiomyocytes against death, increasing their proliferation could also ameliorate cell loss during MI. However, even in response to injury, mature cardiomyocytes have a limited capacity to proliferate thereby restricting regeneration in the adult heart. Hippo signaling influences both cell survival and cell cycle progression, and growing evidence indicates that it also modulates mammalian heart regeneration.

In contrast to adult, the postnatal mouse heart retains its regenerative capacity up to 7 days after birth, and can fully recover from severe injury including MI and resection of the cardiac apex[69]. Lineage tracing experiments have demonstrated that this myocardial renewal is derived from pre-existing cardiomyocytes. To directly test the involvement of Hippo in this phenomenon, inducible cardiomyocyte-specific SAV1 cKO mice were subjected to MI by permanent LAD ligation, or subjected to apical resection, at day P8. In contrast to control mice that can no longer effectively mitigate these injuries, SAV1 cKO mice had increased cardiomyocyte proliferative rates, significantly reduced scarring and improved cardiac function[70]. Similarly, transgenic mice expressing a constitutively active cardiac YAP demonstrated that YAP gain of function is sufficient to elicit increased cardiomyocyte proliferation outside of the P0–P7 regenerative window resulting in significantly less fibrotic scarring[65]. In contrast, cardiomyocyte-specific ablation of YAP (driven by α MHC-Cre) resulted in a loss of regenerative potential when mice were subjected to MI at day P2[65]. This work implicates Hippo/YAP signaling as a critical component mediating postnatal heart regeneration, and that targeted manipulation of the Hippo pathway can alter the duration of this renewal period.

To investigate whether Hippo signaling regulates adult cardiomyocyte proliferation, 3–4 month old cardiomyocyte-specific inducible SAV1 cKO or LATS1/2 cKO mice were employed. Acute inactivation of Hippo caused upregulation of cardiomyocyte proliferation as evidenced by increased EdU incorporation, and elevated levels of Ki-67 and AuroraB-positive cardiomyocytes[70]. To determine a functional benefit of increased cardiomyocyte proliferation, 8–10 week old SAV1 cKO mice were administered tamoxifen and subjected to 21 day MI. SAV1 cKO mice showed improved heart function and attenuated infarct size suggesting that enhanced proliferation of cardiomyocytes in adult hearts is protective during MI stress. Additional work has suggested that cytoskeletal remodeling is upregulated due to heightened YAP activation in hearts deficient for Hippo signaling, and this may mediate the observed regenerative effects[37]. While the precise molecular mechanisms responsible for enhanced proliferation and regeneration remain unclear, it is possible that signaling cascades

similar to those affected by Hippo inactivation in the developing heart (i.e. Wnt/ β -catenin, IGF-PI3K-AKT), are also reactivated in the adult to promote cell cycle progression and cell division[35, 42, 43, 65]. Recently, Martin and colleagues identified the transcription factor paired-like homeodomain 2 (Pitx2) as a critical mediator of Hippo-deficient heart regeneration[36]. Pitx2 expression is dramatically downregulated in adult hearts; however, in SAV1 cKO hearts subjected to MI, Pitx2 nuclear presence in border zone cardiomyocytes was enhanced. Importantly, neonatal ablation of Pitx2 disrupted postnatal regeneration following apex resection, whereas overexpression of Pitx2 in adult mice afforded a regenerative response post-MI. Pitx2 was found to associate with YAP in adult mouse myocardium and cardioprotection afforded by Pitx2 gain-of-function was compromised by concomitant YAP downregulation. Further analysis revealed an appreciable overlap between Pitx2 and YAP gene programs and promoter occupancy, strong evidence that Pitx2 works in concert with YAP to promote cardiomyocyte proliferation and heart regeneration.

Conclusions

Is the Hippo pathway a viable therapeutic target? Growing evidence indicates that Hippo signaling has important functions in heart development, homeostasis and response to injury/regeneration. Additionally, components of the pathway have been shown to be altered in human heart failure patients, suggesting translational potential of pathway modulation[46, 47, 62]. These findings imply it is plausible to target Hippo signaling in heart disease, perhaps most obviously in acute MI; however, important considerations remain before transitioning to the clinic. These include questions regarding cell-type specificity of Hippo signaling (e.g. function in cardiomyocytes versus non-myocytes), the potential for off target effects in non-cardiac organs (e.g. liver) where physiological roles for Hippo have been demonstrated, and the optimal duration of pathway manipulation (e.g. acute versus chronic inhibition may lead to different physiological outcomes).

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Figure.

Overview of the core components of canonical Hippo signaling. Activation of MST1/2 kinases, which is facilitated by association with the scaffold SAV1, causes phosphorylation and activation of LATS1/2 kinases. Active LATS1/2 can scaffold with MOB1A and phosphorylate the transcription co-factors YAP/TAZ. This inhibitory phosphorylation promotes binding of YAP/TAZ to 14-3-3 proteins and cytosolic retention, thereby preventing the association of YAP/TAZ with the TEAD family of transcription factors and regulation of gene expression. Ischemia, ischemia/reperfusion (I/R), and pressure overload have been shown to activate MST1 in the heart.

Table

Core components of the Hippo signaling pathway.

Mammals	<i>Drosophila</i>
Mammalian sterile 20-like kinase 1/2 (MST1/2)	Hippo (Hpo)
Large tumor suppressor kinase 1/2 (LATS1/2)	Warts (Wts)
Salvador (SAV1/WW45)	Salvador (Sav)
Mps one binder kinase activator-like 1A (MOB1A)	Mob as tumor suppressor (Mats)
Yes-associated protein (YAP)	Yorkie (Yki)
Transcriptional co-activator with PDZ-binding motif (TAZ)	
TEA domain family member 1–4 (TEAD1–4)	Scalloped (Sd)
Neurofibromin 2 (NF2)	Merlin (Mer)
Ras association domain family member 1A (RASSF1A)	Ras association family member (dRASSF)