Cardiomyocyte-Specific Loss of Neurofibromin Promotes Cardiac Hypertrophy and Dysfunction

Junwang Xu,* Fraz A. Ismat,* Tao Wang, Min Min Lu, Nicole Antonucci, Jonathan A. Epstein

Rationale: Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with a broad array of clinical manifestations, including benign and malignant tumors, and characteristic cutaneous findings. NF1 patients also have an increased incidence of cardiovascular diseases, including obstructive vascular disorders and hypertension. The disease gene, NF1, encodes neurofibromin, a ubiquitously expressed protein that acts, in part, as a Ras-GAP (GTP-ase activating protein), downregulating the activity of activated Ras protooncogenes. In animal models, endothelial and smooth muscle expression of the disease gene is critical for normal heart development and the prevention of vascular disease, respectively.

Objective: To determine the role of NF1 in the postnatal and adult heart.

Methods and Results: We generated mice with homozygous loss of the murine homolog (Nf1mKO) and evaluated their hearts for biochemical, structural, and functional changes. Nf1mKO mice have normal embryonic cardiovascular development but have marked cardiac hypertrophy, progressive cardiomyopathy, and fibrosis in the adult. Hyperactivation of Ras and downstream pathways are seen in the heart with the loss of Nf1, along with activation of a fetal gene program.

Conclusions: This report describes a critical role of Nf1 in the regulation of cardiac growth and function. Activation of pathways known to be involved in cardiac hypertrophy and dysfunction are seen with the loss of myocardial neurofibromin. (Circ Res. 2009;105:304-311.)

Key Words: NF1 ▪ neurofibromatosis ▪ cardiac hypertrophy

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting 1 in 3500 individuals.1 NF1 has a complex array of clinical signs and symptoms, including benign and malignant tumors, cutaneous abnormalities such as café-au-lait spots, Lisch nodules, and learning disabilities.2 In addition to these commonly observed findings, cardiovascular manifestations of this disease are a prominent part of the pathology.3,4 Among these disorders are cardiovascular manifestations of this disease are a prominent part of the pathology.3,4 Among these disorders are cardiac hypertrophy; renovascular and essential hypertension; obstructive vasculopathy; and moyamoya, a cerebral vasculature abnormality that can cause intracranial hemorrhage.

Neurofibromatosis results from mutations in NF1, a tumor suppressor gene that encodes neurofibromin, a protein that acts, in part, as a Ras-GTPase–activating protein (Ras-GAP).5 Neurofibromin downregulates the activity of Ras protooncogenes, a family of genes that are important regulators of cell proliferation, growth, and differentiation.6 The loss of the murine homolog Nf1 in developing mice leads to upregulation of activated Ras and to a series of cardiac defects reminiscent of common forms of congenital heart disease.7,8

We have examined the role of Nf1 and Nf1-mediated Ras regulation in various cardiovascular compartments. Previous work from our laboratory has shown that endothelial expression of Nf1 plays a critical role in cardiac development.9 Nf1 regulation of Ras in the developing endothelium is required for normal development of the endocardial cushions and ventricular myocardium.9,10 Reconstitution of the Ras-GAP function of neurofibromin in those tissues is sufficient to rescue cardiac development.10 Nf1 is also important in regulating Ras signaling in vascular smooth muscle.11,12 Specifically, loss of Nf1 regulation of Ras leads to an abnormal proliferative injury response in vascular smooth muscle. Although Nf1 is expressed in the myocardium, our initial evaluations of myocardial Nf1 in cardiac development did not demonstrate an obvious developmental defect.9 However, to the best of our knowledge, the role of Nf1 in the adult myocardium of murine models has not been previously examined.

Ras activation plays an important role in many forms of cardiac hypertrophy.13,14 Transgenic overexpression of acti-
vated Ras in the heart, for example, leads to cardiac hypertrophy. Activation of a number of transmembrane receptors involved with cardiac hypertrophy are known to activate Ras. Additionally, Ras is intimately linked to signaling pathways known to play a role in cardiac hypertrophy, such as extracellular signal-regulated kinases (Erks) and phosphoinositide 3’-kinase and Akt.

In this report, we examined the role of NF1 in adult myocardium using tissue-specific gene inactivation. Our findings suggest that neurofibromin functions as an important Ras-GAP in adult myocardium and that loss of NF1 leads to activation of the Ras signaling pathway and pathological cardiac hypertrophy and heart failure.

Methods

Generation of NF1 Myocardial-Specific Knockout Mice

NF1 myocardial-specific knockout mice (Nf1mKO) mice were generated through crosses of mice harboring Nf1flox alleles8,19 with α-MHC-cre transgenic mice, which express Cre recombinase under the control of the α-myosin heavy chain (MHC) promoter. In addition, we used the HA-NF1 GAP-related domain (GRD) Rosa knock-in allele10 to reconstitute NF1 Ras-GAP activity in some of these Nf1mKO mice (termed Nf1mKO+GRD). Genotyping of NF1flox and Rosa-GRD was performed as previously described.8,10,12

The α-MHC-cre transgene was detected by using the following primers: 5’-CTGTGGTCCACATTCTTCAGG-3’ and 5’-CTGAAACATGTCATCAGGTTC-3’. Unless otherwise indicated, wild-type, Nf1flox, or Nf1flox/−ittersates were used as controls. All procedures conformed to the guidelines established by the Association for the Assessment and Accreditation of Laboratory Animal Care and were approved by the University of Pennsylvania Animal Care and Use Committees.

Echocardiography

Mice were anesthetized by inhalation of 4% isoflurane in a glass chamber and 1% to 1.5% isoflurane via nose cone to maintain anesthesia. Animals were imaged on a heated platform while monitoring body temperature and ECG. Echocardiography was performed using a Vevo 770 (VisualSonics, Toronto, Ontario, Canada) with a linear 30-MHz probe (RMV 707B). M-mode images were used for measurement of wall thickness, chamber dimension and fractional shortening.

Invasive Hemodynamics

Mice were anesthetized with intraperitoneal ketamine (100 mg/kg), as performed previously.12 A 1.4 French Millar catheter-tip micro-manometer catheter (Millar Instruments, Houston, Tex) was inserted through the right carotid artery into the aorta and then into the left ventricle to record pressures and left ventricular (LV) dP/dt. For generation of pressure–volume (PV) loops, a Millar SPR-839 microtip catheter transducer was inserted into the right carotid artery and advanced into the left ventricle under pressure control. Pressure signals were recorded continuously with an ARIA PV conductance system coupled with a Powerlab/4SP A/D converter. Heart rate, maximal LV systolic and end-diastolic pressures, maximal slope of systolic pressure increment (∆dP/dt) and diastolic pressure decrement (∆dP/dt), stroke work, ejection fraction, and cardiac output were calculated and corrected according to in vitro and in vivo volume calibrations with PVANZ 9.2 software (Millar Instruments). Finally, the catheter was pulled back into the aorta for mean arterial blood pressure (mean blood pressure) measurement.

Histology

Tissue collection, fixation, and staining was performed as previously described.8,10,12 Briefly, hearts were collected in ice-cold PBS, fixed overnight in 4% paraformaldehyde at 4°C, washed with PBS, and dehydrated through an ethanol series before paraffin embedding. Masson’s trichrome and wheat germ agglutinin (WGA) staining was performed as described.21 Staining and quantification details are available in the Online Data Supplement at http://circres.ahajournals.org.

RT-PCR and Real-Time PCR

Total RNA was extracted from adult mouse hearts using TRIzol (Invitrogen, Carlsbad, Calif). Transcripts were then amplified from reverse-transcribed cDNA using SYBR Green (Applied Biosystems, Foster City, Calif). Relative gene expression levels were quantified using the comparative threshold method, with GAPDH serving as the endogenous reference gene. Primer sequences are in the Online Data Supplement.

Tissue Extraction and Immunoblotting

Mouse heart lysates were prepared in lysis buffer consisting of 20 mmol/L HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1 μg/ml leupeptin, 1 mmol/L Na3VO4, 2.5 mmol/L NaF, and 1 mmol/L β-glycerophosphate. Samples were separated by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. Antibodies for p44/42 (Erk), phospho-p44/42 (Thr202/Tyr204), Akt, phospho-Akt (Ser473), glycogen synthase kinase (GSK)3β, phospho-GSK3β (Ser9), mTOR, phospho-mTOR (Ser2481) (Cell Signaling Technologies, Beverly, Mass), GAPDH (Chemicon International, Temecula, Calif), and neurofibromin (sc-67; Santa Cruz Biotechnology, Santa Cruz, Calif) were used at manufacturer-suggested concentrations. For the Ras activation assays, tissue lysates were precleared with glutathione agarose, incubated with Raf-1 RBD (Ras-binding domain) agarose beads (Upstate USA Inc), and blotted with anti-Ras monoclonal antibody (Upstate USA Inc). These blots of activated Ras (Ras-GTP) were compared with blots of the same samples for total Ras before Raf-1 RBD pull down to quantify Ras activation.

Statistical Analysis

All data are expressed as the means ± SEM. Unpaired Student t test was used for all significance testing. Probability values of <0.05 were considered statistically significant.

Results

Loss of Myocardial NF1 Leads to Ras Hyperactivation, Intracellular Signaling Abnormalities, and Late Mortality

We generated Nf1mKO mice using the Nf1flox allele19 and the α-MHC-cre transgene.20 Nf1flox/−α-MHC-cre−/− mice (referred to herein as Nf1mKO) were born alive at expected Mendelian ratios (Table) and appeared as healthy pups and young adults. Cre expression was specific to the myocardium, as shown by β-galactosidase activity in α-MHC-cre− embryos harboring the Rosa26-LacZ reporter. We detected staining as early as embryonic day (E)9.5 (data not shown), and by E12.0, we observed robust staining in the heart without ectopic staining (Figure 1A). Next, we collected hearts from adult (12 week)
**Table.** Loss of Myocardial Nf1 Does Not Lead to Embryonic or Perinatal Lethality

<table>
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<th>Genotype</th>
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<th>Observed (n)</th>
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<tr>
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<td>162</td>
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</table>

Genotypes of adult (8- to 12-week) offspring from Nf1fl/fl; α-MHC-cre × Nf1fl/fl; α-MHC-cre; Nf1+/+; α-MHC-cre; Nf1mKO were observed at the expected Mendelian frequency ($\chi^2 = 8.133; df = 5; P = 0.15$). There were no obvious abnormalities in development.

**Nf1mKO** and control littermates and probed for the presence of neurofibromin protein. There was no detectable neurofibromin in Nf1mKO in contrast to control (Figure 1B).

Next, we collected the hearts of newborn, postpartum day 0 (P0) Nf1mKO and control mice for analysis of Ras activity (Figure 1C and 1D). We also examined hearts from Nf1mKO mice harboring the HA-GRD knock-in allele10 (Nf1mKO+GRD) to delineate the role of the GRD in myocardial Ras activation. Quantification of Ras-GTP/Total Ras ratios demonstrated significant elevations in activated Ras-GTP in Nf1mKO. These elevations were rescued to approximately wild-type levels in Nf1mKO+GRD.

Loss of neurofibromin hyperactivates Ras/Erk signaling.5 Therefore, we examined several downstream effectors of Ras, including phospho-Erk, phospho-Akt, phospho-mTOR, and phospho-GSK3β (Figure 1E). Comparing P0 Nf1mKO hearts with control littermates, we found consistent signaling alterations in Nf1mKO. These changes persisted at 12 weeks of age (Online Figure I). Nf1mKO+GRD showed less activation of these pathways than Nf1mKO. Taken together, these data suggest that inactivation of Nf1 in the myocardium leads to enhanced activation of Ras and downstream effector pathways.

Finally, premature mortality was apparent in Nf1mKO beginning at ~20 weeks; by 32 weeks, one-half were dead (Figure 1F). Necropsy did not reveal any tumors, such as has been reported in Nf1+/− mice at ~2 years of age.5 However, we observed cardiac enlargement in Nf1mKO mice compared to similarly aged controls.

**Loss of Myocardial Nf1 Promotes Progressive Cardiac Hypertrophy, Fibrosis, and Cardiac Myocyte Enlargement**

Heart weights of Nf1mKO and control littermates were compared to their body weights and tibia lengths at 4, 12, and 20 weeks of age. We also examined the hearts histologically with Masson’s trichrome stain looking for fibrosis and wheat-germ agglutinin (WGA) staining to evaluate myocyte size.

We did not observe any significant differences in either index of heart size at 4 weeks (Figure 2A). There were also no observed differences in cardiac fibrosis or myocyte size at 4 weeks (Figure 2B). By 12 weeks, we observed statistically
significant increases in both heart size indices for Nf1mKO mice (Figure 2A). Average myocyte size was also significantly larger in Nf1mKO by 12 weeks (Figure 2C). Although enlarged, Nf1mKO hearts at this age did not have increased fibrosis compared to controls.

At 20 weeks, we added measurements of heart size relative to body size for Nf1mKO+GRD. By this age, the cardiac hypertrophy observed in the Nf1mKO mice was much more pronounced than in controls (Figure 2A). This was significantly ameliorated, although not completely
normalized, in Nf1mKO+GRD animals, suggesting that isolated GRD expression was not able to completely rescue the cardiac hypertrophy resulting from the loss of myocardial Nf1.

We observed significant fibrosis in Nf1mKO hearts that was not seen in controls (Figure 2B). For Nf1mKO, 25.3% (±10.8%) of the area in LV sections stained for fibrous tissues, versus 8.9% (±3.9%) in wild type (P<0.003). There were also even more pronounced increase in myocyte size in Nf1mKO at 20 weeks than in younger ages (Figure 2C). We observed no differences in cell death between Nf1mKO and wild-type hearts (data not shown). In summary, the loss of Nf1 from myocytes leads to progressive cardiac enlargement and myocyte hypertrophy, coupled with the late development of fibrosis.

Progressive Dilated Cardiomyopathy With Systolic and Diastolic Impairment in Nf1mKO

Evaluating the functional impact of the loss of myocardial Nf1, we performed echocardiography on Nf1mKO mice and controls at 12 and 20 weeks (Figure 3A; Online Table I). We observed a progressive increase in the LV dimensions at both end-diastole and end-systole in Nf1mKO as compared to controls. LV dilation was evident by 12 weeks and pronounced by 20 weeks (Figure 3A). Of note, we did not observe any substantial differences in echocardiographic indices between wild-type and α-MHC-cre+ transgenic mice even by 20 weeks (Online Table II), suggesting that cardiac defects were not attributable to transgenic expression of cre recombinase in the heart. In addition, Nf1mKO mice had a reduction in systolic performance as measured by LV ejection fraction and fractional shortening. Low in Nf1mKO at 12 weeks, it declined further by 20 weeks (Figure 3A).

We also performed invasive hemodynamic measurements from the left ventricles of wild-type, Nf1mKO, and Nf1mKO+GRD mice (Figure 3B through 3D; Online Table III). Representative PV loops and LV pressure tracings from Nf1mKO and controls are shown from 12 and 20 weeks of

![Image](http://circres.ahajournals.org/)

**Figure 3.** Nf1mKO mice develop progressive dilated cardiomyopathy with impairment of systolic and diastolic function. A, Progressive decreased fractional shortening of Nf1mKO. Echocardiographic measurement of fractional shortening (FS) in Nf1mKO and control mice at 12 and 20 weeks (±SEM) shows a small decrease in FS at 12 weeks that is markedly worsened by 20 weeks. Representative 20 week M-mode echocardiogram of control (top) and Nf1mKO (bottom) showing LV chamber dilation and reduced shortening in Nf1mKO.

B, Reduced LV contractility and impaired relaxation in Nf1mKO. Representative PV loops from Nf1mKO and control mice at 12 weeks (left) and 20 weeks (right) showing the relationship between LV pressure and volume throughout the cardiac cycle. At 12 weeks, there is a mild increase in LV end-diastolic volume in Nf1mKO, with a concomitant decrease in stroke volume (reduced PV loop width). Systolic pressure is reduced in Nf1mKO, but diastolic pressure is preserved. By 20 weeks, there is a rightward shift of the Nf1mKO PV loop, indicating LV dilation. The narrow loop indicates a reduced stroke volume, and the LV end-diastolic pressure (arrow) is elevated in Nf1mKO. C, Impaired contractility and relaxation in Nf1mKO hearts. Representative tracings of LV pressure (top) and dP/dt (bottom) at 12 weeks. There is both reduced peak LV pressure and maximal dP/dt in Nf1mKO, indicating systolic dysfunction. Furthermore, there is elevated LV end-diastolic pressure and minimal dP/dt in Nf1mKO, indicating diastolic dysfunction and impaired myocardial relaxation. D, Quantification of maximal and minimal dP/dt in 12 week old Nf1mKO (n=3) and control (n=4) adult mice. The reduction in maximal and elevation in minimal dP/dt in Nf1mKO were both statistically significant and indicative of early systolic and diastolic heart failure.
age (Figure 3B and 3C). At 12 weeks, Nf1mKO showed a similar isovolumetric contraction volume to controls (right side of the PV loop) but with a reduced systolic pressure. In addition, Nf1mKO mice show an increased isovolumetric relaxation volume (left side of the PV loop), resulting in a reduced stroke volume (PV loop width). End-diastolic pressure (lower right corner) remained preserved in Nf1mKO at 12 weeks. Thus, Nf1mKO mice showed impaired systolic and diastolic function by 12 weeks.

Invasive hemodynamic studies at 20 weeks were consistent with the pathological and echocardiographic findings (Figure 3B, right). First, there is a marked rightward shift of the Nf1mKO PV loop, indicating LV dilation. The loop is also narrowed, demonstrating a reduced stroke volume and severe systolic dysfunction. Finally, end-diastolic pressures were elevated in Nf1mKO (arrows, Figure 3B). As shown in Figure 3D, the maximum and minimum dP/dt, already abnormal at 12 weeks, is severely worsened by 20 weeks. Detailed review of the hemodynamic data (Online Table III) confirmed progressive worsening of all parameters of systolic and diastolic cardiovascular physiology in Nf1mKO.

Finally, to see the effects of rescuing myocardial Ras-GAP activity on hemodynamics and cardiac function, we performed invasive hemodynamic assessments of Nf1mKO+GRD mice at 20 weeks. Consistent with the partial rescue of cardiac hypertrophy, we found a partial rescue of all of the major parameters of systolic and diastolic function that were altered in Nf1mKO. This suggests that Ras regulation in cardiomyocytes is at least partially responsible for the activity of neurofibromin in the functioning heart.

Taken together, these assessments of the cardiovascular physiology of the Nf1mKO mice show a progressive impairment of both systolic and diastolic function, leading to a dilated cardiomyopathy. This dysfunction is markedly rescued by myocardial expression of the isolated NF1 GRD. Combined with the pathological assessment of the hearts shown earlier, it suggests that the loss of myocardial NF1 leads to a fatal dysregulation of myocardial cell signaling that manifests as gross cardiovascular physiological derangement and the pathological picture of heart failure.

**Loss of NF1 Leads to Progressive Activation of a Fetal Gene Program in Adulthood**

A common feature of cardiovascular failure is the activation of a “fetal gene program” in adulthood.22,23 Our assessment of fetal gene program activation in the heart confirmed a dramatic increase in expression of these genes in Nf1mKO over age-matched control hearts (Figure 4). We performed RT-PCR on RNA from the hearts of Nf1mKO and littermate controls at 4, 12, and 20 weeks of age, assaying for atrial natriuretic peptide, brain-type natriuretic peptide, and β-MHC. Messenger RNA levels for all 3 genes were close to normal in Nf1mKO hearts at 4 weeks but were elevated at 12 weeks compared to control. By 20 weeks, there was a dramatic elevation in the transcript levels in Nf1mKO.

**Discussion**

Our results demonstrate a critical role for NF1 in the adult myocardium. Using α-MHC-cre to delete NF1 in the heart during midgestation, we observed no discernible phenotype at birth or in young adults, consistent with our previous observations.9 However, because these cardiac NF1-deficient mice aged, we documented progressive cardiac hypertrophy, fibrosis, dilatation, and failure associated with premature mortality. Reconstitution of Ras-GAP activity in NF1-deficient myocardial cells using the Rosa-GRD knock-in was, to the limits of our ability to measure, able to return Ras activity to wild-type levels. Furthermore, important downstream effectors of Ras were similarly rescued in Nf1mKO+GRD. With these measures of biochemical rescue, we also showed that there was a significant reduction in the severity of the cardiac dysfunction and hypertrophy, although rescue of the cardiac hypertrophy and heart failure phenotype was incomplete.

Neurofibromin functions as a Ras-GAP,5 and our results are consistent with hyperactivation of the Ras-Erk pathway in Nf1mKO hearts. Ras activation has been previously implicated in cardiac hypertrophy and Ras functions downstream of several known cardiac agonists (such as β-adrenergic agonists) that stimulate hypertrophic responsiveness.15,24,25 However, we are not aware of previous reports that demonstrate a role for neurofibromin as a critical modulator of Ras activity in cardiomyocytes of the adult heart.

Hypertrophic cardiomyopathy is not a ubiquitous feature of NF1, although case reports have documented the coexistence of these disorders.3,26,27 Nevertheless, available data are insufficient to determine whether cardiac hypertrophy occurs more commonly in NF1 patients than in the general population. NF1 individuals inherit one mutated copy of NF1,
whereas the animals evaluated in our study were completely, or nearly completely, deficient in neurofibromin within cardiac myocytes.

A role for Ras activation in humans with cardiac hypertrophy is more clearly evident from the evaluation of patients with Noonan, Costello, Leopard, and cardiofacial cutaneous syndromes. Each of these syndromes is strongly associated with cardiovascular defects including hypertrophic cardiomyopathy, and each can be caused by activating mutations in components of the Ras-ERK pathway.28–30 In fact, these syndromes together with NF1 have been termed the “Ras/Mapk syndromes” to indicate their mechanistic and phenotypic overlap.31 Interestingly, several of these syndromes share with NF1 the common occurrence of additional features thought to result directly from Ras activation, including, for example, juvenile myelomonocytic leukemia.32 In addition to Ras pathway mutations, Noonan, Leopard, and cardiofacial cutaneous syndromes can also be caused by mutations in PTPN11 encoding the tyrosine phosphatase Shp2, and it is therefore likely that Shp2 functions in the Ras/ERK pathway in multiple tissues including cardiac myocytes, consistent with animal studies.33

The Ras/ERK signaling pathway undoubtedly interacts with other signaling pathways within cardiac myocytes to allow for integrated responses to extracellular stimuli, including stretch and receptor-mediated activation. For example, we noted activation of the Akt, GSK3β, and mTOR in Nf1 mKO hearts. Neurofibromin has previously been shown to regulate Akt and mTOR in other tissues,34 and these pathways have been implicated in the regulation of cardiac hypertrophy by numerous investigators.13,35 Ras signaling has also been shown to regulate NFAT activity in cardiac myocytes,36 and NFAT/calcineurin signaling has been suggested as a therapeutic target for cardiac hypertrophy and heart failure.13,37 Our data are strongly suggestive of an important role for neurofibromin in cardiac myocytes as a modifier of cardiac hypertrophy by numerous investigators.13,35 Ras activity is modified by 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (ie, statins). These medications have been shown to inhibit Ras activity through prevention of its normal lipid modification38 and have been considered as treatment options for Ras-related pathologies in NF1.39 Typically used to treat hypercholesterolemia and atherosclerosis, preliminary work suggests that statins may have a role in heart failure therapies.40 It will be interesting to determine whether statins function in cardiac myocytes to modulate NFI-mediated Ras activation.

Our evaluation of cardiac-specific NFI knockout mice, combined with genetic rescue, suggests that neurofibromin is an important regulator of Ras signaling in cardiac myocytes and that Ras activation can lead to progressive cardiac hypertrophy with associated pathological changes, including fibrosis, dilatation, and decreased systolic and diastolic function over time. Loss of NFI may sensitize the heart to the naturally occurring external stimuli of daily activities and physiological responses such as adrenergic tone and circulating growth factors. This could account for the gradual onset of cardiac pathology in adult mice despite loss of NFI during embryogenesis. Our data provide further evidence for commonalities among the Ras/Mapk syndromes31 and suggest that hypertrophic cardiomyopathy can result from loss of function of neurofibromin. Additionally, this work provides further evidence for concerns regarding potential cardiovascular side effects of drugs that target the Ras pathway (such as farnesyl-transferase inhibitors). Conversely, appropriate modulation of Ras signaling or neurofibromin function may offer therapeutic opportunities in patients with hypertrophic cardiomyopathies and heart failure.

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Disclosures
None.

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Supplement Material

Histology

Masson’s Trichrome stain was used for histology and assessment of cardiac fibrosis, and wheat germ agglutinin (WGA) staining was used to highlight the sarcolemma. For WGA staining, 100 µg/ml of TRITC-labeled WGA (Sigma) was used to stain paraffin-fixed sections of hearts from wild type and Nf1mKO mice.

For fibrosis quantification, 5-10 randomly selected high-power (20×) fields were selected from the ventricles of control and Nf1mKO mice after Trichrome staining. For myocyte cross-sectional area measurements, ~1000 WGA-stained cardiac myocytes were measured from the ventricles of control and Nf1mKO mice. NIH ImageJ software version 1.40g (http://rsb.info.nih.gov/ij/) was used for both histological quantifications.

RT-PCR and Real-time PCR primer sequences

The following primer sets were used for RT-PCR and Real-time PCR: atrial naturetic peptide (ANP): 5’ GCTTCCAGGCCATATGGAGCAAA 3’ (forward) and 5’ TGACCTCATCTTCTACCGCATCT 3’ (reverse); brain-type naturetic peptide (BNP): 5’ AATGGCCCAGAGACAGCTCTTGAA 3’ (forward) and 5’ CTTGTGCCCCAAAGCAGCTTGGAGAT 3’ (reverse); and β-myosin heavy chain (βMHC): 5’ CCCTCCTCACATCTTCTCCATCTCTG 3’ (forward) and 5’ CCCTCCTTAGTGACAGCTTCCCAGC 3’ (reverse); GAPDH: 5’
CGGAGTCAACGGATTTGGTCGTAT 3’ (forward) and 5’ AGCCTTCTCCATGGTGGAAGAC 3’ (reverse).

**Online Figure I:** Hyperactivation of Erk, Akt, and GSK3β pathways in *Nf1* mKO adult hearts

**Online Figure I:** Hyperactivation of Erk, Akt, and GSK3β pathways in *Nf1* mKO adult hearts. Immunoblot of total and phospho-Erk, Akt, and GSK3β of 3 independent whole-heart homogenates from adult (12 week) control and *Nf1* mKO mice. There is increased phospho-Erk and Akt, consistent with hyperactivation of the Ras signaling pathway in *Nf1* mKO. There is also increased phospho-GSK3β in *Nf1* mKO, consistent with decreased activation of the GSK3β pathway. GAPDH (loading control) is equivalent in all samples.
Online Table I: \textit{Nf1}mKO mice develop progressive ventricular chamber enlargement and dysfunction: echocardiographic analysis

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<tr>
<td>LVEF (%)</td>
<td>64±7</td>
<td>50±7*</td>
<td>62±3</td>
<td>32±7*</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>34±5</td>
<td>25±7*</td>
<td>32±2</td>
<td>16±5**</td>
</tr>
</tbody>
</table>

*p<0.01

**p<0.03
Online Table I: *Nf1* mKO mice develop progressive ventricular chamber enlargement and dysfunction. Echocardiographic analysis was performed on *Nf1* mKO and control mice at 12 and 20 weeks. We did not observe significant differences in interventricular septum (IVSd and IVSs) or left ventricular posterior wall (LVPWd and LVPWs) thickness between *Nf1* mKO and control. However, we observed a significantly enlarged left ventricular chamber at both end-systole (LVIDs) and end-diastole (LVIDd), as well as reduced left ventricular ejection (LVEF) and shortening (LVSF) in *Nf1* mKO. These differences are significant at 12 weeks, and marked by 20 weeks.
Online Table II: No substantial differences between wild type and α-MHC-cre mice echocardiographic measures at 20 weeks

<table>
<thead>
<tr>
<th></th>
<th>20 weeks</th>
<th></th>
<th></th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type (n=5)</td>
<td>α-MHC-cre (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.84±0.07</td>
<td>0.67±0.01</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>0.95±0.07</td>
<td>0.84±0.09</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.75±0.08</td>
<td>0.73±0.07</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>0.93±0.06</td>
<td>0.98±0.01</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.69±0.09</td>
<td>4.16±0.17</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.49±0.08</td>
<td>2.94±0.19</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>62±3</td>
<td>54±1</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>32±2</td>
<td>28±1</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>81.5±11</td>
<td>83.9±5.2</td>
<td>0.84</td>
<td></td>
</tr>
</tbody>
</table>
Online Table II: No substantial differences between wild type and α-MHC-cre mice echocardiographic measures at 20 weeks. Echocardiographic comparison of wild type (n=5) and α-MHC-cre (n=3) mice at 20 weeks. There did not appear to be any significant differences in interventricular septum (IVSd and IVSs) and left ventricular posterior wall (LVPWd and LVPWs) thickness, as well as left ventricular systolic and diastolic dimensions (LVIDs and LVIDd). Left ventricular ejection fraction (LVEF) was slightly lower in α-MHC-cre (p=0.04), but fractional shortening (LVFS) was not significantly different between wild type and α-MHC-cre.
**Online Table III: Nf1mKO mice develop progressive systolic and diastolic dysfunction: invasive hemodynamic analysis**

<table>
<thead>
<tr>
<th></th>
<th>12 weeks control (n=4)</th>
<th>12 weeks Nf1mKO (n=4)</th>
<th>20 weeks control (n=7)</th>
<th>20 weeks Nf1mKO (n=5)</th>
<th>Nf1mKO +GRD (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>532±21</td>
<td>503±12</td>
<td>516±6</td>
<td>489±17</td>
<td>503±3</td>
</tr>
<tr>
<td>Maximum Pressure (mmHg)</td>
<td>127±5</td>
<td>106±5*</td>
<td>119±5</td>
<td>97±5</td>
<td>102±13</td>
</tr>
<tr>
<td>End-systolic Pressure (mmHg)</td>
<td>115±5</td>
<td>95±5</td>
<td>109±5</td>
<td>96±9</td>
<td>97±14</td>
</tr>
<tr>
<td>End-diastolic Pressure (mmHg)</td>
<td>12±2</td>
<td>11±2</td>
<td>12±2</td>
<td>16±2</td>
<td>9±2****</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>70±1</td>
<td>59±10</td>
<td>62±6</td>
<td>21±6*</td>
<td>44±6****</td>
</tr>
<tr>
<td>Cardiac Output (µl/min)</td>
<td>12537±492</td>
<td>8756±1889</td>
<td>10349±1648</td>
<td>6287±1157*</td>
<td>9171±1257</td>
</tr>
<tr>
<td>Stroke Work (mmHg×µl)</td>
<td>2443±72</td>
<td>1499±382</td>
<td>1858±212</td>
<td>922±223*</td>
<td>1449±329</td>
</tr>
<tr>
<td>dP/dt max (mmHg/sec)</td>
<td>14162±1219</td>
<td>9990±1010*</td>
<td>10396±592</td>
<td>4949±886*</td>
<td>7374±1363</td>
</tr>
<tr>
<td>dP/dt min (mmHg/sec)</td>
<td>-10197±310</td>
<td>-6927±530**</td>
<td>-9151±688</td>
<td>-4039±719*</td>
<td>-6619±1103</td>
</tr>
<tr>
<td>τ (msec)</td>
<td>8.4±0.2</td>
<td>11.4±0.7**</td>
<td>9.8±0.5</td>
<td>24.8±3.5**</td>
<td>11.5±0.8***</td>
</tr>
</tbody>
</table>
*p<0.04

**p<0.01

*** control vs. NflmKO+GRD p<0.06, NflmKO vs. NflmKO+GRD p<0.01

**** control vs. NflmKO+GRD p<0.03, NflmKO vs. NflmKO+GRD p<0.01

***** NflmKO vs. NflmKO+GRD p<0.04

**Online Table III:** *NflmKO* mice develop progressive systolic and diastolic dysfunction partially rescued by GRD. Invasive hemodynamic assessment of control and *NflmKO* mice at 12 weeks and control, *NflmKO*, and *NflmKO*+GRD mice 20 weeks demonstrates progressive loss of both systolic and diastolic performance in *NflmKO* that is partially rescued in *NflmKO*+GRD. At 12 weeks, *NflmKO* shows statistically significant decreases in maximal dp/dt and increases in minimal dp/dt and τ. There was also a trend toward a reduced ejection fraction and total cardiac output. By 20 weeks the changes in both maximal dp/dt, minimal dp/dt, and τ in *NflmKO* had exacerbated and were accompanied by statistically significant reductions in ejection fraction, cardiac output, and stroke work. For *NflmKO*+GRD at 20 weeks there was phenotype that was intermediate to control and *NflmKO*, as demonstrated by a slightly reduced ejection fraction with a preserved cardiac output, and values for maximal dp/dt, minimal dp/dt, and τ that were also between control and *NflmKO*. 