H-Ras Isoform Mediates Protection Against Pressure Overload–Induced Cardiac Dysfunction in Part Through Activation of AKT

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Background—In general, Ras proteins are thought to promote cardiac hypertrophy, an important risk factor for cardiovascular disease and heart failure. However, the contribution of different Ras isoforms has not been investigated. The objective of this study was to define the role of H- and K-Ras in modulating stress-induced myocardial hypertrophy and failure.

Methods and Results—We used H- and K-Ras gene knockout mice and subjected them to pressure overload to induce cardiac hypertrophy and dysfunction. We observed a worsened cardiac phenotype in Hras−/− mice, while outcomes were improved in Kras−/− mice. We also used a neonatal rat cardiomyocyte culture system to elucidate the mechanisms underlying these observations. Our findings demonstrate that H-Ras, but not K-Ras, promotes cardiomyocyte hypertrophy both in vivo and in vitro. This response was mediated in part through the phosphoinositide 3-kinase-AKT signaling pathway. Adeno-associated virus–mediated increase in AKT activation improved the cardiac function in pressure overloaded Hras null hearts in vivo. These findings further support engagement of the phosphoinositide 3-kinase-AKT signaling axis by H-Ras.

Conclusions—Taken together, these findings indicate that H- and K-Ras have divergent effects on cardiac hypertrophy and heart failure in response to pressure overload stress. (Circ Heart Fail. 2017;10:e003658. DOI: 10.1161/CIRCHEARTFAILURE.116.003658.)

Key Words: cardiomyocytes ■ heart failure ■ hypertension ■ hypertrophy ■ Ras proteins

The Ras family of small GTPases consists of ubiquitously expressed signal transducers that relay extracellular cues inside the cell, thereby, regulating a host of signaling pathways and cellular responses.1 Activation of Ras proteins is modulated through engagement of transmembrane receptors, both receptor tyrosine kinases and G-protein–coupled receptors, and through mechanical forces, that is, cell stretch/strain. These signals lead to guanine nucleotide exchange factor activation and Ras GTP loading. GTP-bound Ras is active and is able to bind to and signal through downstream effectors, the most established being Raf,2–3 phosphoinositide 3-kinase (PI3K),4 RaIGDS,5,7 PLCe10 and Tiam1.11 Signal pathways elicited via Ras can modulate a variety of responses, including gene expression, growth, survival, proliferation, endocytosis, and cell motility.

See Clinical Perspective

Ras proteins are highly relevant to human disease, and myriad studies have demonstrated Ras mutations in many types of cancers.12 Human germ line mutations in Ras proteins, and known modulators of Ras signaling pathways, have been linked to the developmental disorders Neurofibromatosis type 1, cardio-facio-cutaneous,13,14 Noonan,15–17 Costello,18 and LEOPARD syndromes. Collectively referred to as RASopathies, these genetic disorders share dysregulation of Ras/mitogen-activated protein kinase (MAPK) signaling and phenotypic overlap, including craniofacial abnormalities, cardiac malformations, impaired cognitive ability, and increased cancer risk. Specifically, these patients present with cardiovascular abnormalities, including hypertrophic cardiomyopathy, atrial-septal defects, pulmonic stenosis, and tachycardia. Although the nature of these cardiac defects is heterogeneous and the cause of these differences remains unclear, these syndromes provide strong evidence of the importance of Ras proteins in human myocardial pathophysiology.

To date, studies investigating the role of Ras signaling in a cardiac context have almost exclusively focused on the H-Ras isoform. Early work demonstrated that agonists and interventions that promote cardiomyocyte hypertrophy, characterized by increased cell size and activation of embryonic gene expression, also elicit activation of Ras.19,20 This growth response was demonstrated to require Ras activity, and later, the expression of activated H-Ras was shown to be sufficient to promote cardiomyocyte hypertrophy.19,21,22 Myocardial expression of activated H-Ras12V driven by the myosin light chain 2v promoter was shown to cause increased left ventricular (LV) mass, hypertrophic gene expression, and functional decompensation.
in transgenic animals, further supporting the hypothesis that H-Ras promotes cardiomyocyte hypertrophy, as well as suggesting a maladaptive response to increased cardiac H-Ras activity. Additional studies suggested that this response was reversible and may involve altered sarcoplasmic reticulum Ca2+-ATPase 2a (SERCA2a) function and calcium handling.

The objective of the current study was to determine the role of endogenous H- and K-Ras in pressure overload–induced cardiac hypertrophy and heart failure. We used genetic loss-of-function mouse models and cultured neonatal rat cardiomyocytes (NRCMs) to interrogate each isoform in vivo and in vitro. Our findings indicate that H-Ras promotes cardiomyocyte hypertrophy and is cardioprotective during chronic pressure overload, whereas K-Ras does not promote growth and serves a deleterious function. We observed markedly less phosphorylated AKT and extracellular signal-regulated kinase (ERK) in pressure-overloaded Hras null hearts. Inhibition of AKT activation attenuated H-Ras-induced cardiomyocyte hypertrophy in vitro, and restoration of AKT signaling in vivo was able to rescue cardiac function in pressure-overloaded Hras null hearts. These results suggest that endogenous H-Ras mediates hemodynamic stress-induced cardiac hypertrophy and affords a cardioprotective function in the murine heart in part through activation of AKT.

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

Animals
Kras+/− and Hras+/− mice have been described previously. All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at Rutgers, New Jersey Medical School.

Transverse Aortic Constriction
Mice were anesthetized and pressure overload induced by ligation of the transverse thoracic aorta. Sham operation was performed without aortic constriction.

Histology
Hearts were analyzed for interstitial fibrosis using Masson’s Trichrome, myocyte cross-sectional area (CSA) using wheat germ agglutinin, and apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

RBD Pull-Down Assay
Homogenates were incubated with Raf-1 RBD agarose (Upstate, Millipore) for 40 minutes at 4°C to precipitate GTP-bound Ras according to manufacturer’s instructions.

Statistical Analysis
All data are reported as mean±standard error of the mean. Student’s t test was used to evaluate the difference in means between 2 groups. Multiple groups were analyzed using Levene’s test to determine heterogeneity of variances, followed by Welch’s analysis of variance. Post hoc multiple comparisons were performed using Tukey’s test. Statistical analyses were performed using SPSS v24 and Graph Pad Prism 6.0. A P value <0.05 was considered significant.

Results
To determine the role of endogenous Ras isoforms on basal cardiac structure and function, we used H- and K-Ras loss-of-function mouse lines. Homozygous deletion of Kras is embryonic lethal; therefore, we used Kras+/− mice for this study. Kras+/− mice are viable and showed no obvious cardiac abnormalities in heart size, structure, and function compared with WT controls at baseline (10–12 weeks; Figure 1A through 1D). Interestingly, homozygous disruption of Hras is tolerated, and mice are born at expected Mendelian ratios. However, by 10 to 12 weeks of age, Hras+/− mice developed a mild but significant reduction in cardiac function, as determined by echocardiographic analysis (percent LV ejection fraction [%LVEF]; Figure 1E through 1H). Hras+/− mice also had a modest but significant reduction in LV mass when compared with WT controls. In contrast, Hras+/− mice showed no difference in cardiac size or function compared with WT mice at baseline (Figure 1E through 1H).

Mechanical stretch is known to activate Ras proteins in cardiomyocytes. We, therefore, subjected WT mice to 1 and 7 days of pressure overload by transverse aortic constriction (TAC) and determined H- and K-Ras activation in the myocardium. We found that both isoforms were activated at 1 day post-TAC; however, only K-Ras activation
was increased at 7 days post-TAC (Figure 2A and 2B). To determine the role of these Ras isoforms in mediating pressure overload–induced cardiac hypertrophy and dysfunction, H- and K-Ras mutant mice were subjected to 4-week TAC. There was no significant difference in pressure gradients achieved in all TAC experiments (Figure I in the Data Supplement). In response to pressure overload, Kras+/− mice showed a similar increase in cardiac hypertrophy, as determined by LV weight/tibia length, cardiomyocyte CSA, and fetal gene expression, when compared with WT littermates (Figure 2C through 2E; Figure II in the Data Supplement). However, we observed a trend toward reduced myocardial fibrosis and a significant reduction in TUNEL-positive cells in Kras+/− hearts after TAC compared with those of WT mice (Figure 2F through 2H). Importantly, %LVEF was significantly greater in Kras+/− mice compared with that in WT mice after 4 weeks of TAC (Figure 2I and Table 1).

Taken together, these results indicate that reduced K-Ras expression does not alter the hypertrophic capability of the mouse heart but does attenuate cardiac maladaptation to pressure overload stress.

H-Ras mutant mice were also subjected to pressure overload and assessed. Pressure overload–induced increases in LV weight/tibia length and cardiomyocyte CSA were not significantly different between Hras+/− mice and WT controls. However, Hras−/− mice had significantly attenuated LV weight/...
tibia length and cardiomyocyte CSA after 4 weeks of TAC compared with WT controls (Figure 3A through 3C). Hras<sup>−/−</sup> hearts also had significantly increased TUNEL-positive cells and myocardial fibrosis after 4 weeks of TAC compared with WT controls, whereas Hras<sup>+/−</sup> mice did not (Figure 3D through 3F). Cardiac function (%LVEF) in Hras<sup>+/−</sup> mice declined to a similar extent as in WT mice after 4 weeks of TAC. However, %LVEF was significantly further reduced in the Hras<sup>−/−</sup> mice after pressure overload (Figure 3G and Table 2). Moreover, septal wall thickness was significantly smaller in Hras<sup>−/−</sup> and Hras<sup>+/−</sup> mice after TAC compared with that in WT mice. These results suggest that H-Ras may contribute to the hypertrophic process in response to pressure overload stress.

To further investigate the underlying mechanism, we used the culture of NRCMs. We ectopically expressed activated H- and K-Ras12V in cultured cardiomyocytes using adenoviral gene transfer. We found that expression of activated H-Ras12V elicited a significant increase in cell surface area as well as protein:DNA content compared with that in LacZ control. In contrast, K-Ras12V expression did not alter cell surface area or protein:DNA content compared with that in LacZ control, suggesting that H-Ras selectively promotes cardiomyocyte hypertrophy in a cell autonomous manner (Figure 4A through 4C). We further evaluated hypertrophy by measuring fetal gene induction. By quantitative reverse transcriptase polymerase chain reaction analysis, we determined that H-Ras expression induced significant increases in ANF, BNP, and β-MHC mRNA expression, whereas K-Ras expression did not significantly alter atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), or β-myosin heavy chain (MHC) expression, compared with those in LacZ control–infected NRCMs (Figure 4D through 4F). These results are in agreement with our previous findings that showed differential signaling elicited by H- and K-Ras in cardiomyocytes<sup>27</sup> and demonstrate that H-Ras activation is sufficient to induce a hypertrophic response in NRCMs. We speculate that differences in isoform signaling may be because of differences in subcellular localization, which were observed by fractionation of ventricular tissue (Figure III in the Data Supplement). Importantly, a complimentary approach using RNAi-mediated depletion of endogenous H-Ras completely abolished the phenylephrine-induced expression of the hypertrophic markers ANF and BNP, indicating that H-Ras

![Figure 3](https://example.com/image3.png)

**Figure 3.** Inhibition of endogenous H-Ras attenuates hypertrophy and exacerbates pressure overload–induced cardiac dysfunction. A, Left ventricular weight/tibia length (LVW/TL) was determined in transverse aortic constriction (TAC) and sham-operated groups. B, Cardiomyocyte cross-sectional area was determined by wheat germ agglutinin (WGA) staining. C, Representative WGA images. D, Fibrosis was determined by Masson’s Trichrome staining. E, Representative images shown. Scale bar, 100 μm. F, Extent of apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. G, Echocardiographic analysis was performed to determine left ventricular ejection fraction (%LVEF). N=4 to 11 per group. Data are mean±SEM. *P<0.05.
is an important mediator of hypertrophy elicited by this α1 adrenergic agonist (Figure 4G and 4H).

Our recent work demonstrated that activation of K-Ras by oxidative stress in cardiomyocytes promotes the phosphorylation and activation of the proapoptotic kinase Mst1.27 Similarly, during pressure overload, K-Ras appeared to mediate Mst1 activation because phosphorylation of Mst1 was attenuated in Kras<sup>−/−</sup>, but not in Hras<sup>−/−</sup>, hearts after TAC (Figure 5A and 5B). Interestingly, phosphorylation of AKT and ERK were not altered in Hras<sup>−/−</sup> hearts but were strikingly inhibited in Hras<sup>−/−</sup> heart samples. Examination of downstream signaling in cultured cardiomyocytes revealed that H-Ras12V expression engaged PI3K-AKT, leading to increased phosphorylation of AKT and glycogen synthase kinase (GSK)-3β (an established physiological substrate of β), increased phosphorylation of AKT and glycogen synthase, and augmented cardiac fibrosis and apoptosis in Hras<sup>−/−</sup> mice (Figure VIII in the Data Supplement). We also found that H-Ras knockdown (si-Hras) abolished phenylephrine-induced activation of AKT and ERK, yet si-Kras treatment had a less pronounced effect (Figure 5A and 5B). Mechanistically, we observed activation of AKT and ERK, yet si-Kras treatment had a less pronounced effect (Figure 5A and 5B). We also found that H-Ras knockdown (si-Hras) abolished phenylephrine-induced activation of AKT and ERK, yet si-Kras treatment had a less pronounced effect (Figure 5A and 5B). We also found that H-Ras knockdown (si-Hras) abolished phenylephrine-induced activation of AKT and ERK, yet si-Kras treatment had a less pronounced effect (Figure 5A and 5B).

**Table 2. Echocardiographic Analysis of H-Ras Mutant Mice**

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<th>Hras&lt;sup&gt;−/−&lt;/sup&gt; TAC</th>
<th>Hras&lt;sup&gt;−/−&lt;/sup&gt; Sham</th>
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<td>26.8±0.6†</td>
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Data are presented as mean±SEM. BW indicates body weight; DPW WT, diastolic posterior wall thickness; DSEP WT, diastolic septum wall thickness; FS, fractional shortening; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; LVEFD, left ventricular end-systolic dimension; SPW WT, systolic posterior wall thickness; SSEP WT, systolic septum wall thickness; TAC, transverse aortic constriction; and WT, wild type.

*P<0.05 vs WT TAC.
†P<0.05 vs WT Sham.
‡P<0.05 vs Hras<sup>−/−</sup> Sham.
§P<0.05 vs Hras<sup>−/−</sup> TAC.

AKT has been implicated in cardiomyocyte hypertrophy and survival.28–31 Based on our mouse studies and cardiomyocyte results, we hypothesized that restoration of AKT activity might ameliorate the deleterious phenotype observed in Hras<sup>−/−</sup> mice after pressure overload. To test this hypothesis, we generated adeno-associated virus (AAV) to express exogenous AKT, or green fluorescent protein (GFP) as a control, and treated mice 2 weeks prior to TAC operation. Under basal conditions, LV mass, cardiomyocyte CSA, and %LVEF were modestly reduced in Hras<sup>−/−</sup> compared with those in WT mice (Figure 6A through 6D and 6H). Similarly to the results shown in Figure 3, we also observed attenuated hypertrophy, augmented fibrosis and apoptosis, and exacerbated cardiac dysfunction in Hras<sup>−/−</sup> mice after TAC compared with WT (Figure 6A through 6H and Table 3). TAC-induced fetal gene expression was attenuated in Hras<sup>−/−</sup> mice when compared with WT mice and was reversed to varying degrees by AKT-AAV, but not GFP-AAV (Figure VII in the Data Supplement). Administration of AKT-AAV, but not GFP-AAV, significantly ameliorated the enhanced fibrosis, apoptosis, and cardiac dysfunction observed in Hras<sup>−/−</sup> mice (Figure 6A through 6H and Table 3). Somewhat unexpectedly, we observed a modest attenuation of the hypertrophic response as determined by LV mass and cardiomyocyte CSA in Hras<sup>−/−</sup> mice supplemented with AKT-AAV after TAC (Figure 6B through 6D and Table 3). However, AKT normalization also significantly attenuated both TAC-induced lung congestion and increased wall stress in Hras<sup>−/−</sup> mice (Figure VIII in the Data Supplement). We hypothesize that the reduction in stress achieved by AKT-AAV, and the diminished need for compensatory hypertrophy, may explain why LV mass and cardiomyocyte CSA were not normalized in AKT-AAV-treated Hras<sup>−/−</sup> mice.
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Discussion
Prior to this report, studies using a loss-of-function approach to investigate the role of Ras isoforms in mediating cardiac hypertrophy and dysfunction in response to hemodynamic stress were lacking. Using H- and K-Ras mutant mice, we observed a beneficial function of endogenous H-Ras that seems to promote hypertrophy and cardioprotection, whereas Kras$^{+/−}$ mice had an improved cardiac phenotype after TAC, suggesting that K-Ras contributes to dysfunction in response to chronic pressure overload. Our previous work demonstrated divergent outcomes downstream of H- and K-Ras during myocardial ischemia/reperfusion. In response to ischemia/reperfusion, Hras$^{−/−}$ mice had greater injury likely because of attenuated PI3K-AKT activation, while Kras$^{−/−}$ mice were protected because of inhibition of Mst1 and reduced mitochondria-mediated cardiomyocyte apoptosis. This study is consistent with those findings and implicates PI3K-AKT signaling as an important mechanism downstream of H-Ras that is needed for compensation in response to TAC-induced cardiac stress.

Mechanisms that mediate cardiac hypertrophy are complex and many. For Ras proteins alone, there are several well-established downstream signaling pathways that have the potential to alter heart growth and function, including Raf-MEK-ERK, PI3K-AKT, and mitogen-activated protein kinase kinase kinase (MEKK) 1-c-Jun N-terminal kinase (JNK). Investigation into each of these cascades has provided a wealth of information; yet, the contribution of each to pathophysiology and disease remains somewhat unclear. Despite studies implicating a role for MEKK1-JNK in cardiomyocyte hypertrophy in vitro, genetic deletion of MEKK1 in mice, the upstream kinase responsible for activating JNK, did not affect cardiac hypertrophy after pressure overload, suggesting that this pathway may not be critical for pathological heart growth. Inhibition of Raf through cardiac expression of a dominant-negative mutant was shown to attenuate hypertrophy in response to pressure overload in mice, implicating this signaling pathway as a mediator of heart growth. However, ERK1$^{−/−}$ ERK2$^{−/−}$ double mutant mice showed no change in hypertrophy after pressure

Figure 4. H-Ras, but not K-Ras, promotes cardiomyocyte hypertrophy in vitro. A-F, Neonatal rat cardiomyocytes (NRCMs) were treated with LacZ, H-Ras12V, or K-Ras12V adenovirus and assayed 48 hours later. A, NRCMs were stained with troponin T to visualize cardiomyocytes and cell surface area determined. B, Cells were collected, and protein and DNA concentrations were determined. C, Representative images shown. Scale bar, 30 μm. mRNA was isolated and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) performed to determine Nppa (D), Nppb (E), and Myh7 (F) levels. NRCMs were treated with siRNA to deplete endogenous H-Ras (si-Hras) or control siRNA (si-CTRL). Seventy-two hours later, cells were treated with phenylephrine (PE; 100 μM; 24 hours) or vehicle and qRT-PCR performed to determine Nppa (G) and Nppb (H) levels. N=3. Data are means±SEM. *P<0.05. NS indicates not significant.
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Taken together, these results suggest that Raf signaling may diverge upstream of ERK to modulate the hypertrophic response. Forced expression of active PI3K (p110α) caused increased heart growth, whereas dominant-negative PI3K transgenic mice had smaller hearts at baseline and showed blunted hypertrophy after exercise but not pressure overload.35,36 Similarly, AKT transgenic mice had concentric hypertrophy and maintained systolic function, whereas Akt1 null mice were resistant to swimming-induced hypertrophy but showed exacerbated responses to pressure overload.36,37 These studies suggest that basal and physiological heart growth rely on PI3K-AKT signaling, whereas pathological hypertrophy elicited by hemodynamic stress may be mediated through alternate mechanisms.

Based on this background, we sought to determine the mechanism underlying H-Ras-mediated heart growth. Our results revealed a clear downregulation of AKT activation after TAC in hearts lacking H-Ras. Moreover, we used AAV-mediated gene expression in vivo to show that restoration of AKT function was sufficient to ameliorate the detrimental effects of H-Ras deletion after pressure overload.28,29 These studies suggest that basal and physiological heart growth rely on PI3K-AKT signaling, whereas pathological hypertrophy elicited by hemodynamic stress may be mediated through alternate mechanisms.

In addition to modulating cardiomyocyte growth and survival, AKT can also regulate angiogenesis and cardiomyocyte contractility, which may indirectly influence myocardial hypertrophy. Akt1−/− mice showed reduced angiogenesis, as well as reduced endothelial nitric oxide synthase (eNOS) activation and nitric oxide production, after ischemia.47 Akt1 null mice also had impaired vascular maturation, leading to leaky vessels, when compared with WT counterparts.48 On the other hand, mice engineered to express AKT in cardiomyocytes,49 or selectively in endothelial cells in an inducible manner,50 had increases in angiogenesis, capillary density, NO production, and heart function after pressure overload, while endothelial cell apoptosis was attenuated. These findings demonstrate that enhanced vascular AKT activity can promote vessel formation, maturation, and protection against insult, indicating its importance for adaptation to pathological stress.

AKT also modulates calcium handling and contractility of cardiomyocytes. Gain of function AKT transgenic mice had significantly increased contractility, concomitant with increases in SERCA expression, SR Ca2+ load, and phosphorylation of phospholamban (PLN).28,42,43 Adenoviral AKT transduction of rat hearts showed similar results.44 However, prolonged expression of AKT elicited cardiac dysfunction, which was attributed to insufficient coupling of angiogenesis, indicating that the duration of AKT activation as well as myocyte/vessel growth are critical determinants of heart function.45 Although the heart typically responds to increased demand via enlargement, it is plausible that AAV-AKT administration may increase cardiac contractility or angiogenesis independent of growth and could explain why we observed attenuated hypertrophy in Hras−/−+AAV-AKT mice but improved cardiac function.

We have shown previously that H- and K-Ras isoforms can have divergent signaling and outcomes in cardiomyocytes.27 Our prior findings, and current data, point to differences in subcellular localization of Ras isoforms that may be responsible for different effects of each in cardiomyocytes. In other cell types, Ras isoforms have been shown to localize to different cellular compartments, which can modulate association with downstream effectors and altered
Interestingly, differences in posttranslational modification and intracellular processing of H- and K-Ras are established and can affect protein trafficking, localization, and signaling. Although less is known in cardiomyocytes, this is one possible explanation for the disparities observed between these 2 isoforms and warrants further exploration.

Interestingly, previous work demonstrated that levels of H-Ras expression correlated with cardiomyocyte size in patients with hypertrophic cardiomyopathy. It has also been shown that the developmental disorders termed RASopathies, which are caused by dysfunctional Ras signaling, share phenotypic overlap, including cardiovascular defects such as hypertrophic cardiomyopathy among others. These findings suggest that H-Ras may be an important modulator of cardiac growth in human disease. Furthermore, our data demonstrate that H-Ras activation is increased by acute pressure overload (1 day) but not by 7 days post-TAC in the murine heart, whereas K-Ras activation seems to be maintained. Because endogenous H-Ras seems to counteract the progression to heart failure, it may be of interest to determine whether maintenance of physiological H-Ras activity could prove beneficial in the face of hemodynamic stress. On the other hand, because K-Ras disruption afforded cardioprotection, perhaps selective inhibition of this isoform could improve outcomes during hypertension.

Acknowledgments

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Sources of Funding

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Disclosures

None.
Table 3. Echocardiographic Analysis of Adeno-Associated Virus-Treated H-Ras+/− Mice

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<td>1.39±0.07</td>
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<td>LVEF, %</td>
<td>45.7±2.6</td>
<td>31.3±4.7†</td>
<td>35.1±3.4</td>
<td>17.2±1.4†</td>
<td>35.2±3.3</td>
<td>16.1±2.7†</td>
<td>43.5±3.1</td>
<td>29.1±2.3†</td>
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<td>BW, g</td>
<td>24.9±0.4</td>
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<td>25.6±0.7</td>
<td>32.5±1.6†</td>
<td>27.0±0.7</td>
<td>28.9±0.6</td>
<td>28.5±1.3</td>
<td>30.4±1.4</td>
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Data are presented as mean±SEM. BW indicates body weight; DPW WT, diastolic posterior wall thickness; DSEP WT, diastolic septum wall thickness; FS, fractional shortening; GFP, green fluorescent protein; KO, knockout; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; LVEDS, left ventricular end-systolic dimension; SPW WT, systolic posterior wall thickness; SSEP WT, systolic septum wall thickness; TAC, transverse aortic constriction; and WT, wild type.

*P<0.05 vs WT TAC.
†P<0.05 vs respective sham.
‡P<0.05 vs KO+GFP TAC.
§P<0.05 vs KO TAC.
H-Ras Mediates Cardioprotection via AKT


H-Ras Isoform Mediates Protection Against Pressure Overload–Induced Cardiac Dysfunction in Part Through Activation of AKT
Takahisa Matsuda, Jae Im Jeong, Shohei Ikeda, Takanobu Yamamoto, Shumin Gao, Gopal J. Babu, Peiyong Zhai and Dominic P. Del Re

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SUPPLEMENTAL MATERIAL

H-Ras isoform mediates protection against pressure overload-induced cardiac dysfunction in part through activation of AKT

Matsuda – H-Ras mediates cardioprotection via AKT

Takahisa Matsuda, PhD; Jae Im Jeong, BS; Shohei Ikeda, MD, PhD; Takanobu Yamamoto, MD, PhD; Shumin Gao, MD, PhD; Gopal J. Babu, PhD; Peiyong Zhai, MD, PhD; and Dominic P. Del Re, PhD

From the Cardiovascular Research Institute and the Department of Cell Biology and Molecular Medicine, Rutgers, New Jersey Medical School, Newark, NJ
Supplemental Methods

**Transverse aortic constriction.** The method for imposing pressure overload in mice has been described previously\(^1\). Briefly, mice were anesthetized with a mixture of ketamine, xylazine, and acepromazine and mechanically ventilated. The left chest was opened at the second intercostal space. Aortic constriction was performed by ligation of the transverse thoracic aorta with a 27-gauge needle using a 7-0 braided polyester suture. Sham operation was performed without constricting the aorta.

**Echocardiography.** Mice were anesthetized using 12 µl/g body weight of 2.5% tribromoethanol (Avertin, Sigma), and echocardiography was performed as described previously \(^2\), 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: \( \text{LVEF} \text{ (\%)} = 100 \times \frac{( \text{LVEDD}^3 - \text{LVESD}^3)}{\text{LVEDD}^3} \).

**Hemodynamic measurements.** Mice were anesthetized with 2.5% Avertin (0.29 mg/kg i.p.). Simultaneous recording of arterial pressures were obtained by using two pressure transducers, as follows: A 1.4- French catheter-tip micronanometer catheter (Millar Instruments) was inserted through the right femoral artery into the abdominal aorta. Then another 1.4-French Millar catheter was inserted through the right carotid artery into the ascending aorta and advanced into the LV, where pressures and the first
derivative of LV pressure over time (dP/dt) were recorded. Arterial pressure gradients were measured as described previously\(^1\).

**Histological analyses.** Heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-µm thickness. Interstitial fibrosis was evaluated by Masson’s Trichrome staining as described \(^2\). Myocyte cross-sectional area was measured from images captured from wheat germ agglutinin-stained sections as described previously \(^3\).

**Evaluation of apoptosis.** DNA fragmentation was detected *in situ* and in cultured cells using TUNEL as described previously \(^2\). Nuclear density was determined by counting DAPI-stained nuclei in 20 different fields for each sample.

**Cell culture and reagents.** Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl: (WI)BR-Wistar rats and maintained in culture as described previously \(^4\). The PI3K inhibitor LY294002, AKT inhibitor V (triciribine) and MEK1 inhibitor PD98059 were purchased from Calbiochem (EMD Millipore).

**Adenoviral constructs.** Adenoviruses harboring Myc-H-Ras12V and Myc-K-Ras12V were generated as described previously \(^5\). Adenovirus harboring beta-galactosidase (LacZ) was used as a control.

**RNAi.** siRNA-mediated knockdown of endogenous H-Ras and K-Ras 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 H-Ras targeted siRNA duplexes used were: RNC.RNAI.N001098241.12.1, RNC.RNAI.N001098241.12.2 and RNC.RNAI.N001098241.12.3. The K-Ras targeted siRNA duplexes used were:
RNC.RNAI.N031515.12.1, RNC.RNAI.N031515.12.2 and RNC.RNAI.N031515.12.3.

Scrambled siRNA CTRL (NC1, Negative Control Sequence) was used as a control.

**Immunoblotting.** For immunoblot analysis, the antibodies used were H-Ras (Santa Cruz), K-Ras (Sigma), Mst1 (BD Transduction Labs), p-Mst1/2(Thr183/180) (Cell Signaling), tubulin (Sigma), GAPDH (Cell Signaling), ERK1/2 (Cell Signaling), phospho-ERK1/2 (Cell Signaling), AKT (Cell Signaling), p-AKT(Ser473) (Cell Signaling), p-AKT(Thr308) (Cell Signaling), p-GSK-3β(Ser9) (Cell Signaling), GSK-3β (Cell Signaling), Ras (Upstate, Millipore), KDEL (Enzo), N-cadherin (BD Transduction Labs), GFP (Cell Signaling), Myc-tag (Santa Cruz), p110α (Cell Signaling), p85 (Cell Signaling), PTEN (Cell Signaling) and α-actinin (Sigma). Densitometry was performed using ImageJ software.

**RBD Pulldown Assay.** Homogenates were incubated with Raf-1 RBD agarose (Upstate, Millipore) for 40 minutes at 4°C to precipitate GTP-bound Ras according to manufacturer’s instructions.

**Immunostaining.** Cardiomyocytes were plated on gelatin-coated glass coverslips. Cells were fixed in PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.1% Triton-X, and blocked with 5% normal goat serum. Immunostaining was performed using anti-Troponin-T mouse monoclonal antibody (Thermo Scientific), Alexa-fluor 568 goat anti-mouse IgG (Molecular Probes) and Vectashield mounting medium with DAPI (Vector Laboratories). Imaging was performed using a Nikon fluorescence microscope. Cardiomyocyte area was measured using ImageJ software. Total protein concentration/ dish was determined as described previously ¹.
qRT-PCR. RNA was isolated from cardiomyocytes or LV tissue, cDNA was generated, and quantitative real-time PCR performed as described previously\textsuperscript{1}. Primers used to detect rat transcripts were: \textit{Nppa} (sense) 5’ atacagtcgtgccgtcaca, (antisense) 5’ cgagagcaacctcctcttc; \textit{Nppb} (sense) 5’ ggaaaattgcagcagagcagc, (antisense) 5’ cgatccggtcttctctgtc; \textit{Myh7} (sense) 5’ tgtgacactccgaggagcttt, (antisense) 5’ tgacacagaccccttgacag, \textit{Actin} (sense) 5’ agccatgtagccatcag, (antisense) 5’ ctctcagctgtggtggtgaa. Primers used to detect mouse transcripts were: \textit{Nppa} (sense) 5’ gcctccaggcagatggagag, (antisense) 5’ gggggcatgacctcatct; \textit{Nppb} (sense) 5’ gaggtcactctatctctgg, (antisense) 5’ gccatttcctccgactttctc; \textit{Myh7} (sense) 5’ actgtcaacataaggggca, (antisense) 5’ tgtgatgatctgatccaggg; \textit{Gapdh} (sense) 5’ agacagccgcatctttgtt, (antisense) 5’ cttgccgtgagctgactcatc. Results were normalized to \textit{Actin} or \textit{Gapdh} and relative quantitation was determined using the $\Delta\Delta C_T$ method.

AKT Kinase assay. AKT kinase activity was assessed using nonradioactive AKT kinase assay kit according to manufacturer’s protocol (Cell Signaling). Briefly, cardiomyocytes were harvested in Cell Lysis Buffer (20 mmol/L Tris pH7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% triton-X100, 2.5 mmol/L PNPP, 1 mmol/L $\beta$-glycerophosphate, 1 mmol/L Na3VO4 and 10 mg/mL leupeptin), and p-AKT(S473) was immunoprecipitated from cell lysates using an immobilized p-AKT monoclonal antibody bead conjugate (Cell Signaling). Immunocomplexes were centrifuged, washed twice with Cell Lysis Buffer and twice with Kinase Buffer (25 mmol/L Tris pH7.5, 5 mmol/L $\beta$-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4 and 10 mmol/L MgCl2). The immunocomplexes were resuspended in 50 $\mu$l Kinase Buffer with 200 $\mu$mol/L ATP and 1 $\mu$g of a GSK-3 $\alpha/\beta$ GST-fusion protein, corresponding to residues
surrounding GSK-3 α/β (Ser21/9) (CGPKGPGRRGRRRTSSFAEG) as a substrate (Cell Signaling). Following a 30 minute incubation at 30°C, kinase reactions were terminated by adding 25 µl of 3X SDS buffer and boiling for 10 minutes. Samples were subjected to Western blotting using p-GSK-3α/β (Cell Signaling) and anti-GST (Cell Signaling) antibodies.

**Co-immunoprecipitation.** For immunoprecipitation assays, cardiomyocyte lysates 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 Na3VO4, 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 (Myc-tag, Santa Cruz) overnight at 4°C, and immunocomplexes were precipitated following 1 hour of incubation with sepharose A/G beads (Santa Cruz). Following SDS-PAGE, immunoblot was performed using Myc-tag (Santa Cruz) and p110α (Cell Signaling) antibodies.

**Subcellular Fractionation.** Fractions enriched for plasma membrane were prepared from ventricular tissue using the plasma membrane protein extraction kit from Abcam according to manufacturer’s instructions. The resulting cytosolic fraction was further separated to ER/SR enriched fraction by centrifugation at 100,000 × g for 2 hours.

**Adeno-associated virus.** The recombinant adeno-associated virus (rAAV) was produced using the AAV-DJ/8 helper free expression system (Cell Biolabs, Inc.) at the AAV core, Department of Cell Biology and Molecular Medicine, Rutgers, NJMS, Newark, NJ. To produce self-complementary recombinant AAV-DJ/8, the AKT cDNA (Addgene 64831) was cloned to the pAAV-MCS expression vector (Cell Biolabs, Inc.)
using EcoRI and BamHI restriction enzymes. GFP virus was prepared using the pAAV GFP Control Vector (Cell Biolabs, Inc.). The recombinant pAAV vector was used for co-transfection of 293AAV cell line (Cell Biolabs, Inc.) together with pAAV-DJ/8 and pHelper in a 1:1:1 ratio using polyethylenimine (PEI). The rAAV produced was purified, and titered using standard procedures. Briefly, after 72 hours of transfection, cells were harvested and purified by the iodixanol gradient / ultra-centrifugation method, and the AAV fraction was concentrated by the VIVASPIN 20 concentrator (100 kDa cut-off, Satorious, Germany). The virus titer was determined using the Cell Biolabs AAV quantitation kit (Cat. # VPK-145). Tail vein injection was used to deliver $2 \times 10^{11}$ viral genomes/mouse two weeks prior to surgery.
Supplemental References


Supplemental Figure Legends

Supplemental Figure 1. TAC-induced pressure gradients. A, B and C. Pressure overload was achieved by TAC and pressure gradients were determined by invasive pressure catheter measurement after 4 weeks TAC. No significant differences were found between groups.

Supplemental Figure 2. TAC-induced fetal gene expression in K-Ras mutant mice. A, B and C. Pressure overload was achieved by TAC and mRNA expression in left ventricles was determined by qRT-PCR after 4 weeks TAC. *, P<0.05. N.S. = Not significant. N = 3 mice/group.

Supplemental Figure 3. Localization of Ras isoforms in myocardium. Representative western blot demonstrating differing patterns of localization between H- and K-Ras isoforms in mouse myocardium.

Supplemental Figure 4. Activation of PI3K-AKT signaling pathway by active Ras isoforms in cardiomyocytes. A, NRCMs were transduced with LacZ, H-Ras12V or K-Ras12V adenovirus and treated with either vehicle, the PI3K inhibitor LY294002 (LY; 10 µM) or the AKT inhibitor triciribine (V; 10 µM). B, NRCMs were transduced with LacZ or H-Ras12V adenovirus and treated with LY or vehicle control. Cell lysates were immunoprecipitated for active AKT and subjected to kinase assay using GSK-3α/β-GST fusion protein as a substrate. C, NRCMs were transduced with adenovirus and lysates
were subjected to immunoprecipitation using anti-Myc-tag antibody. Complexes were probed for Myc-tagged H-Ras and p110α. Representative images are shown.

Supplemental Figure 5. Expression of PTEN and the PI3K subunits p85 and p110α in WT and Hras-/- hearts and cardiomyocytes. A, Ventricular homogenates were prepared from WT and Hras-/- mice and subjected to SDS-PAGE to determine protein expression. B, Quantification of results in panel A. *, P<0.05. N.S., not significant. N = 3 mice/group. C, NRCMs were treated with control siRNA (si-CTRL), or siRNA targeted to H-Ras (si-Hras) for 72 hours. Protein lysates were subjected to SDS-PAGE to determine protein expression. D, Quantification of results in panel C. *, P<0.05. N = 3 experimental replicates.

Supplemental Figure 6. Role of MEK-ERK signaling in H-Ras-induced hypertrophy. A-C, cardiomyocytes were transduced with control LacZ or active H-Ras adenovirus. Cells were treated subsequently with vehicle control or the MEK1 inhibitor, PD98059 (10 µM). RNA was prepared and gene expression determined. *, P<0.05 versus LacZ + Veh. N.S. = not significant. N = 4 experimental replicates.

Supplemental Figure 7. TAC-induced fetal gene expression in H-Ras mutant mice. A, B and C. Pressure overload was achieved by TAC and mRNA expression in left ventricles was determined by qRT-PCR after 4 weeks TAC. AAV was administered 2 weeks prior to TAC surgery. *, P<0.05. N.S. = Not significant. N = 3 mice/group.
Supplemental Figure 8. Increased lung congestion and wall stress in Hras-/- mice is attenuated by AAV-AKT administration prior to pressure overload. A and B, Pressure overload was achieved by TAC. Hras-/- mice were treated with control AAV-GFP or AAV-AKT 14 days before TAC. Gravimetric and echocardiographic analyses were performed after 4 weeks TAC or Sham operation. *,P<0.05. N = 3-9 mice/group.
Supplemental Figure 1.

A. N.S.

B. N.S.

C. N.S.
Supplemental Figure 2.

(A) Nppa mRNA expression

(B) Nppb mRNA expression

(C) Myh7 mRNA expression

* N.S.
## Supplemental Figure 3.

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Supplemental Figure 4.

A. 

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p-AKT$^{5473}$
AKT
p-GSK-3β
GSK-3β
Ras
Tubulin

B.

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p-GSK-3α/β-GST
GSK-3α/β-GST

C.

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Supplemental Figure 5.

A.WT

Hras-/-

PTEN
p85
p110α
Tubulin

B.

[Bar charts showing protein expression levels for WT and Hras-/- conditions]

C.

si-CTRL

si-Hras

PTEN
H-Ras
Tubulin

D.

[Bar charts showing protein expression levels for si-CTRL and si-Hras conditions]
Supplemental Figure 6.

A. Relative mRNA levels of Nppa

B. Relative mRNA levels of Nppb

C. Relative mRNA levels of Myh7

* Significant difference
N.S. Not significant
Supplemental Figure 7.

A. Nppa mRNA expression

- WT
- Hras-/-
- Hras-/- + GFP
- Hras-/- + AKT

B. Nppb mRNA expression

- WT
- Hras-/-
- Hras-/- + GFP
- Hras-/- + AKT

C. Myh7 mRNA expression

- WT
- Hras-/-
- Hras-/- + GFP
- Hras-/- + AKT