RhoGEF12 controls cardiac remodeling by integrating G protein– and integrin-dependent signaling cascades

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Structural cardiac remodeling, including hypertrophy and fibrosis, plays a crucial role in the pathogenesis of heart failure. In vitro studies suggested a role of the small GTPase RhoA in hypertrophic cardiomyocyte growth, but neither the molecular mechanisms leading to RhoA activation nor their relevance in vivo are known. We use here a mass spectrometric approach to identify Rho guanine nucleotide exchange factors (RhoGEFs) activated during cardiac pressure overload in vivo and show that RhoGEF12 is a central player during cardiac remodeling. We show that RhoGEF12 is required for stretch-induced RhoA activation and hypertrophic gene transcription in vitro and that its activation depends on integrin β1 and heterotrimeric G proteins of the G12/13 family. In vivo, cardiomyocyte–specific deletion of RhoGEF12 protects mice from overload–induced hypertrophy, fibrosis, and development of heart failure. Importantly, in mice with preexisting hypertrophy, induction of RhoGEF12 deficiency protects from cardiac decompensation, resulting in significantly increased long-term survival. Collectively, RhoGEF12 acts as an integrator of stretch–induced signaling cascades in cardiomyocytes and is an interesting new target for therapeutic intervention in patients with pressure overload–induced heart failure.

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integrins, receptor tyrosine kinases, and heterotrimeric G proteins of the families G\textsubscript{i}, G\textsubscript{q/11}, and G\textsubscript{12/13} (Burridge and Wennerberg, 2004). By what mechanisms RhoA is activated in adult cardiomyocytes under conditions of pressure overload, which downstream effectors it controls, and whether these pathways are relevant for cardiac remodeling in vivo are currently unclear.

RESULTS AND DISCUSSION
To impose pressure overload on left cardiac ventricles in vivo, we used transverse aortic constriction (TAC) in mice, which resulted in a rapid and sustained RhoA activation (Fig. 1, A and B). Quantitative RT-PCR (qRT-PCR) revealed that both adult murine cardiomyocytes and whole human hearts expressed various RhoGEFs, most abundant among them Arhgef12, the gene coding for RhoGEF12 (also known as leukemia-associated RhoGEF [LARG]; Fig. 1 C). To investigate which of these RhoGEFs were activated in the mouse heart by TAC, we performed affinity pull-down assays with a nucleotide-free RhoA mutant, a method designed to precipitate active pools of Rho-interacting proteins (Garcia-Mata et al., 2006), followed by mass spectrometry (MS). The two RhoGEFs that showed strongest activation in response to TAC were Mcf2l and RhoGEF12, whereas other RhoGEFs did not show significantly increased RhoA binding after TAC (Fig. 1, D and E). Protein immunoblotting of left ventricular lysates obtained at different time points after TAC confirmed the strong and sustained activation of RhoGEF12, whereas activation of Mcf2l was less prominent (Fig. 1, F and G).

To investigate the role of RhoGEF12-dependent RhoA activation in cardiomyocyte hypertrophy, we studied stretch-induced effects in cultured neonatal rat ventricular myocytes (NRVMs) in vitro. Mechanical stress induced a fast and stable activation of RhoGEF12 (Fig. 2 A) and RhoA (Fig. 2 C) with a maximal response between 3 and 30 min. siRNA-mediated knockdown of RhoGEF12 (Fig. 2 B) strongly reduced stretch-induced RhoA activation (Fig. 2 C) as well as expression of hypertrophy-specific genes such as \( \beta\)-MHC or atrial natriuretic peptide (ANP; Fig. 2 D). Also, stretch-induced increases in cell size were significantly reduced after knockdown of RhoGEF12 (Fig. 2 E). Pretreatment of NRVMs with the RhoA inhibitor C3 exoenzyme or siRNA-mediated knockdown of RhoA fully mimicked the effect of RhoGEF12 knockdown (not depicted), indicating that

Figure 1. Pressure overload–induced RhoGEF activation in vivo. (A and B) RhoA activation in wild-type hearts at different time points after TAC was determined by ELISA (\( n \) = 6; A) or by pull-down assay and consecutive Western blotting (\( n \) = 2; B). (C) qRT-PCR analysis of RhoGEF expression in isolated adult murine cardiomyocytes (cmc; \( n \) = 3) and whole in human hearts (\( n \) = 2). (D and E) Activation of RhoGEF proteins 24 h after TAC was determined by mass spectrometric analysis of proteins coprecipitated with bead-coupled nucleotide-free RhoA. D shows the label–free ratio distribution against the sum of peak intensities, and E depicts the five RhoGEFs that showed at least twofold increase in RhoA binding after TAC (\( n \) = 1; data presented as log2 of the LFQ intensity ratio between TAC sample and sham sample, and significance indicates ratio outliers from the main distribution). (F) Activation of RhoGEF12 and Mcf2l was determined at different time points after TAC by precipitating RhoA-interacting proteins with bead-coupled nucleotide-free RhoA mutant as in D, followed by immunoblotting with antibodies directed against Mcf2l and RhoGEF12 (total cell lysate as loading control; \( n \) = 3). (G) Statistical evaluation of F (basal set to 1). Error bars indicate SEM. *, \( P < 0.05; **, P < 0.01; ***, P < 0.001. 
Figure 2. Mechanism of stretch-induced RhoGEF12 activation in NRVMs. (A) Stretch-induced RhoGEF12 activation was determined by precipitating RhoA-interacting proteins with a bead-coupled nucleotide-free RhoA mutant, followed by immunoblotting with antibodies directed against RhoGEF12 (n = 3). (B) Efficiency of RhoGEF12 knockdown in NRVMs as shown by immunoblotting with anti-RhoGEF12 antibodies (actin as loading control; n = 2). (C) RhoA activation in siRNA-treated NRVMs at different time points after exposure to cyclic stretch (n = 3; basal set to 1). (D) Expression of hypertrophy-specific genes in siRNA-transfected NRVMs was determined by qRT-PCR after 24 h of cyclic stretching (n = 3; data normalized to GAPDH, control values set to 1). (E) Effect of RhoGEF12 (GEF12) knockdown on cell size in the basal state and after 24 h of cyclic stretching (n = 2). (F) Efficiency of siRNA-mediated knockdown of Gα12/13 or Itgβ1 in NRVMs (α-tubulin as loading control; n = 2). (G) Stretch-induced activation of RhoGEF12 after knockdown of Gα12/13 or Itgβ1 was determined by affinity pull-down assay with nucleotide-free RhoA and consecutive immunoblotting (n = 8; basal set to 1; data in B–G were generated using two independent sets of siRNAs). (H) Stretch-induced RhoA activation was determined in NRVMs after siRNA-mediated knockdown of Gα12/13 or Itgβ1 (n = 4). (I and J) RhoGEF12 activation in response to 1 µM ET-1 (5 min; I) or in response to 5 µg/ml of an activating Itgβ1 antibody (Ab; 5 min; J) in siRNA-transfected NRVMs (left, exemplary immunoblot; right, statistical evaluation; n = 3; basal set to 1). (K) RhoGEF12 activation induced by an activating Itgβ1 antibody after siRNA-mediated knockdown of Src (siSrc) or Fyn (siFyn; n = 3). (L) Translocation of MRTF-A from the cytosolic fraction (C) to the nuclear fraction (N) in siRNA-transfected NRVMs after 12 h of stretch. Antibodies against tubulin and histone were used as markers for cytosolic and nuclear fractions, respectively (n = 2). (M) Effect of siRNA-mediated knockdown of MRTF-A on stretch-induced up-regulation of β-MHC expression in NRVMs (n = 3). Error bars indicate SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.
RhoGEF12 controls hypertrophic gene expression through RhoA activation. We next studied the role of potential activators of RhoGEF12 such as integrin β1 (Itgβ1; Guilluy et al., 2011), Gq/11 (Fukuhara et al., 2000), Gq/11 (Booden et al., 2002), or CD44 (Bourguignon et al., 2006). siRNA-mediated knockdown in NRVMs (Fig. 2 F) revealed that inactivation of Itgβ1 or the α-subunits of the G12/13 family (Gα12/13) time-dependently reduced stretch-induced RhoGEF12 activation (Fig. 2 G), and comparable effects were observed on the level of RhoA activation (Fig. 2 H). In contrast, knockdown of Gαq/11, CD44, or Itgβ3 was without effect (not depicted). We next investigated whether Itgβ1 and G proteins of the G12/13 family are independent activators of RhoGEF12 or whether they act in a sequential manner. We found that RhoGEF12 activation in response to endothelin-1 (ET-1), an agonist known to activate G12/13-coupled GPCRs but not integrins, depended on Gαq/11 but not on Itgβ1 (Fig. 2 I), whereas RhoGEF12 stimulation induced by an Itgβ1-activating antibody required Itgβ1 but not Gα12/13 (Fig. 2 J). We furthermore found that knockdown of protein kinases c-Src and Fyn strongly reduced RhoGEF12 activation elicited by direct Itgβ1 stimulation (Fig. 2 K), suggesting that these kinases mediate Itgβ1-dependent RhoGEF12 activation in cardiomyocytes. With respect to the mechanisms linking RhoA activation to transcriptional regulation, we studied the potential involvement of myocardin-related transcription factors (MRTFs), which have been shown to translocate upon RhoA-mediated actin polymerization to the nucleus where they act as coactivators of serum response factor (SRF)-dependent gene transcription (Olson and Nordheim, 2010). We found that stretch-induced translocation of MRTF-A from the cytoplasmic fraction to the nuclear fraction depended on RhoGEF12 (Fig. 2 L) and that stretch-induced expression of hypertrophy-specific genes such as β-MHC depended on MRTF-A (Fig. 2 M).

To study the role of RhoGEF12 in cardiomyocytes in vivo, we generated mice with tamoxifen-inducible, cardiomyocyte-specific RhoGEF12 deficiency (cmc-GEF2-KO). Magnetic resonance imaging (MRI) and histological analyses did not reveal basal differences between the genotypes (not depicted). When pressure overload was induced by TAC, cmc-GEF2-KOs showed reduced RhoA activation compared with control mice (Fig. 3 A). 4 wk after TAC, the left ventricular wall thickness (Fig. 3 B), left ventricular weight/tibia length ratio (Fig. 3 C), and expression of hypertrophy-specific genes (Fig. 3 D) were significantly reduced in mutant mice. What is more, cmc-GEF2-KOs showed lower expression of collagen isoforms (Fig. 3 E) and reduced fibrosis (Fig. 3 F), and the left ventricular ejection fraction was preserved compared with control mice (Fig. 3 G). To investigate whether Gα12/13 and Itgβ1 are also under in vivo conditions relevant for RhoGEF12 activation, we studied TAC-induced RhoGEF12 activation in mice with tamoxifen-inducible, cardiomyocyte-specific Gα12/13 deficiency (cmc-Gα12/13-KO; Takefuji et al., 2012) and tamoxifen-inducible, cardiomyocyte-specific Itgβ1 deficiency (cmc-Itgβ1-KO). We found that in both mutant mouse lines, activation of RhoGEF12 was significantly reduced 24 h after TAC, indicating that also under conditions of pressure overload Itgβ1 and Gα12/13 are crucial for RhoGEF12 activation (Fig. 3 H).

Based on the finding that inactivation of RhoGEF12 in cardiomyocytes prevents not only the development of hypertrophy but also deterioration of cardiac ejection fraction, we next investigated whether inhibition of RhoGEF12 would also prove beneficial if applied in already established hypertrophy. To study this, TAC was performed 2 wk before tamoxifen-mediated induction of RhoGEF12 deficiency, and cardiac performance was followed by MRI for up to 1 yr (Fig. 4 A). Both genotypes behaved similarly during the first 2 wk after surgery, but upon tamoxifen treatment, RhoGEF12-deficient mice were protected from further increases in wall thickness and deterioration of ejection fraction (Fig. 4, B and C). Interestingly, at ~6 mo after TAC, wild-type mice but not cmc-GEF2-KOs started to show progressive ventricular dilation (Fig. 4, D and E), resulting in significantly increased mortality (Fig. 4 F). Hearts of surviving control mice 1 yr after TAC compared with mutants showed significantly higher RhoA activation, stronger fibrosis, elevated expression of heart failure markers ANP and brain natriuretic peptide (BNP), and increased apoptosis (Fig. 4, G–J).

Collectively, we show in this study that RhoGEF12 integrates signals from integrins and GPCRs to control hypertrophic gene expression and the transition to heart failure. Because of their strategic position at the interface between extracellular matrix and intracellular cytoskeleton, integrins have repeatedly been suggested as sensors of mechanical stress (Wang et al., 2009). Evidence for a role of integrins in cardiac hypertrophy mainly stems from studies in NRVMs showing that cell growth and ANP production are reduced after blockade of β integrins (Yutao et al., 2006) and that stretch activates integrin-dependent signaling intermediates such as focal adhesion kinase, integrin-linked kinase, or Src (Laser et al., 2000; Torsoni et al., 2003; Lammerding et al., 2004; Brancaccio et al., 2006). Integrins are also well known for their ability to modulate RhoA activity (Burridge and Wennerberg, 2004), but the role of integrin-dependent RhoA activation in the heart is not understood. Our data show that mechanical stress induces Itgβ1-dependent RhoA activation in neonatal cardiomyocytes and that RhoGEF12 is the major RhoGEF contributing to this effect. In line with this, it was recently shown that application of force to β integrins induces in fibroblasts recruitment of RhoGEF12 to focal adhesions, resulting in RhoA activation (Guilluy et al., 2011). The same study identified Arhgef2/GEF-H1 as a second GEF involved in force-induced RhoA activation, but our mass spectrometric analysis suggested that TAC-induced Arhgef2/GEF-H1 activation in adult cardiomyocytes is mild compared with RhoGEF12 activation.

Our data furthermore show that stretch-induced RhoGEF12 activation is not only mediated by Itgβ1, but also requires heterotrimeric G proteins of the G12/13 family, whereas the Gq/11 family seems dispensable. The stretched myocardium is...
known to release several humoral factors that enhance the hypertrophic response through stimulation of GPCRs, for example ET-1 or angiotensin II (Ito et al., 1993; Sadoshima et al., 1993), and so far these effects were generally believed to be mediated by the G\textsubscript{q/11} family (Frey and Olson, 2003; Dorn and Hahn, 2004). However, the receptors in question can also signal through the G\textsubscript{12/13} family (Riobo and Manning, 2005), and our findings clearly suggest a role of G\textsubscript{12/13} in stretch-induced hypertrophy in vitro. This notion is in line with several in vitro studies in NRVMs (Miyamoto et al., 2010) and is furthermore supported by the fact that TAC-induced hypertrophy is strongly reduced in cardiomyocyte-specific G\textsubscript{12/13}-deficient mice (Takefuji et al., 2012). Interestingly, G\textsubscript{12/13} seemed mainly responsible for early stretch-induced RhoGEF12 activation, whereas Itg\textbeta\textsubscript{1} was required for later effects, together creating a unique temporal pattern of RhoA activation in response to stretch.

The mechanisms underlying the transition from hypertrophy to heart failure are not well defined, but cardiomyocyte apoptosis and fibrotic replacement are most likely contributing factors (Dorn and Hahn, 2004; Hill and Olson, 2008; Miyamoto et al., 2010). Our study reveals that inactivation of one particular path of RhoA activation, namely RhoGEF12-dependent RhoA activation, protects the heart not only from hypertrophy development, but also from cardiomyocyte apoptosis, fibrosis, and the development of chronic heart failure. RhoA itself is at the center of several signaling pathways (Burridge and Wennerberg, 2004), and its role in cardiac

Figure 3. RhoGEF12 mediates pressure overload–induced hypertrophy in vivo. (A) RhoA activation in adult hearts from control mice and cmc-GEF12-KOs 3 d and 4 wk after TAC or sham surgery (\(n = 3\)). (B) Left ventricular end-diastolic (LVED) wall thickness (as determined by MRI) 4 wk after sham operation or TAC (\(n = 6–7\)). (C) Cardiac morphology and left ventricular weight/tibia length (LVW/TL) ratio 4 wk after TAC or sham surgery (\(n = 8–12\)). (D and E) Expression of hypertrophy-specific genes (D) and profibrotic genes (E) was determined by qRT-PCR in hearts of control mice and cmc-GEF12-KOs 4 wk after sham or TAC (\(n = 4–6\)). Coll1, 3, and 4: collagen isoforms 1, 3, and 4. (F) TAC-induced fibrosis determined by Picosirius red staining 4 wk after TAC (\(n = 4–6\)). (G) Left ventricular (LV) ejection fraction determined by MRI 4 wk after sham surgery or TAC (\(n = 6–7\)). (H) TAC-induced RhoGEF12 activation in hearts of control mice as well as in hearts of tamoxifen-inducible, cardiomyocyte-specific G\textalpha\textsubscript{12/13}-deficient mice (cmc-G\textalpha\textsubscript{12/13}-KO) and tamoxifen-inducible, cardiomyocyte-specific Itg\textbeta\textsubscript{1}-deficient mice (cmc-Itg\textbeta\textsubscript{1}-KO) was determined by precipitating RhoA-interacting proteins with a bead-coupled nucleotide-free RhoA mutant, followed by immunoblotting with antibodies directed against RhoGEF12 (\(n = 2\)). Error bars indicate SEM. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
Figure 4. Cardiomyocyte-specific inactivation of RhoGEF12 improves cardiac function and survival in preexisting hypertrophy. (A) Experimental design. (B–E) Control mice (blue lines, diamonds) and not yet induced cmc-GEF12-KOs (red lines, squares) were subjected to sham surgery (dashed lines) or TAC (solid lines) at day 0, followed by tamoxifen (Tam) induction of recombination on days 14–18. MRI analysis of left ventricular end-diastolic (LVED) wall thickness (B), left ventricular (LV) ejection fraction (C), and LVED volume (D, exemplary MRI images; E, statistical evaluation) was performed before and after TAC, followed by tamoxifen injection (n = 15–24). (G) RhoA activation in adult hearts from control mice and cmc-GEF12-KOs 1 yr after TAC (n = 3). (H) TAC-induced fibrosis 1 yr after TAC as determined by Picrosirius red staining (n = 3). (I) Expression of ANP and BNP 1 yr after sham surgery or TAC as determined by qRT-PCR in whole hearts (n = 3–5; data normalized to GAPDH, control values set to 1). (J) Apoptosis was determined by TUNEL staining in left ventricles 1 yr after TAC (n = 3). Error bars indicate SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

physiology and pathophysiology is correspondingly complex: although strong RhoA activation in cardiomyocytes has been shown to induce apoptosis and cardiomyopathy (Miyamoto et al., 2010), moderate RhoA activation has cardioprotective effects under conditions of ischemia/reperfusion (Xiang et al., 2011). Whether a general inhibition of RhoA activation would have the same beneficial effects during pressure overload as prevention of RhoGEF12-dependent RhoA activation is currently unclear.

In conclusion, we found that RhoGEF12 plays a unique role in mediating stretch-induced cellular responses by integrating signals from activated GPCRs and integrins. The fact that genetic ablation of RhoGEF12 does not affect basal heart function but efficiently prevents cardiac decompensation even in mice with established hypertrophy makes this signaling pathway a promising target for therapeutic intervention in patients with pressure overload–induced heart failure.

MATERIALS AND METHODS

Materials and chemicals. ET-1 and tamoxifen were purchased from Sigma-Aldrich. C3-exoenzyme (cell-permeable Rho inhibitor [C30]) was obtained from Cytoskeleton. Antibodies to Gα13 (A-20), RhoGEF12 (LARG; N14), Mcf2l (Dbs; L-20), Histone (N-16), MKT-F-A (C-19), and β-actin (C-4) were purchased from Santa Cruz Biotechnology, Inc. Activating Itgβ1 antibodies (clone 18/CD29) were obtained from BD, and α-tubulin antibodies were obtained from Sigma-Aldrich.
Experimental animals. The generation of Arhgef12<sup>fl/fl</sup> mice and αMHC-CreERT2<sup>cre/cre</sup> mice has been reported previously (Herroeder et al., 2009; Takefuji et al., 2012). Cre-mediated recombination of floxed alleles was induced by intraperitoneal injection of 1 mg tamoxifen dissolved in 50 µl Miglyol on five consecutive days. Vehicle-treated mice received Miglyol only. For in vivo experiments, tamoxifen-treated αMHC-CreERT2<sup>cre/cre</sup> Arhgef12<sup>fl/fl</sup> mice were used as controls. Experiments were performed 2 wk after the end of induction and were approved by local authorities (Regierungspräsidium Darmstadt, Hessen).

TAC. For TAC, male mice aged 8–10 wk were anesthetized with 50 mg/kg pentobarbital sodium. After intubation, the chest was opened and the aortic arch was identified. TAC was created by ligating the transverse aorta between the right innominate and the left common carotid artery against a blunted 27-gauge needle using a 7-0 suture. The needle was then gently retracted. The sham procedure was identical except that the aorta was not ligated.

Determination of activated RhoGEF12. Determination of activated RhoGEF12 was performed as described previously (García-Mata et al., 2006) with the following modifications: Hearts were extirpated and frozen in liquid nitrogen and then disrupted by a homogenizer in lysis buffer (0.2% Triton X-100, 20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, and protease inhibitors), and the protein concentration of the supernatants was determined after centrifugation by Precision Red Advanced Protein Assay software; 75 cells were evaluated per experimental condition. To study the cellular effects of stretch, NRVMs were incubated in serum-free medium for 18 h and then stretched by 10% with a 27-gauge needle to allow retrograde perfusion of the coronary arteries. The heart was first washed with 50 ml of perfusion buffer (113 mM NaCl, 4.7 mM KCl, 0.6 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 12 mM NaHCO<sub>3</sub>, 10 mM KHCO<sub>3</sub>, 10 mM Hepes, 30 mM taun, 10 mM 2,3-Butanedione monoxime, and 5.5 mM glucose, pH 7.46) for 10 min and then digested with 75 ml of digesting buffer (perfusion buffer with 0.05 mg/ml Liberase DH [Roche] and 12.5 µM CaCl<sub>2</sub>) for 20 min. The heart was removed from the perfusion apparatus, and the left ventricle was minced with a forceps in digesting buffer. Centrifugation (50 g, 1 min) was performed three times to enrich cardiomyocytes.

Cell culture. To study the cellular effects of stretch, NRVMs were incubated in serum-free medium for 18 h and then stretched by 10% with a frequency of 1 Hz using a Flexcell system. Stretch-induced changes in cardiomyocyte size were quantified using the Cell Death Detection kit (Roche) according to the manufacturer’s instructions. In brief, paraformaldehyde-fixed cells were deparaffinized and rehydrated. The sections were then incubated with Proteinase K at room temperature for 30 min. The sections were incubated with TUNEL reaction mixture at 37°C for 1 h. Nuclear density was determined by manual counting of DAPI-stained nuclei. At least 10,000 DAPI-positive cells were counted in each animal.

Western blotting. Samples were subjected to SDS-PAGE, transferred to nitrocellulose transfer membranes (Whatman), and then incubated with primary antibodies as indicated. Equal loading was checked using antibodies to α-tubulin or actin.

Histological analyses. Freshly dissected heart tissue was fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Embedded hearts were stained with Picosirus red according to standard protocols. 20 randomly chosen frames from the sections were quantified to assess the degree of heart fibrosis using ImageJ software. 75 cells were evaluated per experimental condition. To study the cellular effects of direct stimulation of G<sub>q/11</sub> signaling or IgG<sub>1</sub> signaling, NRVMs were seeded on 6-well tissue culture plates without coating (Greiner Bio-One). NRVMs were incubated in serum-free medium for 18 h and then incubated in 500 µl of serum-free medium containing IgG, 5 µg/ml of a stimulating anti-IgG<sub>1</sub> antibody (clone 18/CD29; BD), or 1 µM ET-1 for 5 min. After 5-min incubation, RhoGEF pull-down assay was performed. To study stretch-induced MRTF-A translocation, isolation of nuclear protein and cytoplasmic protein extraction was performed with DUALXtract (Dualsystems Biotech).

Isolation of neonatal rat ventricular cardiomyocytes. NRVMs were isolated from 1–2-d-old rat neonates using a kit from Worthington Biochemical Corporation. After digestion, cells were plated for 1 h to remove non-myocytes, plated on cell culture dishes, and then cultured in DMEM with 10% fetal bovine serum. The next day, cells were cultured in serum-free DMEM containing 100 µM BrdU (Sigma-Aldrich). NRVMs were transfected with siRNAs (Qiagen or Sigma-Aldrich) using Lipofectamine RNAmax (Invitrogen) 3 and 20 h after plating according to the manufacturer’s instructions. The following siRNA target sequences were used: RhoGEF2-1, 5′-GGTCTCAAGTGTTCTCTGAGTA-3′; RhoGEF2-2, 5′-CCAAGTATCTCTACCCA-3′; ITGB-1-1, 5′-CATGGAGATGAGTTCAA-3′; ITGB-1-2, 5′-GTTGACAATCTCAGCTGCG-3′; αMHC-1, 5′-CAAGCCTGATCAAGGGAT-3′; αMHC-2, 5′-CAAGCTCTCTTCTGAACCATT-3′; αMHC-3, 5′-CACCAACTGTCATCATGAGTA-3′; CD44, 5′-CCAATCCATGAGAGCT-3′; FN1, 5′-GAGATACCTCCGTCGTGGA-3′; SRC2, 5′-CAAGCTTGGGGCTCTAATCACT-3′; RhoA, 5′-CAAGCCTGATGCTTATCACT-3′; and Mdi1 (MRTF-A), 5′-CAATTGGCCTCCATCTGTT-3′.
Mass spectrometric analysis. After immunoprecipitation using bead-coupled GST-RhoA<sup>WT</sup> (see section “Determination of activated RhoGEF12”) from sham- and TAC-operated hearts, proteins were separated by one-dimensional SDS-PAGE (4–12% Novex gels; Invitrogen) and stained with colloidal Coomassie. Gel bands were excised and subjected to gel digest with trypsin. The resulting tryptic peptides were extracted with acetoni-trite and desalted with reversed phase C18 STAGE tips (Luber et al., 2010). Mass spectrometric experiments were performed on a nanoflow HPLC system (Agilent Technologies) connected to an LTQ-Orbitrap instru-ment (Thermo Fisher Scientific) equipped with a nanoelectrospray source (Proxeon). The mass spectrometer was operated in the data-dependent mode to monitor MS and MS/MS spectra. Survey full-scan MS spectra (from m/z 300–2,000) were acquired in the Orbitrap with a resolu-tion of R = 60,000 at m/z 400 after accumulation of 1,000,000 ions. The five most intense ions from the preview survey scan delivered by the Orbitrap were sequenced by collision-induced dissociation in the LTQ. Mass spectra were analyzed using MaxQuant software (version 1.2.2.9; Luber et al., 2010), and all tandem mass spectra were searched against the mouse and rat International Protein Index protein sequence database (mouse IPI version 3.68) and concatenated with reversed copies of all se-quences. The required false positive rate was set to 1% at the protein and peptide level. Maximum allowed mass deviation was set to 0.02 ppm in MS mode and 0.5 D for MS/MS peaks. Cysteine carbamidomethylation was searched as a fixed modification, and N-acetyl protein and oxidized me-thionine were searched as variable modifications. A maximum of three missed cleavages were allowed. Protein quantitation was performed with the MaxQuant label-free option (Luber et al., 2010). Data are presented as the log2 of the LFQ intensity ratio between TAC-operated sample and sham-operated sample. Significance was calculated with the MaxQuant software tool and indicates ratio outliers from the main distribution.

mRNA expression analysis. RNA was extracted from left ventricles with the RNA fibrous tissue kit (QIAGEN) and from NKVMs or adult mouse cardiac myocytes with the RNeasy Mini kit (QIAGEN) according to the manufacturer’s protocol. RT reaction was performed using the QuantiTect Reverse Transcription kit (QIAGEN). Quantification of human and mouse genes was performed using the LightCycler 480 Probe Master System (Roche; for primers and probes, see Tables S1 and S2). Pooled human car-diac RNAs were obtained from Takara Bio Inc. (Caucasian males, 30–39 yr of age, cause of death: trauma). Genomic DNA from mouse tails or HUVECs was used as a universal standard to calculate gene copy number per nanogram of RNA. Was used as a universal standard to calculate gene copy number per nanogram of RNA. Was used as a universal standard to calculate gene copy number per nanogram of RNA. Was used as a universal standard to calculate gene copy number per nanogram of RNA. Was used as a universal standard to calculate gene copy number per nanogram of RNA. Used for hypertype-specific genes and fibrogenic genes, data are pre-sented after normalization to GAPDH, and basal values were set to 1. qRT-PCR for rat genes was performed using the LightCycler 480 SYBR Green Master (Roche; for primer sequences, see Table S3).

MRI. Cardiac MRI measurements were performed on a 7.0 T Bruker Pharmascan, equipped with a 300 mT/m gradient system, using a custom-built circularly polarized birdcage resonator and the Early Access Package for self-gated cardiac Imaging (Bruker; Takefuji et al., 2012). The mice were mea-sured under volatile isoflurane (2.0%) anesthesia. The measurement is based on the gradient echo method (repetition time = 6.2 ms; echo time = 1.6 ms; field of view = 220 × 220 mm; slice thickness = 1.0 mm; matrix = 128 × 128; repetitions = 100). The imaging plane was localized using image guidance showing the two- and four-chamber view of the heart, followed by acquisition in short axis view, orthogonal on the septum in both scouts. Multiple contiguous short-axis slices consisting of 9 or 10 slices were acquired for complete coverage of the left ventricle. MRI data were analyzed using Qmass digital imaging software (Medis).

Generation of tamoxifen-inducible, cardiacmyocyte-specific KO s for G<sup>12/13</sup> (cmc-G<sup>12/13</sup>-Ko) and Itgβ<sub>1</sub> (cmc-Itgβ<sub>1</sub>-KO). The generation and characterization of cmc-G<sup>12/13</sup>-KOs has been described previously (Takefuji et al., 2012). cmc-Itgβ<sub>1</sub>-KOs were generated by intercrossing the tamoxifen-inducible, cardiacmyocyte-specific α-MHC–CreERT2 line to mice carrying a floxed Itgβ<sub>1</sub> allele (B6;129-Itgβ<sub>1</sub>tm15J/J; The Jackson Laboratory). In both mouse lines, Cre-mediated recombination of floxed alleles was in-duced by intraperitoneal injection of 1 mg tamoxifen dissolved in 50 μl Mijglon on five consecutive days. Vehicle-treated mice received Mijglon only; α-MHC–CreERT2<sup>+</sup> mice not carrying floxed alleles were used as controls. Experiments were performed 2 wk after the end of induction.

Statistical analyses. Data are presented as means ± SEM. Comparisons between two groups were performed with unpaired Student’s t test, and comparisons between more than two groups were performed by ANOVA followed by Bonferroni post hoc test. Survival curves were analyzed using Kaplan Meyer estimators and Log-rank (Mantel-Cox) test. Comparisons be-tween more than two groups at different time points were performed by repeated measures ANOVA followed by Bonferroni post hoc test. “n” refers to the number of independent experiments or mice per group. P-values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Online supplemental material. Tables S1–S3 show primer sequences and probes used for qRT-PCR analyses. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122126/DC1.

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