Carabin Protects Against Cardiac Hypertrophy by Blocking Calcineurin, Ras, and Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II Signaling

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**Background**—Cardiac hypertrophy is an early hallmark during the clinical course of heart failure and is regulated by various signaling pathways. However, the molecular mechanisms that negatively regulate these signal transduction pathways remain poorly understood.

**Methods and Results**—Here, we characterized Carabin, a protein expressed in cardiomyocytes that was downregulated in cardiac hypertrophy and human heart failure. Four weeks after transverse aortic constriction, Carabin-deficient (Carabin\textsuperscript{−/−}) mice developed exaggerated cardiac hypertrophy and displayed a strong decrease in fractional shortening (14.6±1.6% versus 27.6±1.4% in wild type plus transverse aortic constriction mice; \(P<0.0001\)). Conversely, compensation of Carabin loss through a cardiotropic adeno-associated viral vector encoding Carabin prevented transverse aortic constriction–induced cardiac hypertrophy with preserved fractional shortening (39.9±1.2% versus 25.9±2.6% in control plus transverse aortic constriction mice; \(P<0.0001\)). Carabin also conferred protection against adrenergic receptor–induced hypertrophy in isolated cardiomyocytes. Mechanistically, Carabin carries out a tripartite suppressive function. Indeed, Carabin, through its calcineurin-interacting site and Ras/Rab GTPase–activating protein domain, functions as an endogenous inhibitor of calcineurin and Ras/extracellular signal-regulated kinase prohypertrophic signaling. Moreover, Carabin reduced Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II activation and prevented nuclear export of histone deacetylase 4 after adrenergic stimulation or myocardial pressure overload. Finally, we showed that Carabin Ras–GTPase–activating protein domain and calcineurin-interacting domain were both involved in the antihypertrophic action of Carabin.

**Conclusions**—Our study identifies Carabin as a negative regulator of key prohypertrophic signaling molecules, calcineurin, Ras, and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II and implicates Carabin in the development of cardiac hypertrophy and failure. (Circulation. 2015;131:390-400. DOI: 10.1161/CIRCULATIONAHA.114.010686.)

**Key Words:** heart failure ■ hypertrophy ■ signal transduction ■ ventricular remodeling

Cardiac hypertrophy, the compensatory response of the heart to stress, is characterized by nonmitotic growth, the addition of new sarcomeres, and fetal gene expression.\(^1\) However, prolonged hypertrophy of the myocardium can progress to heart failure (HF), a leading cause of morbidity and mortality, often associated with sudden cardiac death.\(^2\)\(^-\)\(^4\) Thus, understanding the signaling mechanisms that regulate pathological cardiac hypertrophy may lead to a better treatment for patients with HF.

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The hypertrophic growth of the myocardium is typically initiated by signal transduction pathways in response to...
increased mechanical load on the heart or through the action of neurohumoral mediators. Numerous signaling pathways coordinate the cardiac hypertrophic response. Among them, the Ca²⁺/calmodulin-dependent phosphatase calcineurin is considered as a central prohypertrophic signaling effector in the myocardium. Activated calcineurin directly binds to and dephosphorylates the nuclear factor of activated T cells (NFAT) in the cytoplasm, permitting its translocation to the nucleus, where it participates in hypertrophic gene expression. However, no single pathway seems to regulate cardiac hypertrophy alone. Rather, it appears more likely that each pathway operates as a component of an orchestrated hypertrophic network. For instance, the small G protein Ras, the stress-responsive mitogen-activated protein kinases, and the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) can cross-talk with the calcineurin signaling pathway to regulate hypertrophic processes. CaMKII is known to activate the myocyte enhancer factor 2 (MEF2) on class II histone deacetylases (HDAC) phosphorylation, thereby allowing this transcription factor to induce hypertrophic gene expression.

In contrast to these growth-promoting pathways, several endogenous molecules in the heart have been shown to negatively regulate cardiac hypertrophy. Increasing these negative signaling pathways may be of significant therapeutic value. In this context, Carabin (alias TBC/Rab GTPase activating protein [GAP]), a protein expressed mainly in the immune system and the heart, raised our interest. Carabin has 446 amino acid residues and contains 2 important regions, a putative Ras GTPase-activating protein (GAP) domain at its amino terminus and a carboxy-terminal domain that bears a calcineurin-interacting domain. Here, we provide the first evidence for an antihypertrophic role of Carabin in the heart. Using knockout mice for Carabin (Carabin−/−), we showed a severely impaired cardiac function in response to pressure overload–induced cardiac hypertrophy compared with control wild-type (WT) animals. Carabin was decreased in cardiac hypertrophy and failure. Compensation of its loss of expression using adeno-associated virus serotype 9 (AAV9)–mediated gene transfer in vivo protected the heart against hypertrophy and dysfunction. At the molecular level, Carabin repressed the prohypertrophic signaling of calcineurin, Ras/extracellular signal-regulated kinase (ERK), and CaMKII after cardiac pressure overload or adrenergic stimulation. Thus, this work establishes Carabin as a negative regulator of cardiomyocyte hypertrophy through the inhibition of various components of the intracellular signal-transduction network that coordinates hypertrophic responses.

Methods

The detailed and expanded methodology is provided in the online-only Data Supplement.

Animals

Mice carrying a global heterozygous knockout of Carabin were generated by targeted gene deletion as previously described by Schickel et al (kindly provided by P.S.-S.). Eight-week-old male Carabin−/− mice and littermate controls (WT) used in this study were obtained by breeding Carabin−/− males and Carabin−/− females, both from a C57BL/6 genetic background. Mice were genotyped by polymerase chain reaction with the following 2 sets of primers as previously described: 5′-GCAGCAGCAGCTACAGGTCCC-3′/5′-CCCTCTGCAGACCTCATCGGC-3′ and 5′-GGCCACCAT TGCCACAGCTCA-3′/5′-GCAGACCTCATCAGCGCA G-3′. All mice used in this study were pathogen free and housed at constant (22°C) temperature on 12-hour light/dark cycles with ad libitum access to food and water.

AAV9 Production and Administration

AAV9-Carabin and AAV9–green fluorescent protein (GFP) were generated and constructed by Penn Vector Core (School of Medicine Gene Therapy Program, University of Pennsylvania). Physiological serum solution (100 μL) containing 3×10¹⁰ genome copies per 1 mL AAV9 was injected in the retro-orbital sinus of 4-week-old male mice on a C57BL/6 genetic background (Janvier, France).

Adenoviral Infection

Bicistronic adenoviruses bearing human Carabin under the control of a cytomegalovirus promoter and GFP under internal ribosomal entry site were constructed and amplified by Genecust (Evry, France). One day after plating, cardiomyocytes were incubated for 2 hours with recombinant adenoviruses. After removal of the virus suspension, cells were replaced in maintenance medium for 2 days and then stimulated with the different drugs. Viruses were used at a multiplicity of infection of 10.

Statistical Analysis

Results are expressed as mean±SEM. Differences between groups have been analyzed by an unpaired Student t test and 2-way ANOVA followed by the Bonferroni posttest or Tukey posttest. Differences were considered significant at P<0.05, P<0.01 and P<0.001. Values of P<0.0001 were considered extremely significant. All statistical analyses were performed with GraphPad Prism (version 6.01).

Study Approval

All animal experiments were performed under European laws on the protection of animals of the University of Toulouse outlined in Council Directive 86/609/EEC. Mouse and rat experiments were approved and performed according to the guidelines of the Ethics and Animal Safety Committee of INSERM Toulouse/ENVT (agreement No. C3155507).

Results

Carabin Deficiency Potentiates the Progression of HF Under Hemodynamic Stress

To study the role of Carabin in the development of cardiac hypertrophy and HF, we first analyzed cardiac Carabin expression in mice and rats subjected to pressure overload induced by thoracic aortic constriction (TAC) and in failing human heart samples. In both rodent and human hearts, Carabin mRNA and protein amounts were markedly reduced compared with control hearts, suggesting that Carabin deficiency is associated with the development of cardiac hypertrophy and failure (Figure 1A).

Next, to test whether Carabin contributes to heart disease, we analyzed the hearts of Carabin−/− mice. This mouse line developed and reproduced normally, as previously described. We found that Carabin was expressed in cardiac myocytes and not in cardiac fibroblasts isolated from the heart of WT animals (Figure 1A in the online-only Data Supplement). Western blot analysis failed to detect any cardiac expression of Carabin in Carabin−/− mice (Figure IB in the online-only Data Supplement). Cardiac function (Table 1) and the ratios of heart...
weight to tibia length and left ventricular weight to tibia length (Figure 1B) were not different between WT and Carabin-deficient mice. In addition, hearts from Carabin-deficient mice were macroscopically indistinguishable from those of WT animals (Figure 1C). However, 4 weeks after TAC, Carabin−/− mice showed a decline in cardiac contractility, as indicated by the reduced fractional shortening (Table 1). Left ventricular end-diastolic and end-systolic internal diameters were largely increased in Carabin−/− mice after TAC (Table 1), indicating LV dilatation. Notably, Carabin deficiency aggravated TAC-induced hypertrophic response compared with WT mice, as shown by increased ratios of heart weight to tibia length and left ventricular weight to tibia length and hematoxylin and eosin–stained heart sections (Figure 1B and 1C). These changes were accompanied by an increased cardiomyocyte cross-sectional area and induction of interstitial fibrosis (Figure 1C). In addition, the development of pulmonary congestion (ratio of lung weight to tibia length) as an indirect indicator of HF was also significantly increased in Carabin−/− mice (Figure II in the online-only Data Supplement). Consistently, the expression of cardiac fetal genes (atrial natriuretic factor, brain natriuretic peptide, β-myosin heavy chain) and fibrotic markers (procollagen, type I, α1 and collagen type III) was

### Table 1. Echocardiographic Analysis of WT and Carabin−/− Mice After Sham and TAC Operations

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<th>Carabin−/− TAC</th>
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<tr>
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<tr>
<td>FS, %</td>
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Statistical significance was determined by 2-way ANOVA with Tukey post-test.

P<0.05, †P<0.001, ‡P<0.0001 vs the respective control group; §P<0.001, ||P<0.0001 vs the WT-TAC animal group.

### Figure 1. Carabin deficiency accelerates pressure overload–induced heart failure (HF).

A. Analysis of myocardial Carabin expression by quantitative polymerase chain reaction (qPCR) and Western blot. Left and Middle. Quantification of Carabin in mouse or rat left ventricular myocardium from thoracic aortic constriction (TAC; mouse n=6; rat n=10) or sham-operated controls (mouse, n=7; rat n=10). Right. Quantification of Carabin in left ventricular myocardium from patients with dilated cardiomyopathy (DCM; n=4) or nonfailing (NF) control biopsies (n=4). Representative immunoblots are shown. B. Heart weight (HW)/tibia length (TL) and left ventricular weight (LVW)/TL ratios of control and Carabin−/− mice after sham and TAC operation (n=10–12 per group). C. Top, Representative hematoxylin and eosin–stained heart sections of wild-type (WT) or Carabin−/− mice subjected or not to TAC (scale bar, 2 mm). Vinculin staining (scale bar, 100 μm) and histological characterization of fibrosis (scale bar, 100 μm) in cardiac sections of WT and Carabin−/− mice. The graph represents quantification of cardiomyocyte cross-sectional area (right) and the collagen content (left; n=6–8 per group). D. Levels of cardiac hypertrophy–related transcripts (atrial natriuretic factor [ANF], brain natriuretic peptide [BNP], β-myosin heavy chain [β-MHC]) and fibrosis markers (procollagen, type I, α1 [COL1], collagen type III [COL3]) were quantified by qPCR (n=6–8 per group). Statistical significance was determined by the Student t test (A) or by 2-way ANOVA with interaction analysis and Tukey posttest (B–D). RQ indicates relative quantity. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs the indicated value.
significantly augmented in Carabin-deficient mice after TAC (Figure 1D). Thus, Carabin deficiency does not cause baseline cardiac abnormalities but remarkably renders the heart more susceptible to stress-induced pathological cardiac remodeling.

**Cardiotropic Expression of Carabin Prevents Cardiac Myocyte Hypertrophy and Preserves Cardiac Function After Pressure Overload**

We first examined the effect of Carabin overexpression in isolated cardiac myocytes. We infected or transfected neonatal rat ventricular myocytes (NRVMs) with either a control vector or a vector encoding Carabin (Figure 2A and 2B). Carabin-overexpressing cells had a dampened hypertrophic response to phenylephrine (10 μmol/L) treatment on the basis of analysis of cell surface area, sarcomeric organization, or the expression of the atrial natriuretic factor, which are hallmarks of cardiomyocyte hypertrophy (Figure 2A and 2B). Conversely, knockdown of Carabin with specific siRNA (Si-Carabin) further amplified the effect of phenylephrine on the expression of atrial natriuretic factor and cardiomyocyte surface area (Figure III in the online-only Data Supplement). These results indicate an antihypertrophic effect of Carabin in cardiomyocytes.

Because Carabin was decreased in cardiac remodeling and HF, we tested whether its cardiac overexpression would prevent hypertrophy. For that, we chose a gene transfer approach using AAV9, which shows strong tropism for cardiac myocytes. An AAV9 encoding Carabin or its control (GFP) was injected into the retro-orbital sinus of mice, and 5 weeks later, TAC was performed (Figure IV in the online-only Data Supplement). Mice treated with AAV9-Carabin and subjected to TAC showed a level of cardiac Carabin similar to that observed in the basal condition (Figure 2C). Remarkably, restoration of Carabin expression with AAV9-Carabin significantly prevented cardiac hypertrophy, as judged by the ratios of heart weight to tibia length and left ventricular weight.

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**Figure 2.** Increased Carabin expression blocks the cardiac hypertrophic response. **A.** Left, Sarcomere organization, as determined by immunostaining for sarcomeric α-actinin (red) in neonatal rat ventricular myocytes (NRVMs) infected with the indicated adenoviruses (Ad.) and stimulated or not with phenylephrine (PE; 10 μmol/L). Scale bar, 10 μm. **Right,** Quantification of cell surface area. **B.** Relative luciferase activity of the atrial natriuretic factor (ANF) promoter in NRVMs cotransfected with the indicated plasmids. Bar graphs show results from 8 independent experiments performed in triplicate. The immunoblot shows the expression of recombinant cMyc-Carabin. **C,** Immunoblot analysis of Carabin was performed on left ventricular (LV) homogenates from mice injected with AAV9–green fluorescent protein (GFP) and AAV9-Carabin (Carabin) and subjected or not to TAC (n=5 per group). GAPDH expression was used as loading control. The graph represents quantification of Carabin levels. **D,** Heart weight (HW)/tibia length (TL) and LV weight (LVW)/TL ratios of AAV9-GFP (GFP)– and AAV9-Carabin (Carabin)–treated mice after sham and thoracic aortic constriction (TAC) operations (n=10–12 per group). **E,** Heart sections of AAV9-GFP (GFP) and AAV9-Carabin (Carabin) mice were stained with either vinculin to demarcate cell boundaries (green; scale bar, 50 μm) or Masson trichrome to detect fibrosis (blue; scale bar, 100 μm). The bar graphs show the quantification of cardiomyocyte cross-sectional areas and fibrosis (n=8–12 per group). **F,** Relative expression of cardiac hypertrophic (atrial natriuretic factor [ANF], brain natriuretic peptide [BNP], β-myosin heavy chain [β-MHC]) and fibrosis (procollagen, type I, α1 [COL1], collagen type III [COL3]) markers genes of heart samples from AAV9-GFP (GFP) and AAV9-Carabin (Carabin) after sham and TAC (n=6–8 per group). All data were evaluated by 2-way ANOVA/Tukey posttest. RQ indicates relative quantity. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs the indicated value.
to tibia length (Figure 2D). Importantly, histological analysis showed a reduction in cardiomyocyte size and fibrosis in the TAC group treated with AAV9-Carabin (Figure 2E). In addition, upregulation of the hypertrophic gene markers and fibrotic markers was compromised in AAV9-Carabin (Figure 2F). This effect of Carabin was associated with a preserved cardiac function, as demonstrated by the echocardiographic data on left ventricular end-systolic internal diameter, left ventricular end-diastolic internal diameter, and fractional shortening (Table 2). None of these effects occurred in the AAV9-GFP control group. These data strongly suggest that Carabin overexpression protects against the development of pathological cardiac hypertrophy.

**Carabin Suppresses the Calcineurin/NFAT Signaling Pathway in a Ras-GAP–Independent Fashion**

Next, we sought to determine the signaling mechanisms by which Carabin represses hypertrophy. Because Carabin contains a calcineurin-interacting domain, we analyzed its ability to regulate calcineurin activity in NRVMs (Figure 3A). Cyclosporine A, a well-known inhibitor of calcineurin, was used as a positive control. Phenylephrine-induced calcineurin activation was significantly decreased in NRVMs infected with an adenovirus encoding Carabin (Ad.Carabin) compared with control cells (Ad.GFP; Figure 3A), indicating that the phosphatase is a target of Carabin. Conversely, Si-Carabin potentiated phenylephrine-induced calcineurin activation and NFAT nuclear translocation (Figure VA and VB in the online-only Data Supplement). In line with this result, phenylephrine and the calcium ionophore ionomycin failed to induce NFAT nuclear translocation in NRVMs transfected with Carabin (Figure 3B and Figure VI in the online-only Data Supplement). Accordingly, the expression of the 1.4 isoform of the calcineurin-interacting protein (MCIP 1.4), a downstream effector for calcineurin-dependent activity that can serve as a highly sensitive readout for calcineurin activity in vivo, was prevented in left ventricular lysates of AAV9-Carabin–treated mice after TAC compared with control animals. In contrast, cardiac MCIP 1.4 expression was potentiated in Carabin−/− mice subjected to pressure overload (Figure 3C).

The interaction of Carabin with calcineurin/NFAT signaling was further confirmed by cotransfecting Si-Carabin and NFAT response elements coupled to the luciferase gene (NFAT-Luc) in NRVMs (Figure 3D). As expected, we observed a significant increase in phenylephrine-induced NFAT-Luc transcriptional activity in Si-Carabin–transfected cells compared with control siRNA stimulated with phenylephrine. A similar inhibitory effect of Si-Carabin on NFAT-Luc activity was observed under stimulation of a nonselective β-adrenergic receptor agonist, isoproterenol, indicating that Carabin inhibited NFAT activation in response not only to α-adrenergic receptor but also to β-adrenergic receptor activation (Figure VII in the online-only Data Supplement). The presence of the C-terminal calcineurin-interacting domain in Carabin raised the possibility that this domain might be involved in the regulation of calcineurin/NFAT signaling. Transient transfection experiments showed that indeed the inhibitory effect of Carabin on NFAT activity was dependent on its calcineurin-binding domain but independent of the Ras-GAP domain because mutations in the calcineurin-binding domain of Carabin (CarabinAAVGA mutant) or inactivation of the Carabin Ras-GAP domain (CarabinRI41A mutant; Figure 3E) abolished or mimicked the effect of Carabin, respectively (Figure 3F). Together, these data show that Carabin specifically inhibits the calcineurin/NFAT signaling pathway through its calcineurin-interacting domain and independently of its Ras-GAP activity.

**Carabin Blocks Ras and ERK Activation Via Its Ras-GAP Domain**

The fact that Carabin presents a Ras-GAP domain led us to investigate a potential Ras/Carabin interaction. Com Immunoprecipitation experiments, followed by immunoblotting, showed that Carabin interacted with Ras in NRVMs (Figure 4A). Knockdown of Carabin confirmed the specificity of this association (Figure 4A). The Ras/Carabin interaction was constitutive and was not influenced by adrenergic stimulation (Figure 4A). Consistent with its antihypertrophic action, Carabin decreased the amount of the active form of Ras that Ras-GTP produced under phenylephrine treatment, whereas CarabinRI41A failed to inhibit Ras activation (Figure 4B). To further substantiate the inhibitory action of Carabin on Ras signaling, we analyzed its effect on the activation of a downstream target of Ras, the mitogen-activated protein kinase ERK. Si-Carabin enhanced phenylephrine-induced ERK activation by ~70% (Figure VIII in the online-only Data Supplement), whereas transfected Carabin decreased phosphorylated ERK in cardiac myocytes stimulated with phenylephrine compared with control cells (Figure 4C). Similarly, loss or gain of function of Carabin potentiated or prevented TAC-induced ERK activation in left ventricular homogenates, respectively (Figure 4D and 4E). Of particular interest, phenylephrine-induced ERK was still observed in the presence of CarabinRI41A, confirming the involvement of the Carabin Ras-GAP domain on the inhibition of the Ras/ERK pathway (Figure 4F). These data demonstrate that Carabin, via its interaction with Ras and

### Table 2. Echocardiographic Analysis of AAV9-GFP or AAV9-Carabin–Treated Mice After Sham and TAC Operations

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Statistical significance was determined by 2-way ANOVA with interaction analysis and the Tukey posttest. AAV9 indicates adeno-associated virus serotype 9; EDV, end-diastolic volume; ESV, end-systolic volume; FS, fractional shortening; GFP, green fluorescent protein; LVIDd, left ventricular end-diastolic internal diameter; LVIDs, left ventricular end-systolic internal diameter; and TAC, thoracic aortic constriction.

*P<0.01, †P<0.001 vs the WT control group; §P<0.001, ‡P<0.0001 vs the WT-TAC animal group.
its Ras-GAP domain, inhibits Ras and ERK hypertrophic signaling.

**Carabin Regulates HDAC4 Nucleocytoplasmic Shuttling**

We previously showed that the small GTPase Ras regulates cardiac myocyte hypertrophy in coordination with CaMKII.\(^5\,^6\) Hence, to further analyze Carabin antihypertrophic signaling, we investigated its effect on CaMKII. Carabin prevented phenylephrine- or TAC-induced CaMKII autophosphorylation state (indicative of CaMKII activation), whereas CaMKII was activated more in heart samples of Carabin\(^{-/-}\) mice subjected to TAC compared with WT animals (Figures 5A–5C). In contrast to Carabin and Carabin\(^{AAVGA}\), Carabin\(^{R141A}\) failed to inhibit CaMKII activation, indicating that the effect of Carabin on CaMKII was Ras dependent (Figure 5A).

Once phosphorylated by CaMKII, HDAC proteins are translocated out of the nucleus, thereby allowing MEF2 to activate its target genes.\(^1\) Therefore, to ensure the relationship between Carabin and CaMKII hypertrophic signaling, we tested whether Carabin could influence HDAC4 nucleocytoplasmic shuttling and subsequent MEF2 transcriptional activation (Figure 5D–5G). First, we examined HDAC4 nuclear export after TAC in Carabin\(^{-/-}\) and AAV9-Carabin–treated mice. Although the myocardium of Carabin\(^{-/-}\) mice showed an increase in the cytosolic/nuclear ratio of HDAC4 protein expression after TAC compared with WT mice (Figure 5D), cardiac overexpression of Carabin prevented the nucleocytoplasmic shuttling of HDAC4 (Figure 5E). Second, we
Antihypertrophic Action of Carabin
Calcineurin-Interacting Domain Are Involved in the

Both the Carabin Ras-GAP Domain and Calcineurin-Interacting Domain Are Involved in the Antihypertrophic Action of Carabin

Because Carabin regulates both calcineurin and Ras/CaMKII pathways, we assessed their relative contributions to the regulation of the cardiac myocyte hypertrophy of Carabin. Carabin<sup>R141A</sup> and Carabin<sup>A16GA</sup> mutants were significantly less potent than the wild-type form of Carabin for inhibiting cell surface area, sarcomeric organization, or the atrial natriuretic factor promoter transcriptional activity (Figures X and XI in the online-only Data Supplement). The 2 Carabin mutants had a similar effect on cardiomyocyte hypertrophy markers. Transfection of Carabin<sup>R141A</sup> or Carabin<sup>A16GA</sup> in the presence of either a pharmacological inhibitor of CaMKII, KN93, or calcineurin, cyclosporine A mimicked the effect of Carabin on phenylephrine-induced myocyte hypertrophy (Figures X and XI in the online-only Data Supplement). These data demonstrate that both the Carabin Ras-GAP domain and calcineurin-interacting domain are involved in the antihypertrophic action of Carabin.

determined the expression level of cytoplasmic HDAC4 and MEF2 transcriptional activity in NRVMs transfected with Carabin and treated with phenylephrine. The results show that Carabin inhibited phenylephrine-induced HDAC4 nuclear export and MEF2 activation (Figure 5F and 5G). Inversely, Si-Carabin potentiated the upregulation of MEF2 transcriptional activity induced by phenylephrine (Figure IX in the online-only Data Supplement).

Next, to find out which domain of Carabin is important for its effect on HDAC4 and MEF2, we performed transient transfection in NRVMs using Carabin<sup>R141A</sup> and Carabin<sup>A16GA</sup> mutants. KN93, a pharmacological inhibitor of CaMKII, was used as positive control. Both mutants failed to block HDAC4 translocation, suggesting that Carabin calcineurin-interacting and Ras-GAP domains are involved in HDAC4 nucleocytoplasmic shuttling (Figure 5F). In agreement with its effect on HDAC4, Carabin<sup>R141A</sup> did not prevent MEF2 activation in cells stimulated with phenylephrine (Figure 5G). We obtained an intermediate effect with Carabin<sup>A16GA</sup>, suggesting that the calcineurin domain of Carabin is partially involved in the regulation of MEF2 transcriptional activity (Figure 5G). Taken together, these data show that, in response to adrenergic stimulation, Carabin, mainly through its Ras-GAP domain, prevents the activation of the prohypertrophic transcription factor MEF2 via the inhibition of CaMKII and HDAC nuclear export.
Discussion

This study is the first to show that Carabin is a negative regulator of cardiac hypertrophy under pathological conditions. This is supported by both gain-of-function and loss-of-function studies. Because Carabin was downregulated in cardiac remodeling and failure, we sought to compensate for its loss through an in vivo gene transfer approach using AAV9-Carabin. Restoration of Carabin expression prevented the hypertrophic response to TAC and improved cardiac function, as evidenced by gravimetric, echocardiographic, fetal program, and cell size analysis. Consistent with the in vivo data, transfected Carabin inhibited phenylephrine-induced hypertrophy in NRVMs. Conversely, Si-Carabin potentiated cardiac hypertrophic markers. Importantly, Carabin-deficient mice developed a severe cardiac dysfunction in response to pressure overload, associated with altered contractile function, increased left ventricular dilation, and cardiac fibrosis. Furthermore, we observed severe HF with lung edema developed in Carabin-deficient mice after TAC. Together, these data suggest that Carabin plays a key role in the regulation of the transition from hypertrophy to HF in response to pathological stress.

Several endogenous repressors of cardiac hypertrophy have previously been identified. Among them, Cabin 1, Calsarcin-1, and PICOT (protein kinase C–interacting couster of thioredoxin) have previously been identified. Among them, Cabin 1, Calsarcin-1, and PICOT (protein kinase C–interacting couster of thioredoxin) have been shown to prevent pathological cardiac hypertrophy mainly through modulation of calcineurin activity. Although Carabin is another negative regulator of cardiac hypertrophy, this protein takes a special place among the others for 2 reasons. First, Carabin represents a promising target for preventing maladaptive cardiac hypertrophy. In contrast to other hypertrophic signaling molecules, Carabin does not seem to be required for normal development. Rather, Carabin gains functional importance during the cardiac stress response resulting from hypertrophic signaling and mechanical overload. This is supported by the absence of myocardial atrophy in Carabin-overexpressing mice and by data from Carabin-deficient mice that show no overt cardiac phenotype under basal conditions. Second, mechanistically, Carabin carries out a remarkable tripartite suppressive
function. Indeed, Carabin exhibits inhibitory activity not only on calcineurin/NFAT (by its carboxy-terminal domain of interaction with calcineurin) and Ras/ERK (by its amino-terminus Ras-GAP domain) pathways but also on CaMKII and HDAC4 nuclear export.

Importantly, we noticed that Carabin is expressed in both the cytoplasm and nucleus of cardiac myocytes and induces the inhibition of these prohypertrophic signaling proteins, whatever the stimuli. Indeed, Carabin prevents NFAT transcriptional activity under α1- or β-adrenergic receptor stimulation. Expression of the gene encoding MCIP1.4, which is under tight control by calcineurin activity, was further augmented or decreased in hearts of Carabin−/− or AAV9-Carabin− injected mice on pressure overload, respectively. Likewise, the effect of Carabin on CaMKII activity was observed in vivo in the TAC model and in NRVMs treated with either phenylephrine or 8-pCPT (Figure XIIA in the online-only Data Supplement), an exchange protein directly activated by cAMP–selective agonist, which is a potent activator of cAMP-dependent cardiomyocyte hypertrophy.26,31 Phosphorylation of the sarcoplasmic ryanodine receptor, which is another effector of CaMKII, was inhibited by Carabin after activation of the prohypertrophic factor exchange protein directly activated by cAMP (Figure XIIIB in the online-only Data Supplement). Together, these data suggest that Carabin does not belong to a protein complex specifically associated with a subcellular compartment or a signaling pathway but most probably exerts its functions in concert with various proteins localized in distinct intracellular compartments.

Because Carabin expression was decreased in human failing heart and in TAC-induced hypertrophy in rodents, we speculate that this downregulation of Carabin may play an active role in promoting cardiac hypertrophy under stress conditions. In support of this hypothesis, Carabin overexpression attenuated hypertrophic signaling and protected the heart against hypertrophic remodeling. Cardiac gene therapy has emerged as a promising approach for treating HF, supported by a growing number of preclinical studies and a recent successful result in phase II study (the Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease [CUPID] trial) targeting the cardiac sarcoplasmic/ endoplasmic reticulum Ca2+ ATPase pump.33 Our findings that restoration of Carabin expression with AAV9-Carabin improves cardiac function in mice with HF suggest that Carabin gene transfer may provide a novel therapeutic strategy for the treatment of HF. As a next step, it will be important to test the beneficial impact of Carabin gene transfer in a preclinical study using a large-animal model of HF.

Although our data obtained both in vitro and in vivo support a cardioprotective role of Carabin, we found a 50% increase in the expression of Carabin in response to 1 day of treatment with phenylephrine in vitro (Figure IIIB in the online-only Data Supplement). Accordingly, the proximal promoter region of Carabin revealed the presence of multiple consensus NFAT-binding sites, suggesting that the expression of Carabin might be regulated by the calcineurin signaling pathway. Therefore, one can imagine a scenario in which the upregulation of Carabin at the early phase of hypertrophy development may counteract the progression of pathological cardiac growth, and when the stress persists as observed 4 weeks after TAC, Carabin may be degraded, thereby contributing to disease progression. However, to validate this hypothesis, further experiments are required to show the upregulation of Carabin at the early phase of pressure overload–induced cardiac hypertrophy. Interestingly, bioinformatic analysis of the Carabin sequence showed many potential phosphorylation sites, mainly for protein kinase A and C (analysis with NetPhosK, Expasy software), which are implicated in cardiac hypertrophy signaling, suggesting that Carabin may be tightly regulated by phosphorylation and dephosphorylation, which can then influence its proteolytic degradation.

Calcineurin, CaMKII, and Ras signaling pathways may converge at the same downstream effectors such as the transcriptional factors NFAT and MEF2 to regulate cardiac growth. For instance, studies in transgenic mice showed that inhibition of MEF2 abrogated the effects of calcineurin on cardiac dilation and dysfunction, suggesting that this transcription factor was a target of calcineurin. Yet, Li and collaborators reported that calcineurin regulates MEF2 transcriptional activity in C2C12 myoblasts. In contrast, CaMKII is also known to negatively regulate calcineurin/NFAT signaling by phosphorylation. These different signaling pathways are therefore tightly linked, and our finding on Carabin adds complexity to this cross-talk.

As expected, mutations in the calcineurin-binding domain of Carabin (CarabinAAVGA) prevent NFAT activation. These data, combined with the observation that NFAT transcriptional activity was unaffected by the CarabinR141A mutant, indicate that Carabin inhibits calcineurin/NFAT signaling pathways only via its calcineurin-binding domain and in a Ras-independent fashion. On the contrary, the inhibitory effect of Carabin on CaMKII involves its Ras-GAP function because CarabinR141A fails to prevent HDAC4 nuclear translocation and subsequent MEF2 transcriptional activation. This finding is in agreement with a previous report showing that increased MEF2 activity in response to hypertrophic stimuli was dependent on Ras. The calcineurin-binding domain of Carabin is also involved in the inhibitory effect of Carabin on the CaMKII pathway because the mutant CarabinAAVGA behaves as the vector control on HDAC4 nucleocytoplasmic shuttling. The fact that CarabinAAVGA had a slight tendency to inhibit MEF2 activation could be explained by a differential interaction between calcineurin/HDAC4 and calcineurin/MEF2. This hypothesis is strengthened by the study of Grégoire and collaborators, who showed that calcineurin interacted with HDAC4 more strongly than MEF2.

Finally, we found that the antihypertrophic action of Carabin was dependent on its Ras-GAP domain and calcineurin-interacting domain. This makes sense for 2 reasons: The Ras-GAP domain of Carabin prevents the activation of Ras/CaMKII and the calcineurin-interacting domain of Carabin specifically inhibits calcineurin/NFAT signaling, and both Ras/CaMKII signaling and calcineurin signaling are involved in cardiac hypertrophy. Our finding that the calcineurin-binding domain of Carabin is implicated in the inhibitory effect of Carabin on HDAC translocation and MEF2 activation may justify why CarabinAAVGA is less potent than KN93 in inhibiting phenylephrine-induced cardiomyocyte hypertrophy.
This cooperation between the Carabin Ras-GAP domain and calcineurin-interacting domain to exert their inhibitory action on hypertrophy may explain why CarabinR141A is less effective than cyclosporine A in preventing cardiomyocyte hypertrophy.

Conclusions

Our study has revealed Carabin as a novel and negative regulatory point for modulating calcineurin/NFAT, Ras/ERK, and CaMKII signaling. Hypertrophic remodeling of the heart under chronic stress appears to result, at least in part, from downregulation of Carabin and thus from insufficient control by this protein. Our finding that Carabin has a protective function in the heart in response to hemodynamic stress suggests that Carabin may be a potential diagnostic and therapeutic target for heart disease.

Acknowledgments

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Prolonged hypertrophy of the myocardium can progress to heart failure, a leading cause of morbidity and mortality, often associated with sudden cardiac death. Understanding the underlying molecular and cellular mechanisms of cardiac hypertrophy is crucial for developing new therapeutic strategies. Among the numerous signaling pathways that coordinate the cardiac hypertrophic response, the Ca\(^{2+}\)/calmodulin-dependent phosphatase calcineurin, the small G protein Ras, and the Ca\(^{2+}\)/calmodulin-dependent protein kinase II are considered central prohypertrophic signaling effectors in the myocardium. Here, we characterized Carabin, a protein expressed in cardiomyocytes that functions as an endogenous inhibitor of calcineurin, Ras, and Ca\(^{2+}\)/calmodulin-dependent protein kinase II prohypertrophic signaling. Carabin-deficient mice developed a severe cardiac dysfunction in response to myocardial pressure overload, associated with altered contractile function, increased hypertrophic markers, and cardiac fibrosis. Interestingly, Carabin was downregulated both in animal models of cardiac hypertrophy and in human failing myocardium. Compensation for Carabin downregulation in a cardiac disease model using viral gene transfer in vivo protected the heart against hypertrophy and dysfunction. Together, these data indicate that Carabin acts as a negative regulator of pathological cardiac hypertrophy through the inhibition of various components of the intracellular signal-transduction network that coordinates hypertrophic responses. We believe that Carabin gene transfer may provide a novel therapeutic strategy for the treatment of heart failure.
Carabin Protects Against Cardiac Hypertrophy by Blocking Calcineurin, Ras, and Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II Signaling
Malik Bissierer, Magali Berthouze-Duquesnes, Magali Breckler, Florence Tortosa, Loubina Fazal, Annélie de Régibus, Anne-Coline Laurent, Audrey Varin, Alexandre Lucas, Maxime Branchereau, Pauline Marck, Jean-Nicolas Schickel, Claudine Deloménie, Olivier Cazorla, Pauline Soulais-Spraul, Bertrand Crozatier, Eric Morel, Christophe Heymes and Frank Lezoualc’h

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SUPPLEMENTAL MATERIAL

Expanded Methods

Reagents

Phenylephrine (PE), Isoprenaline (Iso) and cyclosporin A (CsA), ionomycin and KN93 were obtained from Sigma-Aldrich. The non-hydrolysable Epac agonist, Sp-8-(4-chlorophenylthio)-2′-O-methyl-cAMP (8-pCPT) was from BioLog.

Human heart tissues

All studies are conformed to the Declaration of Helsinki and institutional ethical regulations. Human failing hearts samples were a kind gift from Dr J.L Samuel (Lariboisiere Hospital, France).

Explanted failing hearts were obtained from patients undergoing cardiac transplantation for end-stage cardiac HF secondary to idiopathic dilated cardiomyopathy. All patients had New York Heart Association (NYHA) Class IV HF, with a mean pretransplant left ventricular ejection fraction of 22±4 %. None had received chronic intravenous inotropic support over at least 7 days immediately prior to transplantation. Heart failure therapy consisted of angiotensin converting enzyme inhibitors and diuretics in all patients. Non-failing hearts were obtained from prospective multiorgan donors who had died from head trauma or intracranial bleeds; these hearts were unsuitable for transplantation for technical reasons. All tissues were stored at −80 °C until further analyses.

Cell Culture

All procedures for cardiac myocyte isolation were performed in accordance with the Guide for the care and use of laboratory animals and the veterinary committee has been informed of
the myocyte isolation protocol used. Neonatal rat ventricular myocytes, mouse and rat adult cardiac myocytes, and cardiac fibroblasts were isolated as previously described by Wollert et al.¹, and Verde et al.², respectively. All media, sera and antibiotics used in cell culture were purchased from Invitrogen (Cergy Pontoise, France).

**Plasmid construct, siRNA and transfection**

The plasmid constructs were generously provided by the following: Carabin by Dr J.O Liu (University School of Medicine, Baltimore), the rat ANF promoter fused to the luciferase reporter gene (ANF-Luc) by Dr K. Knowlton (University of California, U. S. A). The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) was purchased from Stratagene. The luciferase reporter plasmid driven by three MEF2 binding sites (MEF2-Luc) was a kind gift from Dr K.C. Wollert (Hanover Medical School, Germany). Transient transfection experiments of cardiac myocytes were performed using Lipofectamine 2000 (Invitrogen Life Technologies, France) in Optimem medium in the presence of various quantities of plasmid constructs according to the manufacturer’s instructions. The mutants of Carabin, Carabin\textsuperscript{R141A} and Carabin\textsuperscript{AAVG} were generated by site directed mutagenesis using the quickchange site-directed mutagenesis kit (Stratagene). Calcineurin binding domain mutant was made changing PPIEG in AAVGA from amino acid 416 to 420. All plasmid constructs were verified by DNA sequencing. Rat Carabin siRNA (5’-GAGAUGAAAUGGGGUAGAAATT-3’) and control siRNA (5’-GUGGACCCGUGAUGGGCGTT-3’) were purchased from Ambion.

**Thoracic aortic constriction and echocardiographic assessment**

Thoracic aortic constriction (TAC) was performed on mice anesthetized with 1% isofluorane. After endotracheal intubation, mice were connected to a rodent ventilator. The transverse
aorta was isolated and TAC was performed by tying a nylon suture ligature against a 26-gauge needle, the latter being promptly removed to induce pressure overload cardiac hypertrophy. After aortic constriction, the chest was closed, the pneumothorax was evacuated, and the mice were extubated and allowed to recover from the anesthesia. Sham operated animals underwent the same operation except for aortic constriction. Echocardiography was carried out on lightly anesthetized (1% isoflurane in air) mice placed on a heating pad. The left ventricle dimensions were obtained during TM mode acquisition from the parasternal short axis view at the level of the papillary muscles using a Vivid7 echograph and a 14 MHz transducer (i3L, GEHealthcare). Images were transferred and analyzed off line with EchoPAC (GEHealthcare). Echocardiographies were performed with protocols blinded with respect to genotype. TAC was performed in rats as previously described.

Total RNA isolation and real-time quantitative RT-PCR

Extraction of RNA from left ventricles was performed using a QIAGEN RNeasy Mini Kit (Qiagen), as described by the manufacturer. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad). Negative controls without reverse transcriptase were made to verify the absence of genomic DNA contamination. Real-time quantitative PCR was performed in an ABI 7500 Fast (Applied Biosystems) in 96-well plates with 1.5 ng of cDNA from RT reaction, specific primers and SsoFast™ EvaGreen Supermix (Bio-Rad). Rpl32 and GBA were measured as a reference gene for mouse and rat, respectively. See table below for the sequences of the different primer sets. mRNA Carabin expression was analyzed on 4.5 ng cDNA by qRT-PCR using SYBR® Green-based RT² qPCR Primer Assays (SA Biosciences/Qiagen). Primers for mouse and human Carabin were obtained from Qiagen (Mm.288312 and Hs.534648, respectively). Q-PCR was performed using a StepOne Fast Real-Time PCR System (Applied Biosystems). PCR reactions were performed in triplicates.
Results were expressed as the average of relative gene expression normalized for mouse RPL32 mRNA and human GAPDH expressions for each sample and the ∆∆CT method was used for data analysis.

**Primers used for quantitative polymerase chain reaction**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Gene</th>
<th><strong>Sequence 5′- 3′</strong></th>
<th>Species</th>
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<td>ANF</td>
<td>Nppa</td>
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<td>Mouse</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: CTCCTCCAGGTGGTCTAGCA</td>
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<tr>
<td>BNP</td>
<td>Nppb</td>
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<td>Mouse</td>
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<td></td>
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<td>Carabin</td>
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<td>Rat</td>
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<td></td>
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<tr>
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<td></td>
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<td>Reverse: GCACCCAGGAGACCATAATTTC</td>
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<td>GBA</td>
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<td>Forward: TAGGCTCCTGGGATCGAG</td>
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<td></td>
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<td>MCIP1.4</td>
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<td>Mouse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGGATTGGTGAATCTGGATG</td>
<td></td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; Col1A1, pro-collagen, type I, alpha 1; Carabin; Col3, collagen type 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBA, glucocerebrosidase; MCIP 1.4, modulatory calcineurin-interaction protein 1.4; β-MHC, beta-myosin heavy-chains; RPL32, ribosomal protein L32.

**Immunoprecipitation and western blot**

After appropriated treatment, cells were lysed in a buffer (buffer A) containing 20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 20 mmol/L MgCl₂, 0.5% NP40, 0.5 mmol/L EDTA, protease and phosphatase cocktail inhibitors (Roche). After centrifugation for 20 min at 15000 g, proteins concentration was determined with the bicinchoninic acid (BCA) protein assay kit.
Soluble extracts were precleared using the mouse ExactaCruz F IP matrix (ExactaCruz F, Santa Cruz Biotechnology) for 30 min at 4°C. Matrix was removed by brief centrifugation and supernatant transferred to a new tube. An IP antibody–IP matrix complex was prepared as per manufacturer's instructions using anti-ras antibody (Clone Ras10, Millipore). The IP antibody–IP matrix complex was incubated with the pre-cleared cell lysate overnight at 4°C. After incubation, the lysate was centrifuged to pellet the IP matrix. The matrix was washed two times with buffer A and bound proteins were eluted with sample buffer containing SDS. The proteins were separated on SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare). After blocking, membranes were hybridized for overnight at 4°C with the following primary antibodies against: Ras (Clone Ras10, Millipore), Carabin (Abdserotec), p-ERK and t-ERK (Cell Signaling Technology and Santa Cruz Biotechnology), HDAC4 (Santa Cruz Biotechnology), β-actin (Santa Cruz Biotechnology), cMyc (Upstate), p-CaMKII<sup>Thr286</sup> and t-CaMKII (Santa-Cruz Biotechnology), GAPDH (Cell Signaling Technology) and Phospho-RyR2 Ser<sup>2815</sup> (Badrilla Ltd). The secondary antibodies were coupled with Horseradish Peroxidase (Cell Signaling Technology). Immunoblots were revealed by enhanced chemiluminescence (ECL plus, GE Healthcare).

**HDAC translocation**

Neonatal rat cardiac myocytes were collected and harvested in ice cold PBS. After centrifugation at 5000 rpm for 2 min, cells were lysed on ice (15 min) in a hypotonic buffer (Hepes 20mmol/L; pH 7.5, NaCL 10 mmol/L proteases and phosphatases cocktail inhibitors) supplemented with 10% Nonidet P40. After centrifugation (2500 rpm, 2 min, 4°C), the supernatant containing the cytosolic fraction was collected whereas the pellet was resuspended in a hypertonic buffer (Hepes, 20 mmol/L, pH 7.5; NaCL 400 mmol/L),
supplemented with proteases and phosphatases cocktail inhibitors and leave on ice during 15 min. After centrifugation (10 000 rpm, 10 min, 4°C), the supernatant containing the nucleic fraction was collected.

Left ventricular tissue were lysated in a buffer containing Hepes 10 mmol/L pH 7.9, KCl 10 mmol/L, EDTA 0.1 mmol/L, NP40 0.5 %, proteases and phosphatases cocktail inhibitors. After centrifugation (2500 rpm, 2 min, 4°C), the supernatant containing the cytosolic fraction was collected whereas the pellet was resuspended in nuclear lysis buffer (Hepes, 20 mmol/L, pH 7.9, KCl 10 mmol/L, NaCl 0.4 mmol/L, EDTA 1 mmol/L, SDS 0.3 %), supplemented with proteases and phosphatases cocktail inhibitors. After incubation (30 min, 4°C), nuclear fractions were centrifuged (2000 rpm, 10 min, 4°C) and the supernatant containing the nuclear fraction was collected. Protein concentration of both fractions was determined with a BCA protein assay kit (Sigma-Aldrich). 15 μg of total proteins were boiled for 10 min in reducing Laemmli sample buffer and resolved by SDS-PAGE.

**Ras activation assay**

Ras pull-down experiments were performed using a GST fusion protein containing the Ras Binding Domain of Raf1 as previously described⁴.

**Calcineurin assay**

Calcineurin phosphatase activity was measured on cell extracts using the calbiochem calcineurin cellular activity assay kit (Ref. 207007) according to the manufacturer’s instructions. The fraction of total activity due to calcineurin was determined by detection of free phosphate released in the absence or presence of EGTA buffer. Colorimetric measure was performed at 620 nm on plate reader (Dynatech MR 5000).
**Immunostaining assay**

Cardiomyocytes were fixed with 4% paraformaldehyde for 5 min and permeabilized with 0.5% Triton X-100 for 5 min. After a preincubation in 3% BSA for 1h, myocytes were incubated with an anti-α-sarcomeric actinin (monoclonal, clone EA-53, 1:300, Sigma-Aldrich), anti-NFATc3 (monoclonal, 1:200, Santa Cruz) or anti-myc (monoclonal, 1:200, Santa Cruz) antibodies for 1 h. After washing with PBS, cells were incubated with a secondary antibody coupled to Alexa Fluor®633 or Alexa Fluor®488 (1:200, Molecular probes) for 1h. Coverslides were mounted with vectashield mounting medium (Vector Laboratories). Stained-cardiomyocytes were analysed by confocal laser microscopy (Zeiss LSM510). For each condition, quantification of NFATc3 nuclear translocation was determined as the ratio between cells containing NFATc3 localized in their nucleus and the total number of cells counted. For the determination of cell surface area, photographic images of NRVMs were digitized and the areas of 100 to 200 cells per condition from at least 3 independent experiments were analysed by computer-assisted planimetry (Perkin Elmer).

**Quantitative analysis of cardiomyocyte cross-sectional areas**

Hearts were transversely sectioned at 7 µm thickness, rehydrated in H2O, fixed with 4% paraformaldehyde and permeabilized with Triton (Image-iT® Fix-Perm kit, Molecular Probes). After a pre-incubation in Blocking Solution for 1.5 h, cryoslices were incubated with a mouse monoclonal anti-vinculin antibody (1:100, V9131, Sigma-Aldrich) for 2 h. After washing with PBS, cells were incubated with a goat anti-mouse secondary antibody coupled to Alexa Fluor®488 (1:400, A11029 Molecular Probes) for 1.5 h. To determine cell size, images of stained cardiomyocytes were acquired on Zeiss Observer Z.1 microscope (Carl Zeiss) at ×160 magnification. The outlines of a least 300 circular to oval shaped cardiac
myocytes were traced in 4 fields from at least 3 different mouse samples by using Axio Vision Rel 4.7 software (Carl Zeiss).

**Determination of fibrosis**
Hearts were transversely sectioned at 10 µm thickness, fixed with 4% paraformaldehyde and stained with Masson’s Trichrome. Slides were scanned with NanoZoomer (Hamamatsu v1.2) and fibrosis was measured as positively stained area with Masson’s Trichrome and expressed as percent of total area, using ImageJ software (RSB).

**Hematoxylin and eosin-staining**
Hearts were collected, fixed in 4% paraformaldehyde, dehydrated, and embedded in OCT tissue embedding compound (Tissue Tek, EMS). Longitudinal sections were performed at 10 µm in thickness and stained with hematoxylin and eosin for histological examination.
Supplemental Figure 1. (A) Cardiac Carabin expression in adult myocytes and fibroblasts. Cells were isolated from the heart of knock-out mice for Carabin (Carabin−/) or wild type control animals (WT) and Western blot was performed. A representative immunoblot is shown. (B) Immunoblot analysis of Carabin was performed on left ventricular (LV) homogenates of Carabin knock-out mice (Carabin−/) and wild type (WT) animals and subjected or not to thoracic aortic constriction (TAC). GAPDH expression was used as loading control. A representative immunoblot is shown. The graph represents quantification of Carabin levels (n=5 per group). All data were evaluated by 2-way ANOVA/Tukey post-test. **P<0.01, ***P<0.001, compared with indicated values.
Supplemental Figure 2. Carabin-deficient mice subjected to thoracic aortic constriction (TAC) developed a pulmonary oedema. Lung weight (LuW)/Tibia length (TL) ratios of control and Carabin knock-out (Carabin⁻/⁻) mice 4 weeks after sham and TAC operation (n=10–12 per group). Statistical significance was determined by 2-way ANOVA with interaction analysis and Tukey post-test. ***p<0.001 compared with indicated values; ####p<0.0001 compared with wild type (WT)-TAC animal group.
Supplemental Figure 3. Knock-down of Carabin with specific siRNA (Si-Carabin) potentiates PE-induced ANF promoter transcriptional activity and cardiomyocyte surface area. (A) Carabin siRNA efficiency is demonstrated by immunoblot. Neonatal rat ventricular myocytes (NRVMs) were transfected with either Si-Carabin or the scramble siRNA as control (Si-CT). One day after transfection, cells were treated or not with PE (10 µmol/L) for 24 h and immunoblot was then performed. (B) Effect of Si-Carabin on the ANF promoter fused to the luciferase gene (ANF-Luc) construct in NRVMs stimulated or not with PE (10 µmol/L). NRVMs were transfected with ANF-Luc and Si-CT or Si-Carabin. One day after transfection, cells were treated or not with PE (10 µmol/L) for 24 h. Cells were then assayed for luciferase activity. Results are means ± S.E.M from 5 independent experiments performed in triplicate. (C) Quantification of cell surface area. NRVMs were treated as in (B) and cell surface area was determined by immunostaining for sarcomeric α-actinin. The areas of 100 to 200 cells per condition were analyzed by computer-assisted planimetry. Results are means ± S.E.M from 5 independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to the non-stimulated Si-CT cells; #p<0.05, ##p<0.01, compared with stimulated Si-CT cells, 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 4. Schematic time scale and experimental strategy to evaluate the effect of Carabin expression on pressure overload-induced cardiac hypertrophy. Adeno-associated virus of serotype 9 (AAV9) allowing for GFP (control) or Carabin expression in cardiomyocytes were injected into retro-orbital sinus of mice (3.10^{11} genome copies/mL) at week 0. Five weeks later, transverse aortic constriction was performed. Echocardiography and anatomical parameters were determined 4 weeks after surgery (Week 9).
Supplemental Figure 5. Knock-down of Carabin potentiates PE-induced calcineurin (CaN) activity (A) and NFAT nuclear translocation (B). Neonatal rat ventricular myocytes (NRVMs) were transfected with either siRNA Carabin (Si-Carabin) or the scrambled siRNA as control (Si-CT). Two days after transfection, cells were treated or not with phenylephrine (PE, 10 µmol/L) for either 30 min (A) or 10 min (B). (A) CaN activity was determined on NRVMs stimulated with PE. Cyclosporine A (CsA: 1 µmol/L), was used as a positive control (n=4 per condition). (B) Endogenous NFATc3 was visualized by immunostaining. Representative photographic images of NFATc3 translocation and quantification are shown. DAPI stain marks the position of nuclei. 100 to 200 cells were analyzed per condition and experiments. Scale bar represents 10 µm. Results are expressed as percentage of control value (Si-CT) and are means ± S.E.M from 3 independent experiments performed. *p<0.05, **p<0.01, ***p<0.001 compared with indicated values; 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 6. Carabin prevents ionomycin-induced NFAT nuclear translocation. NRVMs were transfected with either Carabin or the empty vector as control (pSV2). Two days after transfection, cells were treated or not with ionomycin for 30 min (Ionomycin, 1 µmol/L). NFAT nuclear translocation was visualized by immunostaining as described in supplemental Methods. Representative photographic images of NFATc3 translocation and quantifications are shown. DAPI stain marks the position of nuclei. 100 to 200 cells were analyzed per condition and experiments. Scale bar represents 10 µm. Results are expressed as percentage of control value (pSV2) and are means ± S.E.M from 3 independent experiments performed. *p<0.05, ***p<0.001 compared with indicated values, 1-way ANOVA, Bonferroni comparison test.
Supplemental Figure 7. Carabin prevents isoprenaline-induced nuclear factor of activated T cells (NFAT) transcriptional activation. Neonatal rat ventricular myocytes were transfected with either the NFAT response elements coupled to the luciferase gene and Carabin or the empty vector as control (pSV2). One day after transfection, cells were treated or not with isoprenaline (ISO, 10 µmol/L) The day after, cells were assayed for luciferase activity. Results are expressed as percentage of control value (pSV2) and are means ± S.E.M from 5 independent experiments performed in triplicate. **p<0.01, compared with the indicated values; 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 8. SiRNA targeting Carabin (Si-Carabin) enhances phenylephrine (PE)-induced extracellular signal-regulated kinase (ERK) activation. Neonatal rat ventricular myocytes were transfected with either a control siRNA (Si-CT) or Si-Carabin. Two days after transfection, cells were treated or not with PE (10 µmol/L) for 10 min. Phosphorylated-ERK (p-ERK), total-ERK (t-ERK) and Carabin were detected by immunoblot. A representative immunoblot shows Carabin expression after cell transfection with either Si-CT or Si-Carabin. The bar graph represents the mean ± S.E.M. of 4 independent experiments. *p<0.05, ***p<0.001 compared with indicated values; 2-way ANOVA, Bonferroni comparison test. Results are expressed as fold activation of unstimulated Si-CT cells.
Supplemental Figure 9. Carabin siRNA (Si-Carabin) potentiates phenylephrine (PE)-induced the transcriptional activity of myocyte enhancer factor 2 (MEF2). Rat neonatal ventricular myocytes were transfected with a construct containing MEF2 response elements fused to the luciferase gene (MEF2-Luc) and either a control siRNA (Si-CT) or Si-Carabin. Two days after transfection, cells were treated or not with PE (10 µmol/L) for 24 h. Cells were then assayed for luciferase activity. Results are means ± S.E.M from 7 independent experiments performed in triplicate. Lower panel, representative immunoblot showing Carabin expression after cell transfection with either Si-CT or Si-Carabin. *<0.05, ***p<0.001 compared with indicated values, 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 10. Carabin Ras-GAP domain and CaN interacting domain are both involved in the inhibitory effect of Carabin on the transcriptional activity of the ANF promoter. Neonatal rat ventricular myocytes were cotransfected with the ANF promoter fused to the luciferase reporter gene and the indicated constructs. One day after transfection, cells were stimulated or not with PE (10 μmol/L) for 24 h in the presence of the selective inhibitor of CaMKII, KN93 (2 μmol/L) or an inhibitor of CaN, Cyclosporine A (CsA; 1 μmol/L). Cells were then assayed for luciferase activity. Lower panel, representative immunoblots showing cMyc-Carabin and GAPDH expression. Results are expressed as percentage of control value and are means ± S.E.M from 5 independent experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001, compared with indicated values; ##p<0.01, ###p<0.001, compared with stimulated control condition; 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 11. Effects of Carabin mutants on cell size. Neonatal rat ventricular myocytes were cotransfected with GFP and the indicated plasmids and treated or not with PE (10 μmol/L) for 24 h in the presence of the selective inhibitor of CaMKII, KN93 (2 μmol/L) or an inhibitor of CaN, Cyclosporine A (CsA; 1 μmol/L). Cell surface area was determined by immunostaining for sarcomeric α-actinin in cardiac myocytes expressing GFP. 100 to 200 cells were analyzed per condition and experiments. Representative image and quantification of cell surface area are shown. Scale bar represents 10 µm. Results are expressed as percentage of control value and are means ± S.E.M from 3 independent experiments performed. *p<0.05, ***p<0.001 versus the indicated values; ###p<0.001, compared with stimulated control condition; 2-way ANOVA, Bonferroni comparison test.
Supplemental Figure 12. (A) Carabin inhibits calcium/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation. Rat neonatal cardiomyocytes were infected with either an adenovirus encoding GFP (Ad.GFP) or Carabin (Ad.Carabin). Two days after infection, cells were treated or not with the Epac agonist, 8-pCPT (10 µmol/L, 10 min). Phosphorylated CaMKII (p-CaMKII) and total CaMKII (t-CaMKII) were determined as described in Methods. (B) Carabin prevents Epac- induced CaMKII phosphorylation of RyR2. Adult rat cardiomyocytes were infected with either Ad.GFP or Ad.Carabin. Two days after infection, cells were treated or not with 8-pCPT (10 µmol/L, 10 min). RyR2 phosphorylation at CaMKII site (p-RyR2Ser2815), Carabin and GAPDH were determined as described in Methods. Upper panel, the graph represents means ± S.E.M of 4 independent experiments. Lower panel, representative immunoblots are shown. *p<0.05, ***p<0.001, versus the non stimulated control value or indicated values; 2-way ANOVA, Bonferroni comparison test. ns, non-significant.
Supplemental references


