Initially identified in *Drosophila melanogaster*, the Hippo signaling pathway regulates organ size through modulation of cell proliferation, survival and differentiation. This pathway is evolutionarily conserved and canonical signaling involves a kinase cascade that phosphorylates and inhibits the downstream effector Yes-associated protein (YAP). Recent research has demonstrated a fundamental role of Hippo signaling in cardiac development, homeostasis, injury and regeneration, and remains the subject of intense investigation. However, 2 prominent members of this pathway, RASSF1A and Mst1, have been shown to influence heart function and stress responses through YAP-independent mechanisms. This review summarizes non-canonical targets of RASSF1A and Mst1 and discusses their role in the context of cardiac hypertrophy, autophagy, apoptosis and function. (Circ J 2016; 80: 1504–1510)

**Key Words:** Cardiac hypertrophy; Hippo signaling; Signal transduction

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**Overview of the Hippo Pathway**

The initial components of what is now recognized as the Hippo signaling pathway were identified through genetic screens in *Drosophila*. Loss of function of either of the kinases hippo (hpo) or warts (wts), or the adapter proteins salvador (sav) or mob as tumor suppressor (mats), caused a dramatic overgrowth phenotype in the fly. Subsequent work demonstrated that most components of this pathway are conserved from flies to humans, as are many of the functional outputs it dictates, including regulation of cell proliferation, survival and differentiation. The cascade is dependent upon protein-protein interactions that facilitate kinase activation, substrate availability and determine subcellular localization. The core signaling cassette is comprised of a hpo-wts-mats-sav complex (mammalian Mst1/2-Lats1/2-MOB1A/B-Sav1) that promotes phosphorylation and activation of the kinases Mst1/2 and Lats1/2. Active Lats1/2 phosphorylates and inhibits the transcription cofactors YAP/TAZ (*Drosophila yorkie*), the end effectors of canonical Hippo signaling.  

Lats1/2 phosphorylates YAP in several regions including the TEAD binding, 14-3-3 binding, WW1 and the C-terminal transcription activation domains. Phosphorylation at Ser127 promotes 14-3-3 binding and cytoplasmic retention of YAP, thereby preventing modified gene expression. Phosphorylation of Ser94 prevents YAP association with TEAD transcription factors, including TEAD family members 1–4, RUNX1/2, Erb-B4, FoxO1, Tbx5 and SMADs. Perhaps most studied to date, the YAP-TEAD interaction has been shown to modulate many of the biological functions observed downstream of active YAP, including gene expression, proliferation and transformation. 

**Regulation of Hippo Signaling**

Mechanical forces are key regulators of the Hippo effectors YAP/TAZ. Changes in substrate or extracellular matrix stiffness can alter the subcellular localization of YAP/TAZ and subsequent functional outputs. YAP activity is sensitive to actin polymerization, and disruption of F-actin negatively regulates YAP. On the other hand, RhoA activation, which mediates actin cytoskeleton rearrangement and stress fiber formation, has been shown to promote YAP activity. The α-catenin complex, which comprises adherens junctions at the cell membrane, has been shown to associate with YAP/TAZ and inhibit its nuclear localization and activity. Similarly, angiomotin-associated YAP is retracted in the cytosol and functionally inhibited. The angiomotin complex has also been shown to activate Lats2 to further prevent YAP activation. The proto-cadherin, fat, can signal through hpo to inhibit tissue growth in *Drosophila*. Interestingly, the most highly conserved fat ortholog in mammals, FAT4, does not appear to regulate Hippo signaling in higher organisms, because of evolutionary divergence.

Neurofibromin 2 (NF2, also known as merlin) has also been implicated in the regulation of Hippo/YAP in *Drosophila* and mammalian cells. Elegant work has defined a role for NF2, together with Sav1, in coordinating Mst-Lats activation at the...
YAP-Independent Hippo Function in the Heart

Soluble Factors and Receptors

The lipid-derived signaling molecules sphingosine 1-phosphate and lysophosphatidic acid, both of which are known to elicit growth and survival in multiple cell types, were the first identified soluble factors to modulate Hippo signaling. These ligands signal through their cognate G-protein coupled receptors (GPCRs) to negatively regulate the Hippo cascade and promote YAP/TAZ activation. This pathway requires RhoA and actin polymerization, and elicits inhibition of Lats1/2 kinases. Interestingly, receptors that couple strongly to Gα<sub>12/13</sub> and Gα<sub>q</sub> induce robust activation of RhoA and YAP/TAZ, whereas Gα<sub>s</sub> activation inhibits YAP/TAZ activity, potentially through a PKA-dependent mechanism.

Although mechanical stress and GPCR regulation of YAP/TAZ have been demonstrated in multiple mammalian cell types, whether or not these upstream mechanisms are functional in cardiomyocytes remains to be determined.

It is becoming increasingly clear that YAP activity is critically important in many aspects of heart biology, including

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**Table. Cardiac Phenotypes of RASSF1A and Mst1/2 Mutant Mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RASSF1A</td>
<td>KO</td>
<td>No obvious baseline abnormalities. Augmented hypertrophy and fibrosis, attenuated apoptosis, and no functional difference after TAC. Enhanced TNFα expression shown to modulate hypertrophy.</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>CKO</td>
<td>No obvious baseline abnormalities. Attenuated hypertrophy, fibrosis and apoptosis with preserved cardiac function after TAC.</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Tg</td>
<td>No obvious baseline abnormalities. Increased fibrosis and apoptosis, increased p-Mst1 and worsened function after TAC.</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>L308P Tg</td>
<td>No obvious baseline abnormalities. Attenuated fibrosis, apoptosis, and p-Mst1 with preserved cardiac function after TAC.</td>
</tr>
<tr>
<td>Mst1</td>
<td>Tg</td>
<td>Increased apoptosis, fibrosis, and LV dilation without hypertrophy at baseline. Concomitant decreased cardiac function and premature death.</td>
</tr>
<tr>
<td>Mst1</td>
<td>K59R DN-Tg</td>
<td>No obvious baseline abnormalities. Attenuated apoptosis, fibrosis, LV dilation and improved function after MI. Reduced apoptosis and smaller infarct after IR.</td>
</tr>
<tr>
<td>Mst1</td>
<td>KO</td>
<td>No obvious baseline abnormalities. Smaller infarct, improved cardiac function and enhanced autophagy after MI.</td>
</tr>
<tr>
<td>Mst2</td>
<td>KO</td>
<td>No obvious baseline abnormalities. Attenuated hypertrophy, fibrosis and apoptosis with no difference in cardiac function after TAC.</td>
</tr>
</tbody>
</table>

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CKO, cardiomyocyte-specific knockout; IR, ischemia-reperfusion; KO, knockout; MI, myocardial infarction; TAC, transverse aortic constriction; Tg, transgenic; TNF, tumor necrosis factor.
Mst1/2 kinases are downstream targets of RASSF1A. Curiously, RASSF1A promotes activation of Mst1/2 in most mammalian cells, whereas the Drosophila ortholog, dRASSF, shows antagonistic function toward hpo and sav in the fly, indicating evolutionary divergence.

In mammalian cells, RASSF1A binds Mst2 via the SARAH domain and causes the inhibitory displacement of Raf-1 to stimulate Mst2 activation. RASSF1A also binds and activates Mst1. The underlying mechanism likely involves the inhibition of protein phosphatase PP2A and preservation of activation loop phosphorylation.

Cardiovascular Functions of RASSF1A
In 2005, two independent labs generated RASSF1A knockout (KO) mice. These mice were viable, yet developed sporadic tumors with age, further establishing RASSF1A as a bona fide tumor suppressor. They have since been used to examine a wide range of pathologies including cardiovascular disease (Table). To investigate the role of RASSF1A in cardiac hypertrophy and heart failure, RASSF1A−/− mice were subjected to pressure overload via transverse aortic constriction (TAC). Compared with the wild type (WT), the RASSF1A−/− mice showed augmented cardiac hypertrophy after TAC, as determined by increased left ventricle (LV) mass, cardiomyocyte cross-sectional area and fetal gene (ANF, BNP, β-MHC) expression. Cardiac fibrosis was also significantly increased in the RASSF1A−/− mice post-TAC; however, cardiac function was indistinguishable from the WT mice.
able from that of the WT mice. Upregulation of p-ERK1/2 in RASSF1A-/- hearts was observed, and overexpression of RASSF1A in neonatal rat cardiomyocytes (NRCMs) attenuated phenylephrine-induced p-ERK1/2 and hypertrophy, providing mechanistic insights.65

Activation of a paracrine effect between cardiac fibroblasts and cardiomyocytes in RASSF1A-/- hearts has also been reported. Depletion of RASSF1A in isolated cardiac fibroblasts elicited the activation of NF-xB and the upregulation and secretion of tumor necrosis factor-a (TNFa). Compared with WT mice, levels of TNFa were elevated in non-myocytes in RASSF1A-/- hearts.64 Additional work demonstrated that TNFa present in fibroblast-conditioned medium promoted cardiomyocyte hypertrophy in culture, and was an important mediator of cardiomyocyte hypertrophy in vivo. Administration of a TNFa-neutralizing antibody at the onset of TAC was sufficient to prevent the augmented cardiomyocyte hypertrophy and cardiac fibrosis observed in RASSF1A-/- mice.65 These findings demonstrated a role for RASSF1A in modulating cardiomyocyte hypertrophic growth through both cell autonomous and non-autonomous mechanisms. Furthermore, this study identified a novel RASSF1A-mediated paracrine mechanism that modulates pathologic fibrosis during pressure overload and highlights cell-type specificity of RASSF1A signaling.

Cardiomyocyte-specific RASSF1A CKO mice (RASSF1Afl/fl; CREMHC) have also been investigated. In contrast to RASSF1A-/-, RASSF1A CKO mice showed attenuated cardiomyocyte hypertrophy in response to pressure overload, with significantly smaller hearts and reduced cardiomyocyte cross-sectional area vs. controls.61 RASSF1A CKO mice also had attenuated cardiomyocyte apoptosis and reduced cardiac fibrosis with significantly improved cardiac function compared with control mice after TAC. This distinct cardioprotective phenotype further supports the hypothesis of divergent effects of RASSF1A signaling by cell type.

To establish a gain-of-function approach, forced expression of RASSF1A in cardiomyocytes was achieved via transgenesis using the CREMHC promoter. RASSF1A-Tg mice showed augmented apoptosis and cardiac fibrosis with significantly reduced heart function following TAC.64 This was associated with upregulation of active Mst1. Interestingly, mutant Leu308Pro RASSF1A-Tg (L308P-Tg) mice, which were unable to activate Mst1, were cardioprotected against TAC.64 Overall, these findings support the deleterious role of RASSF1A in cardiomyocytes in response to pressure overload. However, they also suggest that RASSF1A could have cardioprotective functions in non-myocytes through paracrine signaling mechanisms.

Recent work has demonstrated additional signaling interactions involving RASSF1A in cardiomyocytes. In response to ischemia-reperfusion (IR) injury, RASSF1A mediates the recruitment and activation of Mst1 at mitochondria, thereby facilitating cardiomyocyte apoptosis and contributing to myocardial infarct.66 RASSF1A can also modulate cardiomyocyte contractility.67 In response to TNFa stimulation, isolated adult cardiomyocytes from WT mice positively modulated calcium transients and contraction, while cardiomyocytes from RASSF1A-/- mice had a blunted response. Pressure-volume analysis indicated a similar phenomenon in the intact mice, revealing a physiological function of RASSF1A. Mechanistically, RASSF1A associated with the TNF receptor complex and mediated the recruitment of TRAF2 and TRADD, a critical step for proper signal transduction and subsequent calcium handling.67 RASSF1A also interacts with the plasma membrane Ca2+ pump, PMCA4b.68 Identified by yeast two-hybrid and shown to colocalize and directly bind to PMCA4b, this interaction caused the inhibition of EGF-induced ERK activation in NRCMs, demonstrating additional implications for cardiomyocyte growth and survival.66 Furthermore, it appears as though RASSF1A does not engage canonical Hippo signaling in cardiomyocytes (ie, YAP modulation), and instead mediates its effects through alternative means66,68 (Figure 2).

**Mst1**

Mst1/2 (also known as Stk4/3) are mammalian orthologs of yeast sterile 20 and belong to the germinal center kinase subfamily II. Mst1 is characterized by an N-terminal catalytic domain, regulatory activation loop phosphorylation site, 2 caspase cleavage sites, and 2 putative NES sequences, followed by a C-terminal SARAH dimerization domain.69 Mst1 is activated by numerous pro-apoptotic stimuli, including staurosporin, TNFa, oxidative stress, UV radiation, retinoic acid, heat shock, and serum starvation, and is an important mediator of regulated cell death in many cell types.70 Activation of Mst1/2 is attained via proteolytic removal of the C-terminus by caspase-3 or activation loop phosphorylation.72,73

One of the first described substrates of Mst1 was Ser14 of histone H2B.74 The mitogen-activated protein kinase (MAPK) JNK (c-Jun N-terminal kinase) was also identified as an Mst1 substrate and may contribute to chromatin remodeling during apoptosis.74 Mst1 has been shown to promote neuronal cell death through direct phosphorylation of FOXO proteins.75 Phosphorylation within the forkhead domain of these transcription factors prevents inhibitory 14-3-3 binding and increases their nuclear translocation and cell death. Perhaps the most established Mst1/2 substrates are the NDR (nuclear dbf2-related) family kinases, which include NDR1/2 and Lats1/2.76 Mst1/2 directly phosphorylate Lats1/2 in the hydrophobic motif to facilitate autophosphorylation and full activation. Consequently, active Lats1/2 directly phosphorylate YAP/TAZ to propagate canonical Hippo signaling.32 Mst1/2-mediated activation of NDR1/2 promotes apoptotic death through mechanisms that require further investigation.

**Cardiovascular Functions of Mst1**

Mst1 is a mediator of cardiomyocyte apoptosis. Overexpression of Mst1 in NRCMs promotes death that utilizes caspases and mitochondria.66,78 Following oxidative stress, a subpopulation of Mst1 translocates to mitochondria, where it associates with RASSF1A and active K-Ras. Complex formation leads to Mst1 activation and the direct phosphorylation of Bcl-XL at Ser14. Phosphorylated Bcl-XL is functionally impaired and exhibits a weakened ability to bind and inhibit the pro-death molecule Bax, which triggers a loss of mitochondrial membrane integrity, caspase activation and apoptosis. Mst1 likely plays an important functional role in IR injury in the heart, a condition that generates high levels of reactive oxygen species, because Bcl-XL phosphorylation increased in human failing hearts, and overexpression of a phospho-resistant mutant Bcl-XL (Ser14Ala) showed enhanced cardioprotection compared with WT Bcl-XL.66 Similarly, inhibition of Mst1 via transgenic expression of a kinase-inactive mutant (DN-Tg-Mst1) attenuated p-Bcl-XL and infarct post-IR.66 Additional work demonstrated that knock-in of Bcl-XL (Ser14Ala) affords cardioprotection against IR injury, further evidence that Mst1-Bcl-XL signaling contributes to heart injury in vivo.79

Increased expression of Mst1 in cardiomyocytes in vivo is maladaptive and caused a profound cardiac phenotype at baseline, characterized by increased apoptosis, fibrosis, LV dilatation, a rapid decline in heart function and premature death.78 Interestingly, these hearts do not undergo hypertrophy despite enhanced cardiomyocyte loss, leading to increased wall stress.
and exacerbated injury. In a chronic MI model, DN-Tg-Mst1 mice displayed attenuated LV dilatation and improved cardiac function compared with WT mice; yet, no difference in the size of the infarct was observed. However, the extent of cardiac fibrosis and inflammation in the remodeled heart was significantly attenuated in the DN-Tg-Mst1 mice and likely contributed to improved heart function. These results imply that Mst1-mediated cardiomyocyte apoptosis may be more important in the reperfused vs. ischemic myocardium. Furthermore, it is suggested that inhibition of Mst1 in cardiomyocytes may alter crosstalk with non-myocytes to modulate inflammation and fibrosis.

Autophagy, the sequestration/degradation of cytosolic components, is a fundamental process that has implications in many pathologies, including heart disease. In particular, it is believed that protein quality control is especially important for the survival of terminally differentiated long-lived cell types (e.g., cardiomyocytes). During chronic MI, autophagy has been shown to play a cardioprotective role. In this setting, Mst1 is activated and inhibits cardiomyocyte autophagy, thereby contributing to heart injury and functional decline. Mechanistically, Mst1 directly phosphorylates Beclin1 at Thr108. This post-translational modification enhanced the association of Beclin1 with Bcl-2 and Bcl-xL, inhibiting the Atg14L-Beclin1-Vps34 complex kinase activity and subsequent autophagy. Importantly, Mst1 mice had attenuated aggresome accumulation, smaller infarcts, and improved function after MI. Mst1-mediated phosphorylation of Beclin1 not only inhibits autophagy, but also augments apoptosis by sequestering Bcl-2 and Bcl-xL from pro-apoptotics (e.g., Bax), leading to enhanced cell death. Given its importance in the stressed myocardium, further investigation into the role of Mst1 in IR and MI-induced injury using tissue-specific mouse models is warranted and should provide more insight into how Mst1 modulates cardiac pathology.

To determine novel Mst1 interaction partners, a yeast two-hybrid screen was performed using Mst1 as bait and a human heart cDNA library. An interaction between Mst1 and troponin I was identified and confirmed in cardiomyocytes. Mst1 was found to directly phosphorylate troponin I in response to oxidative stress. Evidence of troponin I conformational change following Mst1 phosphorylation was determined, and the affinity of troponin I for troponins T and C was reduced, suggesting potential functional consequences (Figure 2). These findings suggest that Mst1 may regulate cardiomyocyte contraction through direct post-translational modification of sarcomere components—an intriguing possibility; however, further investigation is needed to translate the relevance of this mechanism in vivo.

**Mst2**

Relative to Mst1, less is known regarding the function of Mst2 in the adult heart. A recent study investigated the contribution of Mst2 to pressure overload-induced hypertrophy using Mst2–/– mice. Under basal conditions, heart morphology and function were indistinguishable between WT and Mst2–/– mice, and no difference in cardiomyocyte proliferation was observed. Following TAC, cardiac hypertrophy, fibrosis and TUNEL-positive cardiomyocytes were attenuated in Mst2–/– mice compared with WT; however, no difference in function was observed. In vitro experiments demonstrated a positive effect of Mst2 on activation of ERK1/2 and cardiomyocyte enlargement, while ERK1/2 activation was blunted in the Mst2–/– mice, suggesting that Mst2 promotes cardiomyocyte growth via MAPK signaling. Whether Mst1 and Mst2 have functionally overlapping roles in the heart, or distinct substrates that mediate disparate outcomes, remains to be elucidated. Based on our current understanding, it is likely that these kinases, although highly conserved, have specialized roles in the stressed myocardium and that selectively targeting each isoform will be imperative in further investigations.

**Non-Myocyte RASSF1A-Mst1 Signaling and Cardiovascular Disease**

The mammalian heart consists of multiple cell types—cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells—that work in concert to maintain structure and function. Cardiac fibroblasts fundamentally contribute to heart homeostasis and stress responses. As discussed, RASSF1A in non-myocytes modulates remodeling. In the fibroblast, RASSF1A acts as a “brake” on NF-κB activation and TNFα production, thereby affecting cardiomyocytes through a paracrine mechanism. This has important implications for heart function, as demonstrated by the restoration of LV ejection fraction in RASSF1A–/– mice supplemented with TNFα-neutralizing antibody during hemodynamic stress. It is possible that RASSF1A in other non-myocyte cell types can influence the heart’s response to injury and warrants further study.

There is growing evidence that T-cells are important constituents in the injury and healing process that accompanies IR of the heart. The role of Mst1 in modulating T-cell biology is expanding and may strengthen our understanding of inflammation during cardiac injury, repair and remodeling. Mst1 is involved in the polarization, adhesion, chemotaxis and survival of T-cells, and human mutations in Mst1 showed reduced naïve T-cell survival and immunodeficiency. Importantly, however, it remains to be determined if modulation of T-cell survival or function by Mst1 contributes to injury or repair processes following IR or MI in the heart.

Metabolic syndrome and diabetes are established risk factors for cardiovascular disease, and may promote cardiomyopathy. The death of pancreatic β-cells plays an important role in the development of type I and type II diabetes. Mst1 was recently shown to contribute to β-cell apoptosis and the diabetic phenotype. Mst1 was activated in mouse models of diabetes and also impaired β-cell function independent of its apoptotic effect by targeting pancreatic duodenal homeobox-1 (Pdx1) for proteosomal degradation. Additionally, Mst1 mice were protected against streptozotocin- and high-fat-induced diabetes, thereby implicating Mst1 as a regulator of β-cell fate and the development of diabetes in vivo. The ability of Mst1 to confer resistance to metabolic disorder raises the possibility that therapeutic targeting of this kinase could afford decreased cardiovascular risk indirectly through β-cell maintenance.

**Conclusions**

Although much attention has focused on the role of YAP and canonical Hippo signaling in cardiac development, injury and regeneration – and rightly so – it is imperative to remember that additional targets are likely to contribute to these processes as well. Importantly, our understanding of the basic mechanisms of Hippo signaling remain incomplete, not only in cardiomyocytes but all cell types of the heart and vasculature, and it will be important to determine how canonical and non-canonical cascades influence one another during homeostasis and in response to stress. Moving forward it is also of interest to determine how temporal and spatial regulation of Hippo intermediates (eg, Mst1, RASSF1A, NF2) affects both signaling specificity and cellular outcomes. Identification of addi-
tional YAP-independent targets will continue to expand this burgeoning field and likely uncover novel mechanisms that contribute to cardiovascular health and disease.

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