NF2 Activates Hippo Signaling and Promotes Ischemia/Reperfusion Injury in the Heart

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Rationale: NF2 (neurofibromin 2) is an established tumor suppressor that promotes apoptosis and inhibits growth in a variety of cell types, yet its function in cardiomyocytes remains largely unknown.

Objective: We sought to determine the role of NF2 in cardiomyocyte apoptosis and ischemia/reperfusion (I/R) injury in the heart.

Methods and Results: We investigated the function of NF2 in isolated cardiomyocytes and mouse myocardium at baseline and in response to oxidative stress. NF2 was activated in cardiomyocytes subjected to H₂O₂ and in murine hearts subjected to I/R. Increased NF2 expression promoted the activation of Mst1 (mammalian sterile 20–like kinase 1) and the inhibition of Yap (Yes-associated protein), whereas knockdown of NF2 attenuated these responses after oxidative stress. NF2 increased the apoptosis of cardiomyocytes that appeared dependent on Mst1 activity. Mice deficient for NF2 in cardiomyocytes, NF2 cardiomyocyte-specific knockout (CKO), were protected against global I/R ex vivo and showed improved cardiac functional recovery. Moreover, NF2 cardiomyocyte-specific knockout mice were protected against I/R injury in vivo and showed the upregulation of Yap target gene expression. Mechanistically, we observed nuclear association between NF2 and its activator MYPT-1 (myosin phosphatase target subunit 1) in cardiomyocytes, and a subpopulation of stress-induced nuclear Mst1 was diminished in NF2 CKO hearts. Finally, mice deficient for both NF2 and Yap failed to show protection against I/R indicating that Yap is an important target of NF2 in the adult heart.

Conclusions: NF2 is activated by oxidative stress in cardiomyocytes and mouse myocardium and facilitates apoptosis. NF2 promotes I/R injury through the activation of Mst1 and inhibition of Yap, thereby regulating Hippo signaling in the adult heart. (Circ Res. 2016;119:596-606. DOI: 10.1161/CIRCRESAHA.116.308586.)

Key Words: apoptosis ■ cardiomyocyte ■ neurofibromin 2 ■ oxidative stress ■ reperfusion injury
signals through, effector proteins. NF2 structure is regulated by phosphorylation at Ser518, a site that can be phosphorylated by PAK, and PKA, and dephosphorylated by the myosin light chain phosphatase, MYPT-1. When Ser518 is phosphorylated, NF2 assumes an open conformation and can no longer associate with binding partners, effectively inhibiting its function. Conversely, dephosphorylation of NF2 favors a closed conformation that promotes protein interaction and signal transduction. Studies in Drosophila have provided evidence that NF2 can regulate the activity of hippo/yorkie, thereby modulating cell proliferation and survival. Yet, to date, evidence linking NF2 to mammalian cardiomyocytes is limited.

Herein, we demonstrate that NF2 is activated by oxidative stress through phosphorylation by the protein phosphatase–targeting subunit MYPT-1. Active NF2 promotes cardiomyocyte apoptosis through the activation of Mst1 and the engagement of the Hippo signaling pathway. Interestingly, NF2 is present in the cytosol and nuclei of cardiomyocytes and promotes phosphorylation and inactivation of Yap, thereby attenuating Yap target gene expression. Mice deficient for NF2 in cardiomyocytes (NF2-MHC-Cre;Mst1−/−) show diminished Mst1 activation, increased Yap transcriptional activity, and are protected against I/R injury. These results provide evidence that NF2 modulates Hippo signaling in the mammalian heart to promote acute myocardial injury.

Methods
An expanded Methods section is available in the online Data Supplement.

Animal Models
NF2-floxed mice were crossed with α-MHC (alpha myosin heavy chain)-Cre transgenic mice to generate cardiac-specific knockout (NF2 CKO) mice. Yap-floxed mice were bred to NF2 CKO mice to generate NF2CKO;Yaplox/lox;Cre+MHC mice. Mice were housed in a temperature-controlled environment with 12-hour light/dark cycles where they received food and water ad libitum. All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at Rutgers, The State University of New Jersey.

Statistics
All data are reported as mean±SEM. Evaluation between two groups. Statistical analyses were performed using Graph Pad Prism 6.0. A P<0.05 was considered significant.

Results
Regulation of NF2 by Oxidative Stress
Our previous work demonstrated that Mst1 is activated in response to oxidative stress both in cultured cardiomyocytes and mouse myocardium in vivo. Therefore, we first determined the activation status of NF2 using Ser518 phosphorylation as an indicator of the inactive conformation of NF2. Treatment of neonatal rat ventricular myocytes (NRVMs) with H2O2, to mimic oxidative stress during reperfusion, elicited the activation of both NF2 and Mst1 (Figure 1A through 1C). To test the activation of NF2 in vivo, we subjected wild-type C57BL/6 mice to I/R and performed Western blots to assay phosphorylation status. Similarly, we found that both NF2 and Mst1 were activated in the myocardium by I/R (Figure 1D and 1F).

We also evaluated the samples of failing human hearts and observed increased NF2 and Mst1 activation compared with healthy controls (Online Figure I). Because NF2 phosphorylation is decreased in response to oxidative stress, it is possible that increased phosphatase activity is responsible. To test this, we pretreated NRVMs with the phosphatase inhibitors okadaic acid or calyculin A, followed by stimulation with H2O2. The dephosphorylation of NF2 caused by H2O2 was partially attenuated in okadaic acid–treated cells at high concentration, but was fully prevented in calyculin A–treated cells indicating likely involvement of PP1 phosphatase (Online Figure II).

Previous work identified PP1-MYPT-1 as an activator of NF2 through dephosphorylation of Ser518 in mammalian cells and in Drosophila. A lack of phosphorylation of MYPT-1 at Ser696 is indicative of its activation. We observed decreased MYPT-1 phosphorylation in response to oxidative stress both in NRVMs and mouse myocardium (Figure 1G through 1J). To directly test the involvement of MYPT-1 in NF2 regulation, we depleted NRVMs of endogenous MYPT-1 using siRNA. MYPT-1 downregulation was sufficient to attenuate NF2 activation by oxidative stress (Figure 1K). Taken together, these data indicate that MYPT-1 is activated by oxidative stress and mediates the dephosphorylation and activation of NF2 in cardiomyocytes.

NF2 Promotes Mst1 Activation and Cardiomyocyte Apoptosis
NF2 can activate the Hippo pathway in the liver and brain. We sought to determine whether NF2 regulates Hippo signaling in the adult heart. Overexpression of NF2 in NRVMs stimulated Mst1 activation (Figure 2A and B). Mst1 is known to promote cell death; therefore, we evaluated cardiomyocyte apoptosis in response to NF2. We found that increased NF2 expression caused a significant increase in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)–positive cardiomyocytes and that this response was significantly attenuated by the inhibition of Mst1 (Figure 2C and 2D).

Similarly, NF2 elicited caspase-3 activation, which was significantly reduced by the inhibition of Mst1 (Figure 2E and 2F). On the contrary, knockdown of endogenous NF2 using siRNA attenuated both the activation of Mst1 and cardiomyocyte apoptosis driven by H2O2 (Figure 2G and 2H). Taken together, these results indicate that NF2 can engage Hippo signaling at the level of Mst1 to promote cardiomyocyte apoptosis.
Subcellular Localization of NF2 in Cardiomyocytes

Previous work has demonstrated subcellular localization of NF2 at tight junctions, adherens junctions, desmosomes, the plasma membrane, and in the nucleus. We sought to investigate the localization of NF2 in NRVMs. Confocal imaging revealed the distribution of NF2 in the cytosol, at the cell membrane, and a strong nuclear signal in NRVMs (Figure 3A, Online Figure IIIA). Detection of p-NF2 revealed a diffuse cytosolic and nuclear distribution with a relatively more pronounced presence at the plasma membrane (Online Figure IIIB). We also stained mouse heart sections and observed both nuclear and cytosolic localization of NF2 (Online Figure IV A). As a control, we evaluated HEK293 cells and found predominant plasma membrane distribution of NF2 and no appreciable nuclear signal (Online Figure IVC). To investigate this biochemically, we separated mouse ventricular lysates into cytosolic and nuclear-enriched fractions. We detected endogenous NF2 and p-NF2 in both fractions (Figure 3B).

Similarly, NF2 was observed in cytosolic and nuclear-enriched fractions generated from NRVMs and isolated adult mouse cardiomyocytes (Figure 3C; Online Figure IVB). Although p-NF2 was detected in cytosolic and nuclear-enriched fractions of NRVMs, we did not observe an appreciable change in p-NF2 levels in the cytosol after H2O2 treatment, whereas nuclear p-NF2 levels decreased (Figure 3C). We also probed for MYPT-1 and detected it in the nuclear-enriched, but not in the cytosolic-enriched, fractions of heart homogenates and NRVMs (Figure 3B and 3C), consistent with a recent report. Subcellular fractionation of NRVMs into cytosolic and plasma membrane-enriched fractions revealed faintly detectable levels of NF2 at the plasma membrane, whereas p-NF2 was more evident (Online Figure IVC).

NF2-Dependent Mst1 Localization

Because NF2 modified Mst1 activity, we investigated the subcellular localization of the Hippo pathway kinases large tumor suppressor kinase 2 (Lats2) and Mst1. Analysis of Lats2...
subcellular distribution in cardiomyocytes demonstrated a robust nuclear presence (Figure 3C; Online Figure IVA and IVB). Mst1 showed a predominant cytosolic presence in NRVMs, adult mouse cardiomyocytes, and heart sections; however, after oxidative stress we observed a nuclear subpopulation of Mst1 (Figure 3C; Online Figure IVB). Knockdown of either MYPT-1 or NF2 attenuated this response in NRVMs (Figure 3C). Furthermore, we observed an increase in Mst1 levels in nuclear-enriched fractions prepared from wild-type mouse myocardium subjected to I/R (Figure 3D). Nuclear localization of Mst1 was attenuated in hearts deficient for NF2, suggesting a causal role for NF2 in this process in vivo.

Association of NF2 With Hippo Pathway Components

Based on previous work demonstrating complexes comprised of NF2 and hippo in Drosophila39 and NF2 and Lats in mammalian cells,36 we examined whether NF2, Mst1, and Lats2 associate in cardiomyocytes. Co-immunoprecipitation studies showed an interaction between NF2 and Mst1 in hearts subjected to I/R (Figure 3E), and NRVMs subjected to oxidative stress (Online Figure IVA). Knockdown of either MYPT-1 or NF2 attenuated this response in NRVMs (Figure 3C). Furthermore, we observed an increase in Mst1 levels in nuclear-enriched fractions prepared from wild-type mouse myocardium subjected to I/R (Figure 3D). Nuclear localization of Mst1 was attenuated in hearts deficient for NF2, suggesting a causal role for NF2 in this process in vivo.

NF2 Deletion Attenuates Cardiac Injury Ex Vivo

To test the physiological role of NF2, we crossed NF2-floxed mice30 with α-MHC-Cre transgenic mice30 to generate cardiac-specific knockout (NF2<sup>flox/flox</sup>; Cre<sup>α-MHC</sup> (NF2 CKO)) mice. These mice had depleted levels of NF2 protein in the myocardium but not in other tissues tested (Figure 4A). Hearts were isolated from NF2 CKO and control NF2<sup>flox/flox</sup> mice and subjected to global ischemia and reperfusion ex vivo using the Langendorff preparation. We observed that NF2 CKO hearts had significantly smaller infarct regions compared with control NF2<sup>flox/flox</sup> hearts (Figure 4B and 4C). Cardiac function was assessed at baseline prior to ischemia and serially every 10 minutes through 1 hour of reperfusion. While no differences were observed at baseline, we found that NF2 CKO hearts
had significantly improved cardiac function during reperfusion (Figure 4D through 4G), whereas no significant difference in heart rate was observed (Figure 4H). Taken together, these data suggest that the disruption of NF2 in cardiomyocytes confers protection against global I/R and preserves heart function.

Deletion of NF2 Affords Cardioprotection Against I/R Injury

To determine whether the disruption of NF2 also conferred protection of the myocardium in vivo, we subjected NF2 CKO and control NF2flox/flox mice to I/R and determined infarct size 24 hours later. We found no difference in the myocardial area at risk after I/R; however, infarct size was significantly reduced in NF2 CKO mice indicating that NF2 deletion is cardioprotective (Figure 5A through 5C). We also subjected mice to I/R and examined heart function by echocardiography 2 weeks postinfarction. We observed that left ventricular ejection fraction (LVEF%) was significantly greater in NF2 CKO mice compared with controls (Figure 5D). Because NF2 regulates Hippo signaling in mouse liver and brain, we examined the activation of the conserved pathway components Mst1 and Yap. The phosphorylation of both Mst1 and Yap was increased in NF2flox/flox hearts in response to I/R, indicating Hippo pathway activation. However, this response was significantly attenuated in NF2 CKO hearts (Figure 5E through 5G). The activation of known cardioprotective targets AKT and ERK1/2 showed negligible differences between NF2flox/flox and NF2 CKO hearts (Figure 5E, 5H, and 5I). Consistent with these findings, we also observed Yap downregulation in NRVMs overexpressing NF2, and increased Yap levels in NRVMs treated with siRNA-targeting NF2 (Online Figure VI).

Yap Activation is Upregulated in NF2 CKO Myocardium

Yap is a transcriptional cofactor and prosurvival signaling molecule in cardiomyocytes; therefore, we hypothesized that altered gene expression because of Yap modulation might explain the protective effect observed in NF2 CKO mice. We first tested whether established Yap target genes were altered after Yap modification in NRVMs. Overexpression of Yap in NRVMs caused a significant increase in mRNA levels of ctgf, cyr61, fgf2, and birc5 compared with LacZ control (Online Figure VIIA). Conversely, shRNA-mediated knockdown of endogenous Yap significantly reduced Yap target gene expression (Online Figure VIIIB). To test whether NF2 affects Yap transcriptional activity in NRVMs, we used a TEAD luciferase reporter system and either overexpressed or silenced NF2, which resulted in a significant decrease and increase in reporter gene expression, respectively (Online Figure VIIIC and VIID). We also examined the expression of these Yap targets.
by quantitative reverse transcription PCR and observed a significant upregulation of the aforementioned genes in NF2 CKO hearts compared with controls (Figure 5J). Taken together, these results suggest that Yap activates a genetic program in the heart similar to that observed in the liver\(^26\) and brain,\(^27\) and indicate that Yap activity is upregulated in hearts deficient for NF2.

**Genetic Inhibition of Yap Abolishes the Protection Observed in NF2 CKO Mice**

Finally, we tested whether Yap activity is required for the protective effect of NF2 depletion in vivo. NF2 CKO, Yap\(^{flox/+};CRE\(^{MHC}\)\(^{−}\), NF2-Yap double deficient (NF2\(^{flox/flox};Yap^{flox/+};CRE\(^{MHC}\)\(^{−}\)), and control (NF2\(^{flox/flox};Yap^{flox/+}\)) mice were generated. The expected reduction in NF2 and Yap protein was confirmed by Western blot (Figure 6A), and these mice were subjected to I/R. Consistent with our findings above, the NF2 CKO mice had significantly smaller infarcts and reduced TUNEL\(^+\) staining compared with control mice (Figure 6B, 6D, and 6E). There were no differences in area at risk/left ventricle between the groups (Figure 6C). Interestingly, we observed a significant increase in infarct size in the Yap\(^{flox/+};CRE\(^{MHC}\)\(^{−}\), and NF2\(^{flox/flox};Yap^{flox/+};CRE\(^{MHC}\)\(^{−}\) myocardium versus controls (Figure 6B and 6D). Furthermore, we observed a significant increase in
TUNEL+ cardiomyocyte nuclei in Yap^{floxed};Cre^{αMHC} hearts compared with controls as well as in NF2^{floxed};Yap^{floxed};Cre^{αMHC} hearts compared with NF2 CKO hearts after I/R (Figure 6E and 6F). Taken together, these results indicate a loss of cardio-protection in the NF2^{floxed};Yap^{floxed};Cre^{αMHC} mice that is likely because of normalization of Yap activity.

**Discussion**

NF2 is a recognized tumor suppressor that has been shown to promote Hippo signaling in *Drosophila*. Subsequent work has demonstrated that NF2 can modulate mammalian Hippo signaling in the murine liver and brain. In a recent report from Marian and colleagues, NF2 was shown to be activated in a mouse model of arrhythmogenic cardiomyopathy and in heart samples from arrhythmogenic cardiomyopathy patients. Murine models of arrhythmogenic cardiomyopathy displayed increased activation of Hippo signaling and reduced Yap activation, which was shown to contribute to the observed increase in adipogenesis. However, prior to our study, a loss-of-function mouse had not been used to test whether NF2 regulates Hippo signaling and contributes to myocardial injury caused by I/R. We demonstrate here that NF2 is activated in cardiomyocytes and mouse myocardium in response to oxidative stress and I/R, and contributes to cardiac injury through the engagement of the Hippo pathway and subsequent inactivation of the transcriptional cofactor Yap.

Regulation of NF2 through multiple post-translational modifications has been demonstrated previously. NF2 is negatively regulated via Ser518 phosphorylation by PKA and PAK. Alternatively, dephosphorylation of Ser518 mediated by MYPT-1 activates NF2. NF2 function can also be modulated through Akt-mediated phosphorylation at Thr230 and Ser315. More recent work has demonstrated that NF2 is also a substrate of sumoylation. It was reported that SUMO-1 modification at Lys76 led to decreased active conformation status, altered subcellular localization, and impaired tumor suppressor function suggesting that sumoylation is important for proper NF2 signaling.
Whether NF2 is sumoylated in cardiomyocytes, and if this modification modulates NF2 activity in the heart, remains to be determined.

The subcellular localization of NF2 has been investigated in multiple cell types with varied observations. Recent findings from the Pan laboratory demonstrated that plasma membrane association of NF2 and direct binding with Lats1/2 is critical for engagement of Hippo signaling and subsequent inactivation of Yap. In the other work, Li et al have demonstrated that NF2 can shuttle between the cytosol and nucleus, an observation that may be mediated by a nuclear localization motif present in the N-terminal portion of NF2. It is likely that observed differences in subcellular localization of NF2 are due to cell-type specificity and that NF2 has important cellular functions in multiple locations. In NRVMs, adult mouse cardiomyocytes, and mouse heart sections, we observed NF2 in the cytosol and nucleus, and in relatively lower levels in the plasma membrane, using both biochemical fraction enrichment and immunofluorescence-based approaches. In contrast, p-NF2 was detected in cytosolic and nuclear fractions, with relative enrichment at the plasma membrane. Importantly, nuclear p-NF2 levels decreased after oxidative stress indicating that this is an important site of NF2 activation. We also observed a subpopulation of Mst1 in nuclear-enriched fractions of stressed cardiomyocytes and hearts, and Lats2 appeared almost exclusively nuclear, similar to our prior work. The arrhythmogenic cardiomyopathy study mentioned above also examined the distribution of Hippo pathway components by immunostaining heart sections. In mouse myocardium, p-NF2 was reported to localize to cardiomyocyte desmosomes. This is not inconsistent with our current findings, which demonstrate p-NF2 at the plasma membrane. It should be noted that this prior study did not observe the localization of p-Mst1 in the nucleus. It is possible that this apparent discrepancy concerning Mst1 is due to the type of stress imposed or the potential for heterogeneity between cardiomyocytes in the heart. Indeed, our NF2 staining results suggest that nuclear NF2 may be more pronounced in a select subset of cardiomyocytes. We think it is likely that NF2 serves important physiological functions in multiple subcellular regions. Based on our localization and immunoprecipitation results, we propose that the nucleus is one of perhaps several important focal points of NF2-Hippo signaling in the cardiomyocyte.

Our prior work demonstrated a deleterious role for Mst1 during I/R. These studies interrogated both upstream regulation and downstream effectors of Mst1 in this setting. We identified a K-Ras/RASSF1A/Mst1 complex present at mitochondria that mediated the activation of Mst1 at this subcellular locale. We also found that active Mst1 phosphorylates Bcl-xL, thereby attenuating Bcl-xL-Bax interaction and increasing Bax activation and cardiomyocyte apoptosis. Importantly, we demonstrated that this noncanonical Hippo signaling (ie, not through Lats2/Yap) contributes to I/R injury. Our current findings extend this work by demonstrating that NF2 is a regulator of canonical Hippo signaling in the myocardium and implicate the nucleus as a target organelle in which this signaling occurs. Based on our findings, we hypothesize that NF2 and K-Ras/RASSF1A function to activate Mst1 in separate subcellular locations (mitochondria versus nuclei) leading to the engagement of different downstream effectors (Bcl-xL versus Lats2/Yap). Mst1 has also been shown to inhibit cardiomyocyte autophagy and contribute to ischemic injury. Although we found that NF2 promotes Mst1 activation, we did not observe a significant effect of NF2 on

**Figure 6.** Hemizygous deletion of myocardial Yap abrogates the cardioprotection observed in NF2 CKO hearts after I/R. Control (NF2flox/flox;Yapflox/+), NF2 CKO (NF2flox/flox;CreαMHC), Yapflox/+;CreαMHC, and NF2-Yap double deficient mice (NF2flox/flox;Yapflox/+;CreαMHC) were generated. A, Ventricular homogenates from all groups were subjected to western analysis to determine levels of NF2 and Yap protein. B–D, All groups were subjected to I/R (30'/24hr) and infarct size and area at risk (AAR) determined by TTC and Alcian blue staining respectively. Scale bar, 1 mm. E, Representative images from TUNEL staining of heart sections after I/R (30'/24hr) are shown. Sections were counterstained with DAPI (blue) and troponin-T (green) to identify nuclei and cardiomyocytes, respectively (Merged). Scale bar, 50 μm. F, Average data as determined by TUNEL. *, P<0.05. N.S. indicates not significant. n=5 to 8 mice per group.
cardiomyocyte autophagy (Online Figure VIII), although further study is needed to examine this in greater detail. Taken together, we propose that the inhibition of Mst1 likely confers cardioprotection by preventing multiple signaling mechanisms and would be advantageous versus targeting a single pathway in isolation.

Mst1 contains a regulatory phosphorylation site, Thr183, in its N-terminal catalytic domain. Autophosphorylation of Thr183 has been shown to be important for Mst1 activation and homodimerization, the latter mediated by its C-terminal SARAH domain. The SARAH domain also allows for heterodimerization between Mst1 and RASSF1A, NORE1 and Salvador, known activators of the kinase. Structural studies of Mst1 demonstrated the importance of h1 and h2 helices for homodimerization of Mst1 monomers; however, the precise molecular mechanism of Mst1 activation remains unclear and it is possible that additional kinases may play an important role. We observed an association between NF2 and MYPT-1, Mst1 and Lats2, and increased phosphorylation of Mst1 after oxidative stress. Our results lead us to speculate the formation of a nuclear complex that could include additional components (eg, Salvador, MOB1A/B, MAP4K/Happyhour). We hypothesize that the recruitment of Mst1 to this complex may be facilitated by Lats2, as a direct interaction between NF2 and Lats2 has been demonstrated previously.

Based on the literature regarding the function of Yap in the mammalian heart, it is not entirely surprising that ablation of a negative regulator of Yap activity, in this case NF2, would prove cardioprotective against I/R injury. Our previous work demonstrated that the inhibition of Lats2 in the myocardium afforded protection against I/R injury through increased Yap-FoxO1 transcriptional activation. Through loss-of-function studies, Yap has been shown to be critical for adult heart homeostasis and protection against cardiomyocyte apoptosis, as well as a promoter of cardiomyocyte growth and proliferation. Conversely, strategies to increase Yap expression and activity stimulate proliferation of cardiomyocytes and cardioprotection against myocardial infarction and resection of the neonatal mouse heart. Our current study demonstrates that the cardioprotection observed in NF2 CKO mice is abrogated by Yap heterozygosity in cardiomyocytes. We think that this is compelling evidence that Yap functions downstream of NF2; however, we cannot exclude the possibility that Yap acts in a parallel pathway that supersedes the NF2 effect in this setting.

Our results indicate that NF2 is activated by oxidative stress and has a presence in the nuclei of cardiomyocytes where it promotes the activation of Mst1 and inhibitory phosphorylation of Yap (Figure 7). This work further supports the notion that increased Yap activity during acute stress/injury serves to protect the myocardium and highlights Yap as an attractive target for potential future therapies against myocardial infarction.

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Disclosures

None.

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MATERIALS AND METHODS

Animal models. NF2 floxed mice\(^1\) were crossed with \(\alpha\)-MHC-Cre transgenic mice\(^2\) to generate cardiac-specific knockout (NF2 CKO) mice. Yap floxed mice\(^3\) were bred to NF2 CKO mice to generate \(NF2^{\text{flox/flox}},Yap^{\text{flox/+}},\alpha\text{-MHC}^{\text{Cre}}\) mice. Mice were housed in a temperature-controlled environment with 12-hour light/dark cycles where they received food and water \textit{ad libitum}. All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at Rutgers, The State University of New Jersey.

Ischemia/reperfusion injury. Prior to anesthesia, cephazolin (60 mg/kg) was administered (intraperitoneal injection) to prevent infection. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Once anesthetized, mice were intubated and ventilated with a tidal volume of 0.2ml and a respiratory rate of 110 breaths per minute using 65% oxygen (rodent ventilator model 683; Harvard Apparatus Inc.). The animals were kept warm with heat lamps. Rectal temperature was monitored and maintained between 36°C and 37°C. The heart was exposed by a thoracotomy through the 4\(^\text{th}\) and 5\(^\text{th}\) ribs. The left coronary artery was located and a suture was passed under the artery. To occlude the artery, a short length of tubing was threaded through the suture ends and occlusion was effected by placing tension on the suture such that the tube compressed the artery. Ischemia was confirmed by ECG change (ST elevation). After occlusion for 30 minutes, the silicon tubing was removed to achieve reperfusion, and the rib space and overlying muscles were closed in layers using 5.0 nylon sutures. For sham
operation, the same protocol was followed; however, no ligation of the coronary artery was performed. Postoperatively, mice were administered Buprenex-SR (1.2 mg/kg) subcutaneously for analgesia. Mice were then allowed to recover under close monitoring in an incubator. During this time, mice were observed for signs of post-operative complications including pain, pneumothorax, and acute heart failure or sudden death. Animals were housed in the Animal Facility in a climate-controlled environment.

**Measurement of infarct size.** Twenty-four hours after reperfusion, mice were reanesthetized and intubated, and the chest was opened. After the heart was arrested at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The left anterior descending coronary artery was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross sections, and incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 minutes. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured with the use of Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages.

**Langendorff perfusion model.** Mice were anesthetized with pentobarbital (65 mg/kg, i.p.) and treated intraperitoneally with 50 units of heparin. The heart was quickly removed and catheterized with a 22-gauge needle. The hearts were mounted on a Langendorff-type isolated heart perfusion system and subjected to retrograde coronary
artery reperfusion with 37°C oxygenated Krebs-Henseleit bicarbonate buffer (NaCl 120 mmol/L, Glucose 17 mmol/L, NaHCO₃ 25 mmol/L, KCl 5.9 mmol/L, MgCl₂ 1.2 mmol/L, CaCl₂ 2.5 mmol/L, EDTA 0.5 mmol/L), pH 7.4, at a constant pressure of 80 mmHg. A balloon filled with water was introduced into the left ventricle (LV) through the mitral valve orifice and connected to a pressure transducer via a plastic tube primed with water. LV pressures and LV dP/dt were recorded with a strip chart recorder (Astro-Med, Inc). The LV end-diastolic pressure was set at 4-10 mmHg at the beginning of perfusion by adjusting the volume of the balloon in the LV and the volume was kept constant throughout an experiment. After a 30 min equilibration period, the heart was subjected to 30 min of global ischemia (at 37°C) followed by 60 min of reperfusion.

**Echocardiography**, Mice were anesthetized using 12 µl/g body weight of 2.5% tribromoethanol (Avertin, Sigma), and echocardiography was performed as described previously⁵, using a 13-MHz linear ultrasound transducer. Two-dimensional guided M-mode measurements of LV internal diameter were obtained from at least three beats and then averaged. LV end-diastolic dimension (LVEDD) was measured at the time of the apparent maximal LV diastolic dimension, and LV end-systolic dimension (LVESD) was measured at the time of the most anterior systolic excursion of the posterior wall. LVEF was calculated using the following formula:

\[
LVEF (%) = 100 \times \frac{(LVEDD^3 - LVESD^3)}{LVEDD^3}
\]

**Cell culture.** Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl: (WI)BR-Wistar rats and maintained in culture as described previously⁴. The phosphatase inhibitors okadaic acid and calyculin A were purchased from Calbiochem (EMD). Hydrogen peroxide was purchased from Sigma.
Adult mouse cardiomyocyte isolation. Myocytes were enzymatically isolated from mouse hearts as previously described. Briefly, the hearts were removed from mice anesthetized with pentobarbital (65 mg/kg, i.p.), and were retrograde perfused at 37°C using a Langendorff-type apparatus with nominally Ca\(^{2+}\)-free Tyrode’s solution containing 0.5 mg/ml collagenase (Type II; Worthington) and 0.1 mg/ml protease (type XIV, Sigma) for 13-15 minutes. The enzyme solution was then washed out and the hearts were removed from the perfusion apparatus. Ventricles were placed in a petri dish, and gently teased apart with forceps. Finally, the myocytes were filtered through nylon mesh (200 µm) and used immediately for biochemical assays.

Adenoviral constructs. Recombinant adenovirus vectors were constructed as described previously. Briefly, pBHGlloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC316 shuttle vector containing the gene of interest into HEK293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring 3x FLAG-tag. Wild-type NF2 and S518A mutant NF2 were subcloned from Addgene plasmids #19699 and #19700 respectively. Plasmids were sequenced to confirm fidelity. Adenoviruses harboring Mst1, Dn-Mst1(K59R), 3x FLAG-Yap and sh-Yap were generated as described previously. Adenovirus harboring beta-galactosidase (LacZ) was used as a control.

siRNA knockdown. siRNA-mediated knockdown of endogenous NF2 was performed in neonatal rat cardiomyocytes. Cells were transfected with Lipofectamine 2000 Transfection Reagent (Life Technologies) using pre-designed pooled siRNAs (IDT) diluted in OPTIMEM (Gibco). The NF2 targeted siRNA duplexes used were: siNF2-1 sense (5’-GAACCAGUUACCUUCAUUCCUGG-3’) and antisense (5-
CCAGGAAUGAAAGGUAAUCUGGUUCUU-3'); siNF2-10 sense (5’-GGGAGACAGCCUUGGACAUCCUACA-3’) and antisense (5’-UGUAGGAUGUCCAAGGCUGUCUCCCUC-3’); the MYPT-1 targeted duplexes used were: RNC.RNAI.N053890.12.2, RNC.RNAI.N053890.12.6 and RNC.RNAI.N053890.12.10; and siRNA CTRL (NC1, Negative Control Sequence).

Subcellular fractionation. Isolated mouse ventricles or cardiomyocytes were homogenized as described previously\(^\text{10}\). Briefly, homogenates were prepared in ice-cold Buffer A [10mmol/L HEPES pH 7.6, 10mmol/L NaCl, 1.5 mmol/L MgCl\(_2\), 10% glycerol, 0.1% NP40-alternative, 0.1 mmol/L Na\(_3\)VO\(_4\), 5 µg/ml aprotinin, and 5 µg/ml leupeptin] on ice for 15 min. Samples were centrifuged at 13000 × g for 5 min at 4°C and supernatants were collected as cytosolic fractions. The pellets were resuspended in Buffer B [20mmol/L Tris pH 7.6, 3 mmol/L EDTA, 3 mmol/L EGTA, 250 mmol/L NaCl, 1% NP-40 alternative, 0.1 mmol/L Na\(_3\)VO\(_4\), 20 mmol/L β-glycerophosphate, 5 µg/ml aprotinin, and 5 µg/ml leupeptin] and incubated on ice for 10 min. Samples were centrifuged at 2700 × g for 5 min at 4°C, and supernatants were discarded. Pellets were resuspended in RIPA buffer, incubated on ice for 10 min and centrifuged at 13000 × g for 5 min at 4°C. The resultant supernatant was collected as the nuclear fraction.

Plasma membrane isolation. Cell fractions enriched for plasma membrane were prepared using the plasma membrane protein extraction kit from Abcam according to manufacturer’s instructions.

Immunoprecipitation and immunoblotting. For immunoprecipitation assays, left ventricular homogenates or cardiomyocyte extracts were prepared in lysis buffer containing 50 mmol/L Tris·HCl (pH 7.5), 150 mmol/L NaCl, 0.5% IGEPAL CA-630,
0.1% SDS, 0.5% deoxycholic acid, 1 mmol/L EDTA, 0.1 mmol/L Na$_2$VO$_4$, 1 mmol/L NaF, 50 µmol/L phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Samples were incubated with primary antibody overnight at 4°C, and immunocomplexes were precipitated following 1 hour of incubation with sepharose A/G beads (Santa Cruz). For immunoblot analysis, the antibodies used were NF2 (Santa Cruz), p-NF2 (Ser518) (Cell Signaling), Mst1 (BD Transduction Labs), p-Mst1/2(Thr183/180) (Cell Signaling), MYPT-1 (Cell Signaling), p-MYPT-1 (Cell Signaling), Lats2 (Bethyl), tubulin (Sigma), GAPDH (Cell Signaling), ERK1/2 (Cell Signaling), phospho-ERK1/2 (Cell Signaling), AKT (Cell Signaling), p-AKT(Ser473) (Cell Signaling), caspase-3 (Cell Signaling), histone H3 (Cell Signaling), Lamin A/C (Cell Signaling), RhoGDI (Santa Cruz), LC3 (BML), p62 (ORIGENE), N-Cadherin (BD Transduction Labs), DYKDDDDK (Cell Signaling), Yap (Cell Signaling), and p-Yap (Ser127)(Cell Signaling). Densitometry was performed using ImageJ software.

**Immunostaining.** Cardiomyocytes or HEK293 cells were plated on gelatin-coated glass coverslips. Cells were fixed in PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.3% Triton-X, and blocked with 5% normal goat serum. Immunostaining was performed using anti-NF2 rabbit polyclonal antibody (Santa Cruz), anti-p-NF2 (Ser518) rabbit polyclonal antibody (Cell Signaling), anti-Troponin-T mouse monoclonal antibody (Thermo Scientific), Alexa-fluor 488 goat anti-rabbit IgG (Molecular Probes), Alexa-fluor 568 goat anti-mouse IgG (Molecular Probes) and Vectashield mounting medium with DAPI (Vector Laboratories). Mouse left ventricles were fixed in formalin and sectioned at 6-µm thickness. Tissue sections were then subjected to deparaffinization and antigen unmasking using citrate buffer and washed.
with PBS containing 0.3% Triton X-100. Samples were blocked with 5% BSA and incubated with primary antibody overnight and with Alexa 488- and Alexa Fluor 568-conjugated secondary antibodies (Molecular Probes) at room temperature. Primary antibodies used were anti-NF2 rabbit polyclonal antibody (Santa Cruz), anti-Mst1 rabbit polyclonal antibody (Cell Signaling), anti-Lats2 rabbit polyclonal antibody (Bethyl) and anti-Troponin-T mouse monoclonal antibody (Thermo Scientific). Nuclei were stained with DAPI. Imaging was performed using a Nikon A1R confocal or conventional fluorescence microscope.

**Evaluation of apoptosis.** DNA fragmentation was detected in cardiomyocytes using TUNEL (Roche) as described previously. Nuclear density was determined by counting DAPI-stained nuclei in 20 different fields for each sample. For detection of TUNEL+ nuclei in vivo, heart sections were counterstained with Troponin-T to identify cardiomyocytes. TUNEL+ nuclei were assessed in the border zone region of the infarcted myocardium. For each heart, 20 different fields were chosen at random in this region.

**Transfection and luciferase assays.** Cardiomyocytes were transfected using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s instructions. A TEAD luciferase reporter gene (8xGTIIC-luciferase; Addgene plasmid #34615) was used to assess Yap activity. After transfection, cells were lysed with Passive Lysis Buffer (Promega), and transcriptional activity was measured using the luciferase assay system (Promega) with an OPTOCOMP I luminometer (MGM instruments).

**qRT-PCR.** RNA was isolated, cDNA was generated, and quantitative real-time PCR performed as described previously. Primers used to detect rat transcripts were: *Ctgf* (sense) 5’ gagtgtctctgctgatgc, (antisense) 5’ cacagaaacttagccggt, *Cyr61* (sense) 5’
Glucose deprivation. To mimic a starvation condition and elicit autophagy, cardiomyocytes were washed three times with PBS and incubated in glucose-free serum-free DMEM (Invitrogen) at 37 °C as described\textsuperscript{11}.

Human heart samples. The samples from explanted hearts used in this study were obtained from 4 patients who had received heart transplants and 4 age-matched donors at the Taipei Veterans General Hospital. The study was approved by the Institutional Ethics Committee (VGHIRB No.:2012-06-028D), and all patients or their family expressed their willingness to participate through an informed consent form. Myocardial posterior wall samples were collected during preparation of donor hearts for transplantation in the hospital. Myocardial samples from near the mitral annulus were obtained from recipients at the time of therapeutic transplantation. Immediately after tissue procurement, the samples were stored in liquid nitrogen and kept at -80°C.
Statistics. All data are reported as mean ± SEM. Evaluation between three or more groups was done using one-way ANOVA. The statistical significance of the differences between groups was calculated using post hoc comparisons. Student’s $t$ test was used to evaluate the difference in means between two groups. Statistical analyses were performed using Graph Pad Prism 5.0. A $p$ value less than 0.05 was considered significant. The sample size needed to detect changes obtained through preliminary experiments was calculated with Power and Sample Size Analysis (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize), using 80% power and 5% significance level. All mouse studies including assessment of infarct size, echocardiography, TUNEL analysis and hemodynamic measurements, were performed blinded to genotype.
References


proarrhythmic substrate to the heart. *Am J Physiol Heart Circ Physiol.*
2015;308:H240-249


FIGURE LEGENDS

Online Figure I. NF2 activation status during human heart failure. (A) Patient samples were obtained from donor (healthy) and recipient (failing) hearts and subjected to western blot analysis to determine levels of activated Mst1 and NF2. (B and C) Quantification of results shown in A. *, P<0.05. N=4.

Online Figure II. Phosphatase inhibition modulates NF2 phosphorylation in cardiomyocytes. (A) NRVMs were pretreated with vehicle, okadaic acid (OA; 100 or 500 nM) or calyculin A (CalA; 30 or 100 nM) for 1 hour. Cells were then stimulated with H$_2$O$_2$ (100 µM) or vehicle for 1 hour. Westerns were performed to detect phosphorylated and total NF2. (B) Quantification of results in panel A. Vehicle control and H$_2$O$_2$-treated samples with higher inhibitor doses are shown. *, P<0.05. N = 3.

Online Figure III. NF2 localization, as determined by immunostaining, in neonatal rat cardiomyocytes and HEK293 cells. (A and B) NRVMs were treated with control (siCT) or NF2-targeted (siNF2) siRNA. After 72 hours, cells were fixed, permeabilized and stained for endogenous NF2 or p-NF2 Ser518 (green), troponin T (TnT; red) and DAPI (blue). Scale bar, 30 µm. (C) HEK293 cells were fixed, permeabilized and stained for endogenous NF2 (green) and nuclei were visualized with DAPI (blue). Scale bar, 10 µm.

Online Figure IV. NF2, Mst1 and Lats2 localization in primary cardiomyocytes and adult mouse hearts. (A) Wild-type C57BL/6 mouse hearts were fixed, sectioned and
stained to detect endogenous proteins. Nuclei were visualized with DAPI (blue) and cardiomyocytes identified by troponin-T (TnT; red). Scale bar, 50 µm. Images from $NF2^{+/+}$ and $NF2$ CKO hearts at right. (B) Adult mouse cardiomyocytes were isolated from wild-type C57BL/6 mice and subjected to biochemical fractionation to produce cytosolic (cyto) and nuclear (nuc)-enriched fractions. Western blot was performed using these fractions along with unfractionated cardiomyocyte homogenate (Input). (C) NRVMs were treated with $H_2O_2$ (100 uM) or vehicle for 30 minutes, then harvested for subcellular fractionation into cytosolic (cyto) and plasma membrane (PM)-enriched fractions using the plasma membrane extraction kit (Abcam). Western analysis was performed and GAPDH and N-cadherin were used as cytosolic and plasma membrane markers, respectively.

**Online Figure V. Association of NF2 and Mst1 in neonatal rat cardiomyocytes.** (A) NRVMs were treated with $H_2O_2$ (100 uM) or vehicle for 30 minutes and collected for immunoprecipitation using either anti-Mst1 or anti-NF2 primary antibodies or the appropriate control IgG. Captured complexes were subjected to SDS-PAGE and probed for Mst1 and NF2. (B) NRVMs were transduced with control LacZ, wild-type NF2 or mutant NF2 (S518A; SA) adenovirus. Cell lysates were subjected to immunoprecipitation using anti-FLAG antibody or IgG control. Immunocomplexes were resolved by SDS-PAGE.

**Online Figure VI. NF2-associated signaling in cardiomyocytes.** (A) NRVMs were transduced with NF2 or LacZ control adenovirus and western blotting performed to
determine downstream signaling. (B) NRVMs were transfected with siRNA targeted to NF2 (siNF2) or scrambled control (siCTRL). 72 hours later cells were treated with H2O2 (100 µM) or vehicle for 30 minutes prior to assay.

**Online Figure VII. NF2 regulates Yap/TEAD transcription in cardiomyocytes.** (A and B) NRVMs were transduced with Yap, LacZ, short hairpin (sh)-Yap or sh-CTRL adenovirus to overexpress or knockdown endogenous Yap, respectively. qRT-PCR was performed to determine target gene expression. (C and D) NRVMs were transfected with TEAD-luciferase reporter and NF2 overexpression or knockdown to determine TEAD transcriptional activity. *, P<0.05 vs. control. N = 3-4 experiments.

**Online Figure VIII. NF2 and conventional cardiomyocyte autophagy.** (A) NRVMs were transduced with LacZ or NF2 adenovirus. 48 hours later, cells were challenged with glucose deprivation for 4 hours prior to harvest and SDS-PAGE. N = 3 independent experiments. (B) Control or NF2 CKO mice were subjected to sham operation or I/R (30'/2 hr). Hearts were excised and analyzed by SDS-PAGE for markers of autophagy. N = 3 hearts/group.
Online Figure I

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B

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C

\[\text{NF2} \quad \text{DAPI} \quad \text{Merge}\]
Online Figure IV

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MYPT-1
NF2
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Online Figure V

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$H_2O_2$

Mst1

NF2 (short exposure)

NF2 (long exposure)

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Mst1

Lats2

FLAG-NF2
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H2O2 + +
Online Figure VIII

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