Molecular Cardiology

Tumor Suppressor Ras-Association Domain Family 1 Isoform A Is a Novel Regulator of Cardiac Hypertrophy

Delvac Oceandy, MD, PhD*; Adam Pickard, PhD*; Sukhpal Prehar, MSc; Min Zi, MD; Tamer M.A. Mohamed, PhD; Peter J. Stanley, BSc; Florence Baudoin-Stanley, MRes; Raja Nadif, PhD; Stella Tommasi, PhD; Gerd P. Pfeifer, PhD; Angel L. Armesilla, PhD†; Elizabeth J. Cartwright, PhD†; Ludwig Neyses, MD†

**Background**—Ras signaling regulates a number of important processes in the heart, including cell growth and hypertrophy. Although it is known that defective Ras signaling is associated with Noonan, Costello, and other syndromes that are characterized by tumor formation and cardiac hypertrophy, little is known about factors that may control it. Here we investigate the role of Ras effector Ras-association domain family 1 isoform A (RASSF1A) in regulating myocardial hypertrophy.

**Methods and Results**—A significant downregulation of RASSF1A expression was observed in hypertrophic mouse hearts, as well as in failing human hearts. To further investigate the role of RASSF1A in cardiac (patho)physiology, we used RASSF1A knock-out (RASSF1A−/−) mice and neonatal rat cardiomyocytes with adenoviral overexpression of RASSF1A. Ablation of RASSF1A in mice significantly enhanced the hypertrophic response to transverse aortic constriction (64.2% increase in heart weight/body weight ratio in RASSF1A−/− mice compared with 32.4% in wild type). Consistent with the in vivo data, overexpression of RASSF1A in cardiomyocytes markedly reduced the cellular hypertrophic response to phenylephrine stimulation. Analysis of molecular signaling events in isolated cardiomyocytes indicated that RASSF1A inhibited extracellular regulated kinase 1/2 activation, likely by blocking the binding of Raf1 to active Ras.

**Conclusions**—Our data establish RASSF1A as a novel inhibitor of cardiac hypertrophy by modulating the extracellular regulated kinase 1/2 pathway. (Circulation. 2009;120:607-616.)

Key Words: hypertrophy • signal transduction • heart failure

Myocardial hypertrophy is a key event in the pathogenesis of heart failure. This condition has conventionally been regarded as a compensatory mechanism to balance pathological load; however, clinical and basic science evidence suggests that it is a powerful risk factor in the development of heart failure.1 Controlling myocardial hypertrophy and left ventricular (LV) remodeling has been shown to have a beneficial effect, both in an experimental model2 and in patients with chronic hypertensive heart disease and hypertrophy.3 Therefore, numerous studies have focused on characterizing signal transduction pathways that are associated with cardiac hypertrophy.

Clinical Perspective on p 616

Ras, a family of low-molecular-weight guanine nucleotide binding proteins, has long been associated with hypertrophy: many prohypertrophic stimuli activate Ras,4,5 whereas cell transfection/injection with activated Ras will promote changes in gene expression and cell structure, characteristic of cellular hypertrophy.6 Furthermore, transgenic overexpression of active Ras results in cardiac hypertrophy and failure.7 To date, more than 15 Ras effectors/interacting proteins have been identified,8 including the Ras-association domain family 1 (RASSF1). RASSF1 is a member of the Ras family of tumor suppressor proteins (reviewed in9), which consist of at least 10 different proteins, RASSF1 through RASSF10. Seven isoforms of RASSF1 have been recognized, RASSF1A through RASSF1G, which are derived from alternative splicing and different promoter usage. The major splice variants of RASSF1 are RASSF1A and RASSF1C,10 which differ only in their N termini. RASSF1A was first described in lung cancer,11 and its expression is reduced in many cancer types.11

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From the Cardiovascular Medicine Research Group, School of Clinical and Laboratory Sciences, University of Manchester, Manchester Academic Health Science Centre, Manchester, United Kingdom (D.O., A.P., S.P., M.Z., T.M.A.M., P.J.S., F.B.-S., R.N., E.J.C., L.N.); Division of Biology, Beckman Research Institute of the City of Hope, Duarte, Calif (S.T., G.P.P.); Molecular Pharmacology Group, Research Institute in Healthcare Sciences, School of Applied Sciences, University of Wolverhampton, Wolverhampton, United Kingdom (A.L.A.); and Biochemistry Department, Faculty of Pharmacy, Zagazig University, Egypt (T.M.A.M.).

*The first 2 authors contributed equally to this work.
†The last 3 authors are equal senior authors.

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RASSF1A appears to modulate multiple cellular processes, including apoptosis, cell cycle control, cell motility, and maintenance of genomic stability. Recent evidence has suggested that RASSF1A acts as scaffolding protein so that it can assemble and modify the function of the interacting protein complex. Thus despite the lack of its enzymatic activity, the ability to interact with other molecules is very important for RASSF1A to exert its function.11 This molecule is indeed characterized by the presence of a Ras association (RA) domain followed by a Salvador Rassf Hippo (SARAH) domain at the C-terminal segment, which are responsible for protein–protein interaction.8 RASSF1A has been reported to bind to active Ras (Ras.GTP) via heterodimerization with Nore1/RASSF5 (the founding member of the Rassf family).12 indicating a possible role as a Ras effector. In addition, a number of candidate downstream effectors of Rassf1 have been proposed, including the Mst kinases, which interact with RASSF1A via its SARAH domain.13,14

Expression of RASSF1A has been detected in human heart tissue10; however, its role in cardiomyocytes remains unclear. Recently, our group has demonstrated that in cardiomyocytes, RASSF1A is colocalized with the sarcolemmal calcium pump.15 Co-expression of RASSF1A with sarcolemmal calcium pump 4b inhibited extracellular regulated kinase (ERK) activation, a major downstream effector of Ras, in response to epidermal growth factor,15 suggesting a possible role for RASSF1A in regulating the hypertrophic pathways downstream of Ras in cardiomyocytes.

Methods

Animal and Human Subjects

Mice with targeted deletion of exon1α of the Rassf1 gene to specifically knock out RASSF1A expression were used in this study.16 We used 8- to 10-week-old male RASSF1A knock-out mice for pressure-overload induced hypertrophy experiments. For baseline morphology and physiological analyses, 12-week-old animals were used. All animals were maintained on a 129S3/SvImJ background. Age- and sex-matched wild-type litter mates were used as controls. Animal studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. Human heart failure tissue samples and tissues from normal subjects were obtained from Asteraid, who obtained informed consent for the use of human tissue samples and approval by the United Kingdom Human Tissue Authority.

Cardiac Hypertrophy Model

To generate a model of cardiac pressure overload, mice were subjected to transverse aortic constriction (TAC) or a sham operation for 5 weeks using method as previously described.17 TAC was performed using a 7-0 silk suture that was tied to a 27-gauge constriction at the aortic arch, which will produce ~25 to 30 mm Hg of pressure gradient between the left and right carotid artery. Please see the online-only Data Supplement for the histology analysis after TAC.

In Vivo Physiology Analysis

In vivo hemodynamic analyses were performed using a pressure-volume system (Millar Instruments). We used a 1.4F pressure-volume catheter (SPR-839) using a protocol described previously.18 For cardiac morphological analysis, transthoracic 2-dimensional and M-Mode echocardiography were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously.18

Western Blot

Total heart protein lysates were prepared using extraction buffer as described elsewhere.18 Western blot analysis was conducted using a standard protocol as previously described.18 Details of antibodies used for Western blots are provided in the online-only Data Supplement. Also see the online-only Data Supplement for RNA analyses by reverse transcriptase polymerase chain reaction and Northern blot.

Adenovirus

Adenovirus overexpressing RASSF1A was generated by cloning human RASSF1A cDNA (a kind gift from Dr Geoffrey Clark, Louisville, Ky) to pAdEasy using the shuttle system containing the cytomegalovirus (CMV) promoter (pshuttle CMV) as recommended by the manufacturer (Stratagene). The brain natriuretic peptide–luciferase construct was cloned using a similar system.

Neonatal Rat Cardiomyocyte Experiments

Neonatal rat cardiomyocytes (NRCMs) were isolated from 1- to 3-day-old Sprague-Dawley rat neonates as previously described.19 Cells were maintained in medium containing 1% fetal bovine serum before experimentation. Immunofluorescence was conducted using a standard protocol as described.19 Please see the online-only Data Supplement for details of cellular hypertrophy experiments.

RASSF1A Deletion Mutants

We used polymerase chain reaction to generate RASSF1A-ΔC (containing amino acid residue 1 to 289), RASSF1A-ΔC+RA (containing amino acid residue 1 to 139), and RASSF1A-ΔN (containing amino acid residue 132 to 340). Fragments were then cloned to p3XFlagCMV7.1 (Sigma). CMV-RasWT and CMV-RasV12 constructs were obtained from Clontech. Adenoviruses overexpressing RASSF1A deletion mutants were generated using the Gateway system (Invitrogen). Experiments using deletion mutant proteins were conducted in the H9c2 ventricular myoblast cell line and NRCMs. H9c2 cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin. Transfections were conducted using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s protocol. For affinity precipitation analysis, cell lysates were precipitated using Raf1-RBD agarose (glutathione-S-transferase fusion-protein, corresponding to Ras binding domain of Raf-1, bound to glutathione agarose; Upstate) for 24 hours at 4°C following the manufacturer’s recommendation. Precipitated proteins were subjected to Western blot using anti-Ras antibody (Sigma). For the Elk-1 activity assay, we used the PathDetect Elk1 trans-Reporting System (Stratagene) following methods described previously.19

Apoptosis Assay

Apoptosis measurements were conducted by analyzing cleaved caspase-3 and Bak N-terminal conformational change expression using the High Content Assay system (Imagen Biotech, Manchester, United Kingdom).

Data Analysis

Data are expressed as mean±SEM and analyzed using the Student t test or 2-way ANOVA where appropriate. The criterion of statistical significance was P<0.05.

Results

RASSF1A Expression Was Downregulated in Cardiac Hypertrophy and Failure

Expression of RASSF1A was detected in the heart and in isolated NRCMs (online-only Data Supplement Figure I),
where RASSF1A was found by immunofluorescence to be mostly localized in the cytoplasm (Figure 1A). To assess whether RASSF1A was differentially expressed in cardiac hypertrophy, we examined RASSF1A expression in the hearts of wild-type mice subjected to TAC for 12 weeks, which resulted in a 43% increase in heart weight/body weight ratio. Expression of RASSF1A was downregulated by 55% (Figure 1B, n=4, *P=0.02), suggesting the possible involvement of this protein in cardiac cell growth and hypertrophy. However, RASSF1A expression was not changed in NRCMs treated with phenylephrine (30 μmol/L, 72 hours; online-only Data Supplement Figure I).

Consistent with the data from the animal model, RASSF1A expression was downregulated in failing human heart. A reduction by 50% was observed in patients with end-stage heart failure compared with normal controls (Figure 1C, n=3, *P=0.03). Immunofluorescence analysis on adult normal heart sections showed focal and extranuclear expression of RASSF1A, whereas in failing heart sections, less RASSF1A expression was observed (Figure 1D). Expression of the other major splice variant, RASSF1C, was also detected in the heart; however, its expression did not change in the hypertrophic hearts, nor in phenylephrine treated NRCMs (online-only Data Supplement Figure I).

**Rassf1a Gene Deletion Enhanced the Cardiac Hypertrophic Response to Pressure Overload**

To further study whether RASSF1A is involved in the regulation of cardiac hypertrophy, we used mice with a genetic ablation of the *Rassf1a* gene (RASSF1A knock-out mice). RASSF1A knock-out animals did not show any difference in cardiac morphology, size, and hemodynamic function basally (online-only Data Supplement Table I). We then subjected these mice to TAC-induced pressure overload for 5 weeks to look at the possible role of RASSF1A in pathological hypertrophy. As shown in Figure 2A, RASSF1A-deficient animals displayed a marked increase in cardiac hypertrophy compared with wild-type littermates. Measurement of heart weight/body weight ratio indicated that RASSF1A−/− mice showed a 64.2% increase in heart weight/body weight ratio, whereas wild-type mice displayed a 32.4% increase in heart weight/body weight ratio (Figure 2B, n=10 in each group, 2-way ANOVA test indicated that there was a significant effect of TAC (*P<0.001) and an interaction between the 2 factors tested [genotype versus TAC]; *P=0.03). Analysis of cardiomyocyte cross-sectional area from the histological sections revealed a significantly larger cell size in knock-out mice after TAC (Figure 2C and 2D). In addition, pressure overload–induced hypertrophy in knock-out animals was accompanied by extensive fibrosis, as indicated by Masson’s trichrome staining (11.6% of fibrosis in wild-type TAC versus 22.1% in knock-out TAC, Figure 2E and 2F). Furthermore, the expression of β myosin heavy chain, atrial natriuretic peptide, and brain natriuretic peptide (markers of pathological hypertrophy) were significantly higher in TAC-operated knock-out mice compared with TAC-operated wild-type mice (Figure 2G and 2I).

**RASSF1A Deficiency Led to LV Chamber Dilatation After TAC**

Echocardiographic analysis was performed to evaluate LV geometric remodeling in response to TAC (Figure 3A). Although the intraventricular septum and posterior wall thickness were significantly increased in both knock-out and wild-type mice after TAC (Figure 3B and 3C),
RASSF1A knock-out mice displayed substantial LV chamber enlargement 5 weeks after TAC (Figure 3D and 3E). This indicated a pattern of eccentric hypertrophic remodeling in knock-out mice. However, LV fractional shortening was not different between knock-out and wild-type controls after TAC (Figure 3F).

Analysis of Hemodynamic Function in TAC-Operated Mice
Assessment of cardiac function was carried out using invasive hemodynamic analysis. Pressure-volume loops were studied at baseline and after transient inferior cava vein occlusion to measure preload independent contractility indices. As shown in Figure 3G, a more pronounced rightward shift of the pressure-volume loops was observed in TAC-operated knock-out mice compared with wild-type mice, consistent with the LV dilatation and the eccentric hypertrophic remodeling. However, the maximal rate of developed pressure, as well as the speed of relaxation, were not significantly different (Figure 3H and 3I). Also, the end-systolic pressure-volume relationship was not different between knock-out and wild-type mice after TAC (end-systolic pressure-volume relationship values, wild-type TAC, 6.72±1.54 mm Hg/μL, knock-out TAC, 6.19±0.99 mm Hg/μL).

RASSF1A Overexpression Blunted Phenylephrine-Induced Hypertrophy In Vitro
To investigate the mechanism by which RASSF1A regulates cardiac hypertrophy, we studied its effect in NRCMs. We generated an adenovirus that overexpressed the RASSF1A gene under the control of the CMV promoter. Expression of exogenous RASSF1A protein in NRCMs was detected by Western blot (Figure 4A).

Stimulation with 30 μmol/L of phenylephrine for 72 hours induced cellular hypertrophy in NRCMs as indicated by cell size and cellular protein (measured by 3H-leucine incorporation and total protein content). Overexpression of RASSF1A markedly suppressed the hypertrophic response: analyses of cell size, 3H-leucine incorporation, and total protein content showed a significant decrease in cardiomyocyte growth in cells overexpressing RASSF1A (Figure 4B through 4E). Moreover, expression of the...
hypertrophic marker atrial natriuretic peptide was also decreased in RASSF1A-overexpressing cells in response to phenylephrine induction (online-only Data Supplement Figure II). Activation of the brain natriuretic peptide promoter was assessed by coinfecting RASSF1A-overexpressing NRCMs and control cells using an adenovirus-encoding luciferase under the control of the brain natriuretic peptide promoter. Phenylephrine treatment resulted in a 25- to 30-fold induction of luciferase activity in control cells; however, in RASSF1A-overexpressing cells, the induction of luciferase expression was significantly \( (P < 0.04) \) reduced by 43% compared with the control (online-only Data Supplement Figure II). These results are consistent with the in vivo data and suggest the role of RASSF1A as an inhibitor of cardiomyocyte growth.

Inhibition of ERK1/2 Activation by RASSF1A

To further elucidate the signaling pathways regulated by RASSF1A, we investigated the activation of protein kinases downstream of Ras in NRCMs expressing RASSF1A after brief stimulation with phenylephrine (5 to 15 minutes) (Figure 5A). Activation of Raf1 (indicated by the level of phosphorylation at Ser338) was significantly \( (P = 0.04) \) inhibited in cells overexpressing RASSF1A after 15 minutes of stimulation (Figure 5B). Raf1 has a high affinity to bind to active Ras, and this binding will subsequently translocate Raf1 to the membrane, where further interaction and modification (including phosphorylation at Ser338 and Tyr341) leads to its activation.\(^{19}\) Furthermore, the level of phenylephrine-induced phosphorylation of ERK1/2, which is downstream of Raf1, was also reduced in RASSF1A-expressing myocytes (Figure 5C and 5D).

c-Jun N-terminal kinases and Akt activation, which are also downstream of Ras, were also examined. No differences of c-Jun N-terminal kinases and Akt phosphorylation in NRCMs overexpressing RASSF1A were observed (online-only Data Supplement Figure III), suggesting a specific regulation of the Raf1-ERK1/2 pathway by RASSF1A.

We next analyzed the activation of hypertrophic signaling pathways in the TAC-operated mice. We focused on several downstream effectors of Ras, such as mitogen-activated protein kinases and Akt, which are activated during TAC.\(^{20}\) Ablation of RASSF1A slightly but significantly enhanced the activation of Raf1 and ERK1/2 (Figure 5E through 5I, n = 5), whereas activation of c-Jun N-terminal kinases, p38 mitogen-activated protein kinase, and Akt were not different between knock-out and wild-type mice (online-only Data Supplement Figure IV), indicating a specific regulation of the Raf1-ERK1/2 pathway by RASSF1A in the TAC model.
RASSF1A Inhibits the Binding of Raf1-RBD to Active Ras Depending on its N-Terminal Domain

To study the exact mechanism of Raf1-ERK1/2 inhibition by RASSF1A, we generated deletion mutants of RASSF1A proteins. We generated RASSF1A-H9004C which lacks 53 amino acids at the carboxy terminal region, thereby removing the SARAH domain, which is responsible for binding with Mst kinases.13 We also generated RASSF1A-H9004C/H11001RA, which lacks both the RA (Ras binding) and SARAH domains. To look at the importance of the N-termini region, we used the mutant RASSF1A-H9004N, lacking the N-termini region (amino acids 1 to 132), which is responsible for heterodimeriza-
tion with Nore1\textsuperscript{12} and hence binding with Ras.GTP. A schematic diagram of the mutant proteins is presented in Figure 6A. All mutant proteins were tagged with the Flag epitope, and expression was detected using an anti-Flag antibody (Figure 6B).

We conducted affinity precipitation experiments using Raf1-RBD agarose (glutathione-S-transferase fusion-protein, corresponding to Ras binding domain of Raf-1, bound to glutathione agarose) to test whether RASSF1A inhibits signal transmission from Ras to Raf1. We used a ventricular myoblast cell line and transfected it with mutant active RasV12 together with either full-length or truncation mutants of RASSF1A. After 24 hours, cell lysates were precipitated using Raf1-RBD agarose, and the precipitated proteins were subjected to Western blot to detect Ras. We found that full-length RASSF1A, RASSF1A-\texttt{H9004C}, and RASSF1A-\texttt{H9004C/H11001RA} reduced the binding of active Ras to Raf1-RBD, as indicated by a reduction in the amount of precipitated Ras. However, the RASSF1A-\texttt{H9004N} did not inhibit this binding (Figure 6C). Moreover, immunoprecipitation using an anti-Ras antibody followed by Western blot detection using anti-Flag antibody indicated that only the RASSF1A-\texttt{H9004N} mutant did not interact with RasV12 (bottom panel). (D) Western blot to detect ERK1/2 phosphorylation in ventricular myoblast cells transfected with the indicated vectors. (E) To examine Elk-1 activation, ventricular myoblast cells were transfected with pFA2-Elk1 and pRL-Luc reporter system as well as other indicated constructs. Luciferase activity was measured 24 hours after transfection (n=10, **P<0.001).

**Figure 6.** RASSF1A inhibits the binding of Raf1-RBD to active Ras. (A) Schematic diagram of flag-tagged RASSF1A deletion mutants used in affinity precipitation experiments. (B) Western blot (WB) using anti-Flag antibody showing the expression of mutant proteins in ventricular myoblast cells transfected with the indicated constructs. (C) Affinity precipitation using Raf1-RBD agarose in ventricular myoblast cells transfected with the indicated vectors. Precipitated proteins were detected by Western blot with anti-Ras antibody (top panel). Total protein was also analyzed for Ras expression by Western blot (middle panel). Immunoprecipitation using an anti-Ras antibody followed by Western blot detection using anti-Flag antibody indicated that only the RASSF1A-\texttt{H9004N} mutant did not interact with RasV12 (bottom panel). (D) Western blot to detect ERK1/2 phosphorylation in ventricular myoblast cells transfected with the indicated vectors. (E) To examine Elk-1 activation, ventricular myoblast cells were transfected with pFA2-Elk1 and pRL-Luc reporter system as well as other indicated constructs. Luciferase activity was measured 24 hours after transfection (n=10, **P<0.001).

**Inhibition of ERK1/2 Pathway by RASSF1A in Cells Overexpressing Mutant Active RasV12**

To examine the ERK1/2 activation, we analyzed ERK phosphorylation in H9c2 cells overexpressing RasV12 and the RASSF1A truncation mutants. As shown in Figure 6D, ERK1/2 phosphorylation was reduced in cells cotransfected with full-length RASSF1A, RASSF1A-ΔC, and RASSF1A-ΔC+RA, but not in cells expressing RASSF1A-ΔN. Furthermore, a luciferase-based assay system was used to examine the Elk-1 activation. A slight induction of luciferase signal was observed after transfection with RasWT; however, the constitutively active mutant RasV12 induced the luciferase signal by ≈10-fold. Coexpression with full-length RASSF1A or RASSF1A-ΔC inhibited this induction by 65%, whereas RASSF1A-ΔC+RA inhibited it by ≈40%. However, no inhibition was observed when cells were cotransfected with RASSF1A-ΔN (Figure 6E). These data strengthen the results from the affinity precipitation experiment, showing that RASSF1A not only inhibited binding of active Ras to Raf1-RBD, but also reduced the activation of the downstream ERK1/2 pathway.

**Inhibition of Endogenous Ras in NRCM**

Adenoviruses were generated to overexpress truncated RASSF1A mutants in NRCMs (Figure 7A). To examine the effect on endogenous Ras-Raf1 pathway activation, we treated NRCM with phenylephrine (30 μmol/L) for 3 minutes, followed by affinity precipitation of cell lysates using anti-Raf1-RBD agarose. In keeping with the results from the ventricular myoblast cells, NRCMs overexpressing full-length RASSF1A, RASSF1A-ΔC, and RASSF1A-ΔC+RA displayed reduced binding of Ras to Raf1-RBD, both before and after phenylephrine stimulation, where-
as no inhibition was shown in cells overexpressing RASSF1A-N (Figure 7B).

RASSF1A Expression Does Not Promote Apoptosis in NRCM

In noncardiac cells, RASSF1A has been described as a regulator of apoptosis and/or cell death. Cardiomyocytes overexpressing RASSF1A showed no increase in size or protein synthesis in response to phenylephrine. This may indicate an antihypertrophic function of RASSF1A, or it could be attributed to increased cell death. To analyze the level of apoptosis, we stained NRCMs for cleaved caspase-3 and Bak (N-termini conformational change) (online-only Data Supplement Figure VA and VB). We found that overexpression of RASSF1A did not increase the levels of apoptosis (online-only Data Supplement Figure VC). Similarly, RASSF1A overexpressing cells did not show increased susceptibility to the proapoptotic agent staurosporine (online-only Data Supplement Figure VD), as indicated by cleaved caspase-3 detection. Also, no difference in Bak (N-termini conformational change) levels in cells treated with phenylephrine (30 μmol/L, 72 hours) was observed (online-only Data Supplement Figure VE), suggesting that the inhibition of phenylephrine-induced hypertrophy in RASSF1A overexpressing cells is likely due to an alteration in the hypertrophic response and is not due to increased apoptosis.

Discussion

The key finding of our study is that RASSF1A is a pivotal protein in regulating cardiac hypertrophy in mouse and possibly in humans. Its absence causes increased hypertrophy and remodeling under conditions of sustained mechanical stress by increased activation of the ERK1/2 signaling pathway. Essentially, RASSF1A is a potent inhibitor of cardiac hypertrophy, which inhibits the prohypertrophic Raf1-ERK1/2 pathway.

In our present observations, RASSF1A expression is downregulated under conditions of cardiac hypertrophy and failure, both in mouse model and in humans. RASSF1A deficiency enhanced the pressure overload–induced hypertrophy in mice, which is accompanied by extensive fibrosis and increased fetal gene activation. Echocardiography and hemodynamic assessments indicated a significant enlargement of the LV chamber in TAC-stimulated knock-out mice, which is an early sign of dilated cardiomyopathy and could result in heart failure. Consistent with the in vivo data, RASSF1A overexpression in neonatal rat cardiomyocytes inhibited phenylephrine-induced cellular hypertrophy without affecting apoptosis.

Our observations indicate that in cardiomyocytes, RASSF1A regulates the Ras-Raf1-ERK1/2 pathway. Two important findings are as follows: (1) excess RASSF1A expression blocked the binding of active Ras to Raf1-RBD and hence inhibited Raf1-ERK1/2 activation, and (2) the N-terminal region of RASSF1A might be responsible for this inhibitory action. Because the N-terminal region is important for binding/heterodimerization with Nore1/RASSF5, the data indicate that indirect binding with Ras,GTP via Nore1/RASSF5 might be the mechanism of the RASSF1A inhibitory action. Activation of the Raf1-ERK1/2 pathway by Ras,GTP requires complex regulation involving a number of scaffolding molecules, such as suppressor of Ras (SUR-8), kinase suppressor of Ras (KSR), and connector enhancer of kinase suppressor of Ras (CNK). The balance of expression between these molecules appears crucial during the signal transmission; for example, suppressor of Ras, when ectopically expressed, will facilitate the formation of a functional Ras,GTP/Raf complex, whereas another Ras-binding molecule, the impedes mitogenic signal propagation, inhibited Raf-ERK1/2 signaling when overexpressed in noncardiac cells. Our results indicate that in the cardiomyocytes, RASSF1A may be part of the complex assembly during Raf1-ERK1/2 activation, and its level of expression may affect the signal transmission from Ras,GTP to Raf1.

Recent data have shown that inhibition of ERK1/2 activity did not completely abolish cardiac hypertrophy in response to pressure overload. Although this observation suggests that ERK1/2 is not the unique factor and other
signaling pathways might be sufficient to mediate pathological hypertrophy in the absence of ERK1/2, it is important to note that in this study, the authors used transgenic overexpression of dual-specific phosphatases to inhibit ERK1/2, hence any nonspecific effects of dual-specific phosphatases overexpression should be considered. Nevertheless, accumulating evidence that ERK1/2 is sufficient to mediate cardiac hypertrophy both in vivo and in vitro has been thoroughly described (reviewed in\textsuperscript{23}).

Defective Ras signaling in humans, for example in Costello\textsuperscript{26} and Noonan syndromes,\textsuperscript{27} has been associated with susceptibility to cancer and cardiac hypertrophy. Our present results, in both mouse and human cardiac pathology, together with previous observations that have assigned a susceptibility to cancer formation in RASSF1A null mice,\textsuperscript{16} strongly support the idea of an intimate relationship between cardiac anomalies and neoplasia in situations of defective RASSF1 signaling and further underline the relevance of key components of the Ras signaling pathway in the pathophysiology of both conditions. It remains to be seen to what extent modifications of RASSF1A and its interacting proteins (eg, polymorphisms, epigenetic inactivation, posttranslational modifications) contribute to the pathophysiology of more common forms of cardiac hypertrophy and failure as well as neoplasias, but our data clearly demonstrate that RASSF1A knock-out animals are a powerful model to investigate such dual pathologies, which had been entirely unexpected until very recently.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Understanding the molecular mechanisms of hypertrophy is key in elucidating the pathogenesis of heart failure. Our study is based on 2 premises: first, many factors that positively mediate hypertrophy have been identified in recent years, but little is known about molecules that counteract and therefore limit cardiac growth. Second, a series of mutations in genes of the Ras-signaling pathway have recently been identified, which associate certain cancers with hypertrophic cardiomyopathy (eg, in Noonan and Costello syndromes). This led us to hypothesize that negative regulators of the Ras pathway may also limit cardiac growth in common conditions such as pressure overload. One such negative regulator in cancer is the recently described tumor suppressor protein Ras-association domain family 1 isoform A (RASSF1A). We therefore investigated the role of this molecule in negatively regulating myocardial hypertrophy. We subjected mice with genetic ablation of Rassf1a gene to transverse aortic constriction. A massive increase in the hypertrophic response, fibrosis, and left ventricular dilatation were observed in these animals. We further showed that RASSF1A acts through the inhibition of Ras at the level of Ras/Raf1 interaction, thus blocking the downstream extracellular regulated kinase 1/2 signaling pathway. Furthermore, a significant downregulation of RASSF1A expression by \(50\%\) was observed in failing human hearts, indicating its involvement in the development of heart failure. Our findings establish RASSF1A as a novel inhibitor of cardiac hypertrophy both in mouse and human and may provide opportunities for the development of novel therapeutic strategies for heart failure by targeting this molecule.
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SUPPLEMENTAL MATERIALS

Supplementary Methods

Histology
Heart tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin and sectioned at 5 µm thickness. Hematoxylin & eosin and Masson’s trichrome stainings were performed using standard procedures.

Measurement of cardiomyocyte cross sectional area and myocardial fibrosis
Cross-sectional cardiomyocyte size was assessed on hematoxylin & eosin-stained sections using ImageJ software (NIH). Ten randomly chosen cardiomyocytes from 6-9 independent animals in each group (60-90 cells per group in total) were analyzed. Myocardial fibrosis was measured on Masson’s trichrome stained sections. Three randomly chosen frames from each animal (n = 6-9 mice per group) were analyzed.

RNA isolation and real time RT-PCR
Total RNA was prepared from freshly isolated heart tissue or cultured neonatal rat cardiomyocytes using Trizol reagent (Invitrogen) following the manufacturer’s instructions. We used the QuantiTect-SYBR Green RT-PCR system (Qiagen) for real time quantitative RT-PCR analysis. Data were calculated using the $2^{\Delta \Delta Ct}$ method and are presented as fold induction of target gene transcripts relative to sham operated wild type animals. Reactions were performed in an ABI PRISM 7700 (Applied Biosystem).
RT-PCR primers and Northern blot probe

Primers were purchased from Qiagen (Quantitect Primer Assay system): ANP, Mm_LOC230899_1_SG; BNP, Mm_Nppb_1_SG; βMHC, Mm_Myh7_1_SG; GAPDH, Mm_Gapdh_2_SG. Threshold cycle (Ct) values were determined using the Sequence Detection System (SDS) software. GAPDH levels were used as a reference. Qualitative RT-PCR for the detection of Rassf1a and Rassf1c expression was conducted using methods described previously[1]. Northern blot analysis was performed using a standard protocol[2]. The probe for rat ANP was isolated by performing an RT-PCR reaction on rat cardiomyocyte RNA using following primers: forward 5’-ATCACAAGGGCTTCTTCCT3’ and reverse 5’-GCTCCAATCTGTCAAATCTAC-3’.

Antibodies

Primary antibodies used were: anti-human RASSF1A (eBiosciences), anti-RASSF1A N-15 (Santa Cruz), anti-RASSF1C (Santa Cruz), anti-β-galactosidase (Promega), anti-GAPDH (Abcam), anti-pan-Ras antibody (Sigma), anti-Flag-M2-HRP conjugate (Sigma). Antibodies for ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), Raf1, phospho-Raf1 (Ser338), JNK1/2, phospho-JNK1/2 (Thr183/Tyr185), p38-MAPK, phospho-p38-MAPK (Thr180/Tyr182), Akt, phospho-Akt (Ser473) were obtained from Cell Signaling. We used horse radish peroxidase labeled secondary antibodies (Jackson ImmunoResearch/Dako) for visualization. For immunofluorescence in NRCM, anti-RASSF1A N-15 (Santa Cruz) and anti-α-actinin (Sigma) were used. For immunohistochemical staining of human heart tissue sections, anti-human RASSF1A (eBiosciences) was used as the primary antibody. We used Texas red or FITC-labeled secondary antibodies (Jackson ImmunoResearch) for visual detection.
Cellular hypertrophy experiments

For cellular hypertrophy experiments, NRCM were infected with the indicated adenovirus for 24 hours and then stimulated with phenylephrine (30 μM) for 72 hours. NRCM were stained with anti-α-actinin antibody (Sigma). The cell size was then measured using ImageJ software. The BNP promoter activation were analyzed using a BNP-luciferase adenoviral construct and the luciferase activity was determined using luciferase detection reagent (Promega). For $^3$H-leucine incorporation assay, NRCM were infected with the indicated adenovirus for 24 hours in serum free medium and then stimulated with phenylephrine (30 μM) for 24 hours. Then 1.0 μCi/ml of $^3$H-leucine was added for another 24 hours. Cells were washed in ice cold PBS and fixed in 10% trichloroacetic acid (TCA). After washing twice with 5% TCA the radioactivity incorporated in the precipitate was determined by scintillation counting after solubilization in 0.25 M NaOH. Total cell protein was determined using the BCA protein assay kit (Pierce).
### Supplementary Table 1

**Echocardiography and hemodynamic parameters in unstimulated animals (12 weeks of age)**

<table>
<thead>
<tr>
<th></th>
<th>RASSF1A knock out (n=11)</th>
<th>Wild type (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>25.57 ± 2.20</td>
<td>23.55 ± 1.35</td>
</tr>
<tr>
<td><strong>Heart weight (mg)</strong></td>
<td>124.76 ± 10.4</td>
<td>118.48 ± 8.67</td>
</tr>
<tr>
<td><strong>Heart weight/Body weight (mg/g)</strong></td>
<td>4.89 ± 0.12</td>
<td>5.01 ± 0.13</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.92 ± 0.11</td>
<td>4.06 ± 0.09</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.82 ± 0.11</td>
<td>2.85 ± 0.10</td>
</tr>
<tr>
<td>dIVS (mm)</td>
<td>1.06 ± 0.03</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>dPW (mm)</td>
<td>0.77 ± 0.02</td>
<td>0.81 ± 0.07</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>28.1 ± 1.8</td>
<td>29.8 ± 1.3</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>387 ± 11</td>
<td>395 ± 16</td>
</tr>
<tr>
<td><strong>Haemodynamics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dP/dt max (mmHg/sec)</td>
<td>5903 ± 646</td>
<td>7632 ± 641</td>
</tr>
<tr>
<td>dP/dt min (mmHg/sec)</td>
<td>-5187 ± 547</td>
<td>-6210 ± 595</td>
</tr>
<tr>
<td>Tau (msec)</td>
<td>9.24 ± 0.76</td>
<td>7.07 ± 0.58</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>4.6 ± 0.4</td>
<td>4.3 ± 0.4</td>
</tr>
</tbody>
</table>

LVEDD, Left ventricular end diastolic dimension; LVESD, Left ventricular end systolic dimension; dIVS, Interventricular septal wall thickness at diastole; dPW, Posterior wall thickness at diastole

None of the differences observed were statistically significant
## Supplementary Table 2 Heart weight and body weight in TAC-stimulated animals

<table>
<thead>
<tr>
<th></th>
<th>RASSF1A knock out</th>
<th>Wild type</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=10)</td>
<td>TAC (n=10)</td>
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<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
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<tr>
<td>Before surgery</td>
<td>26.0 ± 0.8</td>
<td>26.0 ± 1.48</td>
</tr>
<tr>
<td>5 week after surgery</td>
<td>29.3 ± 0.73</td>
<td>28.73 ± 1.14</td>
</tr>
<tr>
<td><strong>Heart weight (after surgery) (mg)</strong></td>
<td>133.8 ± 3.92</td>
<td>211.73 ± 16.94&lt;sup&gt;§†&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Heart weight/body weight (mg/g)</strong></td>
<td>4.58 ± 0.12</td>
<td>7.52 ± 0.63&lt;sup&gt;§†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* P<0.05 vs wild type-TAC group; †P<0.05 vs sham treated animals from the same genotype
Supplementary Figure 1

A

RT + -
RASSF1A
RASSF1C
GAPDH

B

NRRCM
Rat Heart
Mouse Heart

RASSF1A
RASSF1C

C

sham TAC (12 week)

RASSF1C
GAPDH

D

RASSF1C expression normalized to GAPDH (arbitrary unit)

E

PE + -
RASSF1A
RASSF1C
GAPDH

F

RASSF1A expression normalized to GAPDH (arbitrary unit)

G

RASSF1C expression normalized to GAPDH (arbitrary unit)

H

WT RASSF1A KO

RASSF1C
GAPDH
Supplementary Figure 2

A

<table>
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<tr>
<th>PE</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>ANP</th>
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B

![](chart)

Relative luciferase activity (fold induction of unstimulated cells)

C

![](chart)

Relative luciferase activity (fold induction of unstimulated cells)
Supplementary Figure 3

A

<table>
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<tr>
<th></th>
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<th>Adv-RASSF1A</th>
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B

JNKs

<table>
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<th>Adv-RASSF1A</th>
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<tr>
<td>PE (mins)</td>
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<tr>
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<td>5</td>
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C

Akt

<table>
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<th>Adv-LacZ</th>
<th>Adv-RASSF1A</th>
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<td>PE (mins)</td>
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Supplementary Figure 4

A

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<tbody>
<tr>
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- WT TAC KO TAC
- p-p38
- t-p38

B

<table>
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<tr>
<th></th>
<th>WT</th>
<th>TAC</th>
<th>KO</th>
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<tr>
<td>S</td>
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- WT TAC KO TAC
- p-JNK
- t-JNK

C

<table>
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<tr>
<th></th>
<th>WT</th>
<th>TAC</th>
<th>KO</th>
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<tbody>
<tr>
<td>S</td>
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</table>

- WT TAC KO TAC
- p-Akt
- t-Akt
Supplementary Figure 5

A

Adv-LacZ | Adv-RASSF1A

No treatment

+ staurosporine

B

Adv-LacZ | Adv-RASSF1A

No treatment

+ staurosporine

C

% positive cells

0 5 10 15 20 25

Cleaved Caspase-3 | Bak N-conformational change

D

Cleaved Caspase-3 positive cells (%)

0 2 4 6 8 10 12 14 16 18 20

2 hours | 4 hours

Staurosporine Treatment

E

Bak (N-conformational change)

Relative expression level

0 0.4 0.8 1.2 1.6

Adv-LacZ | Adv-RASSF1A

PE - + - +
Supplementary Figure Legends

Supplementary Figure 1: The major RASSF1 isoforms are expressed in the heart and in isolated cardiomyocytes.
A. Detection of RASSF1A and RASSF1C expression by RT-PCR from 0.2 µg RNA isolated from Balb/c mouse heart, expression was detected in each heart tested (n=6). Products from reactions including (+) or excluding (-) reverse transcriptase are shown.
B. Western blot detection of RASSF1A and RASSF1C in total mouse and rat heart extracts as well as in isolated neonatal rat cardiomyocytes.
C. Expression of RASSF1C in mouse heart extracts after TAC or sham procedure (12 weeks). GAPDH expression served as loading control
D. Quantification of band density revealed that there was no difference in RASSF1C expression between sham and TAC operated mice (n=4).
E. Representative Western blots to detect expression of RASSF1A and RASSF1C in NRCM after treatment with phenylephrine (30 mM, 72 hours). Densitometric analysis showed that there was no change in RASSF1A (F) and RASSF1C (G) expression in NRCM in response to phenylephrine treatment (n=3).
H. Expression of RASSF1C was not altered in RASSF1A knock out mice as indicated by Western blot analysis.

Supplementary Figure 2 Expression of ANP and activation of BNP promoter in NRCM in response to phenylephrine induction
A. ANP expression was detected in total RNA extracts by Northern blot analysis.
B. Quantification of ANP expression normalized to 18s ribosomal RNA levels (n=8 from three independent experiments, *P<0.05).

C. The activation of the BNP promoter in response to phenylephrine stimulation was examined using a BNP-luciferase reporter construct (n=4 independent experiments, *P<0.05).

**Supplementary Figure 3 RASSF1A overexpression in NRCM did not alter JNKs and Akt activation in response to short phenylephrine stimulation**

A) NRCM overexpressing RASSF1A or control were exposed to phenylephrine stimulation (30 mM) for the indicated time. Representative Western blots for the analysis of phosphorylated and total JNKs and Akt from the cell lysates were shown. Activation of B) JNKs and C) Akt were quantified using densitometric analysis. No significant difference was observed between NRCM overexpressing RASSF1A compared to the control cells.

**Supplementary Figure 4 Activation of p38, JNKs and Akt were not altered in RASSF1A null mice following TAC stimulation**

Western blot analysis and the quantification of phosphorylated/total (A) p38, (B) JNKs and (C) Akt indicated that there were no significant differences between RASSF1A knock out mice vs WT control after TAC (n=5 in each group; S=sham).
Supplementary Figure 5 RASSF1A does not sensitize cardiomyocytes to apoptosis

NRCM overexpressing RASSF1A and control were stained with antibodies for (A) Bak (N-conformational change) and (B) cleaved caspase-3 both without and after stimulation with staurosporine.

C) Quantification of % cells positive for cleaved caspase-3 and Bak (N-conformational change) suggested that there is no difference in apoptosis levels between control cells and cells overexpressing RASSF1A (Results from three different stainings, at least 900 cells were counted per staining).

D) Detection of cleaved-caspase-3 in cardiomyocytes overexpressing RASSF1A and control to assess apoptosis after treatment with staurosporine for 2-4 hours. Following treatment with staurosporine the number of cleaved-caspase 3 positive cells was increased, but there were no differences between control and RASSF1A overexpressing cells. Two independent experiments, each conducted in duplicate. At least 250 cells counted per replicate.

E) Apoptosis levels in response to phenylephrine treatment were determined by Bak staining in NRCM overexpressing RASSF1A vs control. No difference in Bak expression (relative to unstimulated control cells) in cells overexpressing RASSF1A vs control (n=3).
SUPPLEMENTARY REFERENCES
