Background—Stromal interaction molecule 1 (STIM1) is a dynamic calcium signal transducer implicated in hypertrophic growth of cardiomyocytes. STIM1 is thought to act as an initiator of cardiac hypertrophic response at the level of the sarcolemma, but the pathways underpinning this effect have not been examined.

Methods and Results—To determine the mechanistic role of STIM1 in cardiac hypertrophy and during the transition to heart failure, we manipulated STIM1 expression in mice cardiomyocytes by using in vivo gene delivery of specific short hairpin RNAs. In 3 different models, we found that Stim1 silencing prevents the development of pressure overload–induced hypertrophy but also reverses preestablished cardiac hypertrophy. Reduction in STIM1 expression promoted a rapid transition to heart failure. We further showed that Stim1 silencing resulted in enhanced activity of the antihypertrophic and proapoptotic GSK-3β molecule. Pharmacological inhibition of glycogen synthase kinase-3 was sufficient to reverse the cardiac phenotype observed after Stim1 silencing. At the level of ventricular myocytes, Stim1 silencing or inhibition abrogated the capacity for phosphorylation of AktS473, a hydrophobic motif of Akt that is directly phosphorylated by mTOR complex 2. We found that Stim1 silencing directly impaired mTOR complex 2 kinase activity, which was supported by a direct interaction between STIM1 and Rictor, a specific component of mTOR complex 2.

Conclusions—These data support a model whereby STIM1 is critical to deactivate a key negative regulator of cardiac hypertrophy.

In cardiomyocytes, STIM1 acts by tuning Akt kinase activity through activation of mTOR complex 2, which further results in repression of GSK-3β activity. (Circulation. 2016;133:1458-1471. DOI: 10.1161/CIRCULATIONAHA.115.020678.)

Key Words: calcium ● genetic therapy ● heart failure ● Stim1 protein, mouse ● TOR complex 2

Cardiac hypertrophy develops in reaction to increased mechanical load or to neurohormonal stimulation as occurs in cardiac dysfunction. Cardiac hypertrophy is a compensatory response that reduces wall stress by increasing wall thickness. Although it represents an initial salutary adaptation to stress, chronic hypertrophic remodeling involves maladaptive changes in cardiac function over the long term.

Clinical Perspective on p 1471

Because of the limited proliferative capacity of adult cardiomyocytes (CMs),1 growth of the cardiac wall is mainly sustained by an increase in CM size. A number of intracellular signal-transduction circuits have been implicated in the hypertrophic remodeling of adult CMs, including calcineurin-nuclear factor of activated T cells (NFAT) and the Akt/protein kinase B mammalian target of rapamycin (mTOR). These signaling circuits directly induce hypertrophic growth by altering gene expression in the nucleus with activation of a defined set of prohypertrophic transcription factors. However, the upstream events at the level of sarcolemma that initiate the cardiac hypertrophic response remain largely unknown.

Among different candidates, it has been observed that local alterations in Ca2+ homeostasis can be an early trigger of intracellular signaling events leading to CM growth.3 In recent years, stromal interaction molecule 1 (STIM1) has been reported as a regulator of CM growth in vitro and in vivo.4–6 STIM1 was initially shown to act as an endoplasmic reticulum (ER) sensor and to mediate store-operated Ca2+ entry, a major mechanism of Ca2+ entry in nearly all nonexcitable cells.7 In these cells, a decrease in ER Ca2+ content triggers STIM1 oligomerization and subsequent interaction with some plasma membrane channels including the highly Ca2+-selective Orai1 channel.8 In this model, the STIM1-Orai1 coupling drives ER-plasma membrane junctions, mediates Ca2+ entry and...
communication among the ER lumen, the cytoplasm, and the extracellular space, and activates Ca\(^{2+}\)-regulatory proteins.\(^8\) Recent information has however shown that STIM1 is a dynamic signal transducer that can interact with a greater diversity of proteins depending on the pathophysiological conditions.\(^9\)

In CMs, a cell type where large fluctuations of sarcoplasmic reticulum and cytosolic Ca\(^{2+}\) occur with each heartbeat, STIM1 not only controls store-operated Ca\(^{2+}\) entry, but also a spontaneous Ca\(^{2+}\) current that occurs independently of Ca\(^{2+}\) store depletion.\(^4\) This spontaneous current is marginal in healthy adult CMs but strengthens during the hypertrophic remodeling of CMs.\(^4\) It has been proposed that an interaction between STIM1 and 2 members of the Orai family, ie, Orai1 and Orai3 channels, occurs in response to hypertrophic stimulation and sustains the emergence of this alternative mechanism of Ca\(^{2+}\) entry.\(^9\) A similar phenomenon has been reported in proliferative smooth muscle cells where STIM1 controls heteromultimers of Orai1 and Orai3 that are regulated by leukotriene C4.\(^9,11\) Interactions with less Ca\(^{2+}\)-selective channels, such as transient receptor potential channels, have also been suggested, and STIM1 overexpression leads to dysregulated calcium transients.\(^5,12\)

All together, these data indicate that STIM1 activates in response to stress and can interact with a diversity of plasma membrane channels that will however mediate highly localized Ca\(^{2+}\) signals.

Importantly, Stim1 silencing has been associated with a reduction in agonist-triggered hypertrophic responses of neonatal and adult CMs in vitro,\(^3,13\) and in the adult heart in vivo, as well.\(^1\) In addition, cardiac-specific deletion of Stim1 triggers ER stress and progressively results in left ventricular dilation under physiological conditions.\(^6,16\) There is thus evidence that STIM1 is involved in cardiac homeostasis and is required for CM hypertrophy, but the pathways underpinning this effect are unknown.

## Methods

All procedures and animal care were approved by our institutional research committee and conformed to the animal care guideline in Mount Sinai School of Medicine in accordance to the following approved protocol #LA08-01064.

### Animal Experiments

Mice underwent transverse aortic constriction (TAC) using a supraventricular construction model as previously described.\(^15\) TAC or sham surgery was performed in 8-week-old male C57Bl/6J mice (body weight 18–22 g). Mice were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) and placed on a ventilator. A longitudinal cut of 2 mm was made in the proximal portion of the sternum, allowing visualization of the aortic arch. The transverse aortic arch was ligated between the innominate and common carotid arteries with an overlying 27-gauge needle. The needle was immediately removed, leaving a discrete region of constriction. The sham group underwent a similar procedure without ligation. Buprenorphine (Buprenex) was used as an analgesic before and 2 days after surgery (0.05 mg/kg intraperitoneally).

T Diaz-Diaz (GSK3β inhibitor, Sigma Aldrich) was dissolved in dimethyl sulfoxide:phosphate-buffered saline (PBS; 1:10) to a concentration of 1.5 mg/mL. Daily intraperitoneal injections were performed during the last 2 weeks of experimental protocol.

### Cardiac Echography

Echocardiography was performed under sedation by intraperitoneal injection of ketamine up to 75 mg/kg. Sedation was optimized by giving the lowest dose of ketamine needed (1) to restrain the animal and prevent motion artifact and (2) to maintain the heart rate in the range of 550 to 650 beats/min. Ketamine was chosen based on our previous experience and considering that alternative agents had either a long duration of action (pentobarbital), were potentially unsafe for heart failure animals, or had a bradycardic effect (isoflurane, ketamine/xylazine) as demonstrated elsewhere.\(^6\) The chest was shaved. Short-axis parasternal views of the left ventricle (LV) at the midpapillary level were obtained using a Vivid 7 echocardiography apparatus with a 13-MHz linear array probe (General Electric, New York, NY). M-mode measurements of the size of the LV walls and cavities were obtained by 2-dimensional guidance from the short-axis view of the LV. Three different measurements in diastole (d) and systole (s) were averaged per animal to estimate LV wall thicknesses (septum and posterior wall) and LV internal diameter (LVID). Fraction shortening (FS) is a way of measuring LV performance. It measures and ratios the change in the diameter of the LV between the contracted and relaxed states with the following formula: FS = (LVIDd – LVIDs) / LVIDd.

### Cardiac Hemodynamic Analysis

Hemodynamic measurements were performed using a 1.2F pressure-volume conductance catheter (Scisense). Pressure-volume loop analysis was performed as previously described.\(^19\) Mice were injected intraperitoneally with urethane (1 g/kg), etomidate (10 mg/kg), and morphine (1 mg/kg) and mechanically ventilated with 7 μL/g stroke volume at 125 respirations per minute. The chest was opened to expose the heart for an apical stab approach.

To determine absolute ventricular volumes via admittance technology, myocardial and blood conductance were obtained before pressure-volume catheter placement in the left ventricle. The inferior vena cava was transiently occluded to reduce ventricular preload to obtain load-independent pressure-volume relationships. Hemodynamic measurements were acquired and analyzed using IOX software (EMKAtech).

### Recombinant AAV Vector Production and Purification

Recombinant AAV9.shStim1 and AAV9.shLuc were produced by using the 2-plasmids protocol as previously described\(^20\) with some modifications. Short hairpin RNA (shRNA) sequences were previously described.\(^2,4\) HEK-293T cells (ATCC) were grown in triple flasks for 24 hours (DMEM, 10% fetal bovine serum) before adding the linear polyethylenimine and the 2 plasmids. Seventy-two hours after transfection (DMEM, 2% fetal bovine serum), the viruses were purified and concentrated from benzene-trease crude cell lysates over an iodixanol density gradient (Optiprep, Greiner Bio-One Inc.). Finally, viruses were formulated into lactated Ringer solution (Baxter Healthcare Corporation) using dialysis membranes Spectra/Por2 MWCO 12 to 14 kDa, 10-mm flat width, 0.32 mL/cm dialysis membranes (Spectrum Labs), titrated by polymerase chain reaction and by Coomassie-stained gels, and then stored at –80°C. For cardiacotropic expression, wild-type male mice received 1E+11 viral genome of AAV9.shStim1 or AAV9.shLuc or PBS by tail vein injection. AAV9.shStim1 was built according to the shRNA sequence that was previously published and validated.\(^4\) To avoid liver toxicity, a novel AAV9.shLuc was redeveloped by using an antiluciferase sequence that was previously described and validated.\(^21\)

### CM Isolation and Treatment

CMs were isolated from C57Bl/6 mouse hearts. Male mice (25–32 g), infected with AAV9.shStim1 or control, were used. In brief, after heparin (50 U) was injected, animals were anesthetized with intraperitoneal injection of ketamine (100 mg/g). The heart was quickly removed from the chest and the aorta was retrogradely perfused at...
for 3 minutes with calcium-free Tyrode buffer (137 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose, 10 mmol/L HEPES [pH 7.4], 10 mmol/L 2,3-butanedione monoxime, and 5 mmol/L taurine) oxygenated with 100% O₂. The enzymatic digestion was initiated by adding collagenase type B (300 U/mL; Worthington) and hyaluronidase (0.1 mg/mL; Worthington) to the perfusion solution. When the heart became swollen after 10 minutes of digestion, the LV was quickly removed and cut into several chunks, and further digested in a shaker (60–70 rpm) for 10 minutes at 37°C in the same enzyme solution. The cell suspension was filtered through a cell strainer (100 μm pore size; BD Falcon) and gently centrifuged at 500 rpm for 1 minute. The pellet containing myocyte fraction was plated on a laminin-coated dish (Life Science). The supernatant containing nonmyocyte fraction was centrifuged at 14,000 rpm for 10 minutes, and the pellet was collected. For in vitro hypertrophic experiments, CMs were starved overnight. The next morning, cells were treated for 48 hours with angiotensin II (100 nmol/L) ± YM-58483 (1 μmol/L). After 48 hours, cells were lysed by using radioimmunoprecipitation assay buffer.

Western Blots and Antibodies

Western blots were performed using 10%, 4% to 20%, or 10% to 20% tris-glycine gels (Invitrogen) regarding the size of the studied proteins. Used primary antibodies were GAPDH (Millipore), STIM1 (Sigma Aldrich), SERCA2a (homemade), phospho and total GSK-3β, Akt (total, S473, T308), mTOR, Rictor, mSN1, mLTS8, Foxo3 (S253), PRAS40 (T246), total, and caspase-3 (all from Cell Signaling). Secondary antibodies were appropriate horseradish peroxidase-linked antibodies (Sigma Aldrich).

Phosphokinase Assay

HEK cells (ATCC) were respectively cultured in DMEM 10% fetal bovine serum, 1% penicillin/streptomycin (Gibco). Proteome Profiler (R&D Systems) was performed on HEK cells according to manufacturer instructions. HEK cells were used at 90% confluence. In brief, selected capture antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysate samples are diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture is then incubated with the array. Any protein/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Streptavidin-horseradish peroxidase and chemiluminescent detection reagents are added, and a signal is produced in proportion to the amount of cytokine bound. Chemiluminescence is detected in the same manner as a Western blot.

Immunoprecipitation

Freshly isolated adult mouse CMs were lysed by using radioimmunoprecipitation assay buffer (Boston Bio Products) with protease and phosphatase inhibitor (Roche). Three hundred microliters of proteins were diluted in a final volume of 600 μL of lysis buffer and incubated 2 hours with rotation at 4°C with either Rictor (1.5 μg) or STIM1 (5 μg) antibody. Control immunoprecipitation was performed by using rabbit IgG (5 μg, Abcam). In the meantime, 10 μg of Protein A-Sepharose beads (Sigma) were washed with PBS and saturated with PBS-bovine serum albumin 1 mg/mL. Cell lysates with primary antibody were mixed with beads and incubated with rotation at 4°C overnight. Beads containing immunoprecipitates were washed 3 times with ice-cold PBS. Proteins were released from beads by adding 25 μL of Laemmli buffer and by agitation for 1 hour at 37°C at 1200 rpm. Supernatant was removed from the protein A-Sepharose, and was analyzed by immunoblotting for Rictor and STIM1.

mTORC2 Kinase Assay

For functional mTOR complex 2 (mTORC2) kinase assay experiments, freshly isolated adult mouse CMs were lysed by using 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate lysis buffer (40 mmol/L HEPES, pH 7.4, 120 mmol/L NaCl, 2 mmol/L EDTA, 0.3% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mmol/L pyrophosphate, 10 mM mmoL/L glycerophosphate, 50 mmol/L NaF, Protein Inhibitor Cocktail [Sigma] at 1:100). Rictor antibody (1.5 μg) was added to the cleared cellular lysates (1 mL lysis buffer) and incubated with rotation at 4°C for 60 minutes. After another 1 hour of incubation with 60 μL of 50% slurry of protein G-agarose (Calbiochem), immunoprecipitates captured by protein G-agarose were washed 3 times with 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate wash buffer (3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate lysis buffer without Protein Inhibitor Cocktail) and once with kinase wash buffer (25 mmol/L HEPES [pH 7.4], 20 mmol/L KCl). For in vitro mTORC2 kinase reactions, immunoprecipitates were incubated in a final volume of 20 μL at 37°C for 30 minutes in mTORC2 kinase buffer (25 mmol/L HEPES [pH 7.4], 100 mmol/L potassium acetate, 1 mmol/L MgCl₂) containing 250 ng of inactive His-Akt1 (Millipore) and 500 μmol/L ATP (Sigma). The reaction was stopped by the addition of 20 μL of Laemmli buffer. Samples were boiled 5 minutes. After a quick spin, the supernatant was removed from the protein G-agarose, and was analyzed by immunoblotting for phosphoSer-Thr Akt and total Akt.

Histological Analysis

At the moment of euthanization, hearts were stopped in diastole by injecting 1 mol/L KCl solution in the left ventricle and then prepared in Tissue-Tek OCT compound (Sakura Fine technical) and sectioned into 8-μm slices (Microm HM560 Cryo-star, Thermo Scientific). Double immunolabeling on cryostat sections allowed the identification of endothelial cells with Caveolin 1 antibody (Santa Cruz) and CMs with vinculin antibody (Sigma-Aldrich). For each animal, 3 sections minimum were taken at different levels of myocardium and were processed. LV fields in which cross sections of capillaries and CMs were clearly detectable (subendocardial area) were recorded by using an Olympus IX71 microscope equipped with an Olympus DP72 camera. A minimum of 6 fields per section was recorded at ×20 magnification (corresponding of a minimum of 1000 cells measured). The cross-sectional area of CM was measured by using Image J software (NIH) by a masked observer. For interstitial fibrosis quantification, heart sections were stained by using the Masson Trichrome staining kit (Sigma Aldrich).

Interstitial Fibrosis Staining and Quantification

In brief, slides were fixed in Bouin solution (Sigma Aldrich) for 15 minutes at 56°C. After washes in tap water (15 minutes) and deionized water, slides were stained with hematoxylin for 10 minutes (Fisher Scientific). After a 5-minute wash in tap water, slides were dipped in a mix solution of phosphotungstic acid and phosphomolybdic acid (2.5%) for 5 minutes, then in aniline solution for 5 minutes, and finally in acetic acid solution (1%) for 2 minutes. Slides were washed in deionized water and then dehydrated by ethanol 95% and 100% bath 2 minutes each. After 2 baths of xylene, slides were mounted with Cytoseal 60 (Thermo Scientific). By using software Spot v 3.5.7.1 (Diagnostic Instruments, Inc), ×20 magnification pictures were taken, and fibrosis was quantified using Image J software (NIH). At least 10 fields per animal were quantified and at least 4 animals per group.

Statistical Analyses

Time-course experiments were analyzed using a 2-way repeated-measures analysis of variance, followed by a Bonferroni post hoc test. Other quantitative data were analyzed using a 1-way analysis of variance followed by a Bonferroni post hoc test. Continuous variables were compared between 2 groups using a Student t test with Welch correction. Exact tests were used for experiments with n<5. All analyses were performed by using GraphPad Prism 6. P values of 0.05 were considered significant. Data are presented as mean±standard error of the mean. Densitometry of Western blots, quantification of cardiac fibrosis, and quantification of cell size were performed by using NIH ImageJ software.
Results

Stim1 Silencing Leads To Progressive Cardiac Dilation and Dysfunction Under Physiological Conditions

We first developed recombinant cardiotropic adenovirus serotype 9 (AAV9) encoding shRNA directed against Stim1 under control of the U6 promoter (Figure IA in the online-only Data Supplement). We used AAV9 because it shows strong tropism for the heart, especially for CMs, after a single tail vein injection.24 We found that cardiac STIM1 expression is reduced by 75% 3 weeks after administration of AAV9.shStim1 in comparison with controls (Figure IB and IC in the online-only Data Supplement). In this first set of experiments, C57/B6J mice were injected with AAV9.shStim1 or an AAV9 encoding a shRNA directed against luciferase or a single PBS injection as negative controls. There were no statistically significant differences in LV wall thickness, volumes, and function between the 2 control groups over the 8-week follow-up period (Figure 1A through 1E). In contrast, we found that 5 weeks after infection mice treated with AAV9.shStim1 started to present significant differences with a progressive decline in fractional shortening and a mild increase in LV diastolic volume, indicative of the development of a mild cardiac dilation and dysfunction (Figure 1A through 1E). At the time of euthanization (8 weeks after injection), AAV9.shStim1–treated animals presented with a slight decrease in heart weights (Figure 1F) and a nonsignificant trend for smaller CM size (Figure 1G).

STIM1 Is Critical for the Development of Adaptive Cardiac Hypertrophy

We then assessed in vivo the contribution of STIM1 in the development of pressure-overload cardiac hypertrophy (LV hypertrophy model). In this experiment, mice were first infected with AAV9.shStim1 or control (1.10^{11} drp) and were subjected to TAC or sham surgery 3 weeks later (Figure 2A). This timing was based on our results showing a reduction in STIM1 expression by 75% in AAV9.shStim1–treated animals in comparison with controls at that time point (Figure IB and IC in the online-only Data Supplement). The animals were then followed up for 3 more weeks before euthanization (Figure 2A). Reduced cardiac STIM1 expression, as achieved after infection with AAV9.shStim1, blunted the development of LV hypertrophy in TAC-treated mice as identified by echocardiographic data on LV wall thickness and measures of heart weights (Figure 2B and 2C). Concordantly, TAC-treated mice that received AAV9.shStim1 had CM sizes almost identical to the one observed in both sham control groups (Figure 2D). However, TAC-treated mice that had received AAV9.shStim1 developed LV dilation and decreased systolic function as assessed by echocardiography (not shown) and hemodynamic recordings (Figure 2E), and a significant increase in lung weights, as well (Figure 2F), indicative of the development of pulmonary edema. Importantly, at that time point, all these parameters were unchanged in sham-treated mice that received AAV9.shStim1 (Figure 2B through 2F), suggesting that the role of Stim1 is exacerbated in response to hypertrophic stimulation.

In another set of experiments, focusing on the transition from cardiac hypertrophy to heart failure (heart failure model), we first subjected mice to pressure overload or sham before randomly assigning mice to AAV9.shStim1 or control administration 3 weeks later (Figure 3A). During the first 3 weeks after surgery, mice developed cardiac hypertrophy that was similar among TAC-treated groups at the time of AAV administration (Figure IIA through IIC in the online-only Data Supplement). Three weeks after randomization, echocardiography was performed on a weekly basis, and final evaluation was performed 5 weeks after AAV9 administration (Figure 3A). Whereas TAC-treated animals showed similar echocardiographic data 3 weeks after injection, a significant decrease in LV ejection fraction, and a marked increase in LV diameter, as well, was observed in TAC-treated animals that received AAV9.shStim1 in comparison with controls (Figure 3B). The progressive development of LV dilation and systolic dysfunction started 3 weeks after AAV9.shStim1 administration in line with the time needed to achieve a significant reduction in cardiac STIM1 expression (Figure IC in the online-only Data Supplement). Hemodynamic recordings confirmed the significant increase in LV volumes and the significant decrease in contractility parameters in TAC-AAV9.shStim1–treated animals in comparison with controls, an effect that was not seen in sham-AAV9.shStim1–treated animals (Figure 3C). In marked contrast to the TAC-control group, TAC-AAV9.shStim1–treated animals presented a significant reduction in hypertrophic markers as assessed by the significant reduction in wall thickness (Figure 3D), heart weights (Figure 3E), and CM area (Figure 3F). By the end of this experiment (ie, 8 weeks after TAC and 5 weeks after AAV9 administration), TAC-AAV9.shStim1–treated animals presented with a CM size that was even smaller than the one measured in sham animals, thus indicating cellular atrophy. These intriguing results were observed despite persistent increased afterload in all TAC-treated groups (Figure 3C). TAC-AAV9.shStim1–treated animals also presented with an increase in lung weights, in line with the rapid worsening of heart failure (Figure 3E).

Overall, these results indicate that STIM1 is critical not only for the development, but also for the persistence of adaptive cardiac hypertrophy. Reduced STIM1 expression promotes cardiac dilation with a rapid transition to heart failure in a context of pressure overload. We performed further histological and biochemical analyses in both LV hypertrophy and heart failure models. We found that, in both control and shStim1 groups, pressure overload was associated with a significant development of interstitial fibrosis in comparison with sham animals (Figure 4A and 4B). Whereas there were no statistically significant differences between sham-control versus sham-AAV9.shStim1–treated animals, there was a trend for higher fibrosis levels in TAC-AAV9.shStim1–treated animals, which, however, did not reach significance (Figure 4B). We also observed a nonsignificant trend for higher activation of apoptosis as assessed by an increase in cleavage of the proapoptotic protease caspase-3 in total heart extracts from AAV9.shStim1–treated animals (Figure 4C). Importantly, a statistically significant difference was observed in CMs...
isolated from the heart of wild-type mice treated with AAV9.shStim1 in comparison with control (Figure 4D).

Stim1 Silencing Is Associated With Increased GSK-3β Activity
Although alternative mechanisms of action have been described,25 STIM1 primarily functions as a dynamic calcium coordinator of cellular calcium signals through interactions with highly calcium-selective plasma membrane channels.8 Therefore, we asked whether the inappropriate reversal of preestablished cardiac hypertrophy after Stim1 silencing might be linked to increases in the activity of some calcium-dependent antihypertrophic molecules. Among different candidates, we detected significant changes in GSK-3β phosphorylation levels following Stim1 silencing (Figure 5A). GSK-3β is constitutively active in unstimulated cells and

Figure 1. Stim1 silencing under physiological conditions. Time-course analysis of A, interventricular septum thickness (IVSd); B, left ventricular internal diastolic diameter (LVIDd); and C, fractional shortening (FS) of wild-type mice treated with AAV9.shStim1 (green), AAV9.shLuciferase (gray), or PBS (white). Mice were followed up to 8 weeks after infection. D, Representative pressure-volume loops in wild-type mice treated with AAV9.shStim1 (green), AAV9.shLuciferase (gray), or PBS (black). E, Hemodynamic parameters (end-diastolic volume [EDV]; end-systolic volume [ESV]; ejection fraction [EF]) assessed 8 weeks after AAV injection. F, Heart weights (HW).
G, Cardiomyocyte areas in the 3 groups. Immunofluorescence analysis was performed on left ventricular sections (8 μm) using an antibody against Vinculin (green) and Caveolin1α (red). Nuclei were stained with DAPI. Images were taken at ×20 magnification. n=5 to 10 animals per group for all experiments; *P<0.05, **P<0.01, ***P<0.001. AAV9 indicates adeno-associated viruses of serotype 9; CM, cardiomyocyte; d, in diastole; DAPI, 4′,6-diamidino-2-phenylindole; and PBS, phosphate-buffered saline.
GSK-3β is expressed in the mammalian heart where it negatively regulates cardiac hypertrophy. GSK-3β is inhibited through specific phosphorylation of serine 9. In line with previous reports, we found that GSK-3β activity is reduced in hearts from TAC-control animals in comparison with sham as indicated by a significant increase in the inactive (ie, phosphorylated) GSK-3β levels (Figure 5A). Stim1 silencing was associated with a significant reduction in GSK-3β activity.

Figure 2. Stim1 silencing before TAC prevented the establishment of cardiac hypertrophy. A, Schematic timeline to study Stim1 silencing effect on TAC-induced left ventricular hypertrophy (LVH). B, Cardiac wall thickness assessed by echocardiography in sham versus TAC animals treated with AAV9.shStim1 (green), or control (white). C, Heart weights. D, Left, Immunofluorescence analysis of left ventricular sections (8 μm) by using an antibody against Vinculin (green). Nuclei were stained with DAPI. Images were taken at ×20 magnification. Right, Quantification of cardiomyocyte area in the 4 groups. E, Cardiac function assessed by hemodynamic measurements. Top left, End-diastolic volume (EDV). Top right, End-systolic volume (ESV). Bottom left, Ejection fraction (EF). Bottom right, Characteristic pressure-volume loops. AAV9.shStim1–treated groups are in green, controls are in white for the bar graphs and in black for the pressure-volume loops. F, Lungs weights. n ≥ 8 per groups. *P<0.05, **P<0.01, ***P<0.001. CM indicates cardiomyocyte; DAPI, 4′,6-diamidino-2-phenylindole; IVS, interventricular septum; IVSd, interventricular septum thickness in diastole; TAC, transverse aortic constriction; and TVI, tail vein injection.
phosphorylation levels in both sham and TAC-treated animals (Figure 5A), indicating a higher level of activity.

We then sought to determine whether the antihypertrophic effects observed after Stim1 silencing were caused by enhanced GSK-3β activity. We thus tested the ability of a pharmacological inhibitor of GSK-3β (TDZD8) to restore hypertrophic responsiveness in AAV9.shStim1-treated animals. We induced pressure-overload heart failure in mice as described in the heart failure model (Figure 3A). However, 6 weeks after TAC surgery and 3 weeks after AAV9.shStim1 administration, we randomly assigned animals to receive the GSK-3β inhibitor (TDZD8 10 mg/kg daily) or vehicle (dimethyl sulfoxide:PBS, 1:10) through intraperitoneal injections during 2 weeks (Figure 5B). We documented that treatment with TDZD8 effectively inhibited cardiac GSK-3β in vivo by examining the ratio of phospho-GSK-3β to total GSK-3β in total

Figure 3. Stim1 silencing after TAC reverted established cardiac hypertrophy. A, Schematic timeline to study Stim1 silencing effect on TAC-induced heart failure (HF). B, Time course of fractional shortening (Left) and left ventricular internal diameter (Right) assessed by echocardiography. Sham (dotted line) versus TAC (straight line) animals treated with AAV9.shStim1 (green) or control (white). C, Cardiac function and volumes assessed by hemodynamic measurements. Top left, End-diastolic volume (EDV). Top right, End-systolic volume (ESV). Bottom left, Ejection fraction (EF). Bottom right, Representative pressure-volume loops in the 4 groups of animals. D, Time course of interventricular septum thickness in diastole (IVSd) assessed by echocardiography in the 4 groups of animals. Sham (dotted line) versus TAC (straight line) animals treated with AAV9.shStim1 (green) or control (white). E, Heart and lung weights at the time of euthanization. F, Left, Immunofluorescence analysis of left ventricular sections (8 μm) using an antibody against Vinculin (green) and Caveolin1α (red). Nuclei were stained with DAPI. Images were taken at ×20 magnification. Right, Quantification of cardiomyocyte area. n≥6 per group. *P<0.05, **P<0.01, ***P<0.0001. d indicates in diastole; DAPI, 4′,6-diamidino-2-phenylindole; and TAC, transverse aortic constriction.
heart extracts. Treatment with TDZD8 resulted in a significant increase of GSK-3\(\beta\) phosphorylation in comparison with treatment with vehicle (Figure 5C). We observed that TDZD8 treatment restored the ability of TAC-AAV9.sh\(\text{Stim1}\) to maintain adaptive cardiac hypertrophy as assessed by echocardiographic (Figure 5D) and morphometric (Figure 5E) analyses. Heart weights were significantly higher in TAC-AAV9.sh\(\text{Stim1}\)–treated animals that received TDZD8 (Figure 5F). In addition, TDZD8 blunts the development of cardiac dilation and reduces LV dysfunction after \(\text{Stim1}\) silencing (Figure 5D). These results indicate that \(\text{STIM1}\) sustains hypertrophic response through the repression of GSK-3\(\beta\) activity.

**STIM1/ORAI-Dependent Modulation of Akt and GSK-3 Activities**

GSK-3\(\beta\) is one of the most studied downstream targets of Akt and the reduced phosphorylation of GSK-3\(\beta\)\(^59\) after \(\text{Stim1}\) silencing suggested a reduction in Akt activity. Concordantly, a significant increase in Akt phosphorylation has been shown to be essential for cardiac hypertrophy after TAC in animals.\(^28\) To analyze the STIM1/ORAI-induced phosphorylation events, we first used a human phosphoantibody array in HEK293 cells (human phosphokinase array) and analyze site-specific phosphorylation of 45 preselected kinases known to be involved in important signaling pathways (listed in Table I in the online-only Data Supplement). In an initial approach, we applied 1 \(\mu\)mol/L of thapsigargin (TG), a classical activator of STIM1/ORAI-mediated store-operated Ca\(^{2+}\) entry in nonexcitable cells. We found that TG application resulted in notable changes in proteins of the Akt pathway (Figure 5D). We observed an important increase in the phosphorylation of glycogen synthase kinase-3 (GSK-3) and \(\beta\)-catenin phosphorylation and Akt\(^S473\), mTOR, and PRAS40, as well (Figure 5A). We then applied 10 \(\mu\)mol/L YM-58483, a potent STIM1/ORAI pharmacological
inhibitor, to HEK293 cells. Reciprocally, we found an important reduction in the phosphorylation of GSK-3, β-catenin, mTOR, and PRAS40 phosphorylation in the minute following YM-58483 application. The phosphorylation of AktS473 was also particularly reduced (Figure 6A). Phosphorylation of AktT308 was minimal under basal conditions and was not affected by TG or YM-58483 application. These data strongly suggest that STIM1 is required for Akt activity and signaling.

**STIM1 Is Required for mTORC2 Signaling in CMs**

After identifying STIM1 as a modulator of Akt phosphorylation on a human cellular model, we further examined whether this
**Figure 6.** STIM1/ORAI–dependent calcium entry regulates Akt and GSK-3β phosphorylation in HEK cells and cardiomyocytes. 

A, Human phosphokinase assay (R&D Systems) in HEK cells. Typical patterns of 5 main targets (AktS473, TOR, PRAS40, GSK-3αS21/βS9, β-catenin) and blotting control in basal conditions (Left), in response to the STIM1 activator thapsigargin (TG; Middle), and to the STIM1/Oral blocker YM-58483 (YM). Right, Quantification of typical changes in phosphorylation of the selected 5 targets in comparison with control condition. 

B, Left, Western blot analysis of SERCA2a and STIM1 on isolated cardiomyocytes and noncardiomyocyte fractions from control and AAV9.shStim1–treated mice. Right, quantification of Western blots. 

C, Left, Western blot analysis of phosphorylation of AktS473, AktT308, GSK-3βS9 on isolated cardiomyocytes from control and AAV9.shStim1–infected mice. Right, quantification of Western blots. 

D, Left, Western blot analysis of mTOR, Rictor, mSN1, and mLTS8 on isolated cardiomyocytes from control and AAV9.shStim1–infected mice. Right, Quantification of Western blots. 

E, STIM1 (Top) or Rictor (Bottom) were immunoprecipitated from isolated mouse cardiomyocytes. Immunoprecipitates with control IgG were used as control. Representative immunoblots of STIM1 and Rictor in both coimmunoprecipitates (as indicated) are shown. 

F, mTORC2 in vitro kinase assay was performed on control and shStim1–treated cardiomyocytes using immunopurified mTORC2 (Rictor) and recombinant kinase-dead Akt as a substrate. 

mechanism also translates into mice CMs. Wild-type mice were treated with AAV9.shStim1 or control, and CMs were isolated 3 weeks later. This strategy resulted in a significant reduction by 90% of STIM1 in isolated CMs (characterized by Serca2a expression), whereas STIM1 expression was not altered in non-CM cells (Figure 6B). Concordant with our phosphokinase array data, we found that Stim1 silencing was associated with a strong reduction in the phosphorylation of Ser473 but not of Thr308 in Akt (Figure 6C). Ser473 in the hydrophobic motif of Akt is directly phosphorylated by mTORC2.29 Our
later results thus suggest an important reduction of mTORC2 activity following Stim1 silencing. The phosphorylation of both S473 and T308 sites is required to support full activation of Akt, and the phosphorylation profile of main Akt substrates (GSK-3, PRAS40, Foxo3) was examined. We observed that Stim1 silencing was mainly associated with a significant reduction in phosphorylation of Ser9 in GSK-3β, whereas there were no significant changes in the phosphorylation levels of Foxo3-S253 or PRAS40-T246 (Figure III in the online-only Data Supplement).

Because mTORC2 is a multiprotein complex composed of several proteins that are required for its activity, we first assessed the expression of mTORC2 main components (mTor, Rictor, mLST8, mSN1) in isolated CMs and did not find any influence of Stim1 silencing on mTORC2 integrity (Figure 6D). We then asked whether STIM1 directly influences mTORC2 activity. We found that STIM1 directly interacts with the mTORC2 complex through a direct physical interaction between STIM1 and Rictor as revealed by specific coimmunoprecipitation experiments (Figure 6E). We then specifically tested mTORC2 activity according to STIM1 expression using an in vitro mTORC2 kinase assay.23 We purified mTORC2 complexes from wild-type and Stim1-deficient CMs by immunoprecipitation Rictor. We then incubated these isolated complexes with kinase-dead Akt as a substrate, thus avoiding Akt autophosphorylation. Akt-pS473 was then measured to directly reflect mTORC2 activity.23,29 We found that Stim1 silencing resulted in a significant reduction in mTORC2 kinase activity toward Akt (Figure 6F). These findings indicate that STIM1 is required for mTORC2 activity and signaling in CMs.

Finally, previous reports have shown that STIM1 activates in response to a mechanical or humoral hypertrophic stressor, which allows gating with plasma membrane Orai channels.9 We thus sought to determine whether inhibition of STIM1/Orai complex at the plasma membrane reproduces...
the effects on mTORC2/Akt activation observed after Stim1 silencing. For this purpose, normal CMs were isolated from wild-type mice and stimulated for 48 hours with angiotensin II or vehicle. Angiotensin II stimulation induced an increase in Stim1 expression, and GSK-3β9 phosphorylation, as well (Figure 7A and 7B). We applied YM-58483, a potent and selective pharmacological inhibitor of Orai channels, and found a deep decrease in Akt577 and GSK-3β9 phosphorylation in angiotensin II–treated CMs (Figure 7B).

**Discussion**

We and others have previously reported that Stim1 is a critical regulator of CM growth.6,12,14–16 These studies were mainly conducted in isolated neonatal and adult CMs where Stim1 silencing was associated with an important reduction in agonist-triggered hypertrophic response. Stim1 was reported as an essential activator of the NFAT transcription factor, a well-known positive regulator of cardiac growth.2 This observation was in accordance with genomic screens that identified Stim1 as a critical regulator of NFAT nuclear translocation in immune cells.30 By activating, Stim1 allows Ca2+ entry that further activates the Ca2+ sensor calmodulin and calcineurin.31,32 This signaling model has not been reproduced in cardiac cells, however.

In our current study, we found that the reduction in Stim1 expression not only prevented the development of cardiac hypertrophy, but also resulted in an inappropriate reversal of established hypertrophy and promotion of LV dilation and dysfunction. Consequently, in response to pressure overload, mice treated with AAV9.shStim1 eventually develop cardiac fibrosis in contrast with our previous observations in rats.3 However, both models differ by the type of constriction (thoracic versus abdominal) and the lack of transition to heart failure in rats. In line with our results, it was reported that Stim1 CM-specific deletion in mice led to cardiac dilation, contractile dysfunction, and wall thinning over the long term under physiological conditions.6 This supports a direct effect of Stim1 on cardiac homeostasis that is enhanced in response to stress as shown in our study.

At the cellular level, Stim1 silencing promoted CM cell atrophy and apoptosis, suggesting that Stim1 activation is rather critical to repress the action of some anti hypertrophic and proapoptotic molecules. Of note, this phenotype was not concordant with current knowledge on calcineurin/NFAT signaling in the heart that is usually associated with reduction of cardiac hypertrophic response but with preservation of systolic function and cardiac volumes.2,31,32 Conversely, accumulating evidence suggests that GSK-3α negatively regulates cardiac hypertrophy and that the inhibition of GSK-3β by hypertrophic stimuli is an important mechanism in the stimulation of cardiac hypertrophy.30 GSK-3 is constitutively active in CMs where it negatively regulates hypertrophic transcriptional effectors such as GATA4, β-catenin, and NFAT. Prohypertrophic stimulation rapidly inactivates GSK-3 through an increase in phosphorylation.27 GSK-3 has 2 mammalian isoforms, GSK-3α and -3β, which are both expressed in the heart. Even if the exact contribution of each isoform in cardiac hypertrophy is not fully understood, studies generally show that hyperactive GSK-3 decrease hypertrophic response by reducing CM size but also by increasing apoptosis.34 Cardiac-specific GSK-3α transgenic mice develop cardiac dysfunction in response to pressure overload.34 Reciprocally, inhibition of GSK-3 during heart failure is protective.35 In our study, we found that the activity of GSK-3β was dramatically enhanced in the absence of Stim1. Of note, pharmacological inhibition of GSK-3 was sufficient to reverse the cardiac phenotype observed after Stim1 silencing. These data support an unanticipated model where Stim1 is critical to deactivate a key negative regulator of cardiac hypertrophy.36

We further define an intracellular pathway for the regulation of cardiac hypertrophic growth that links Stim1 activation to Akt kinase activity through regulation of mTORC2 activity. Akt has long been recognized as a pivotal participant in hypertrophic signaling,28 and GSK-3β is one of its direct downstream targets. In line with our observation, Akt1−/− mice subjected to 7 days of TAC presented LV dilation and contractile dysfunction.37 Reciprocally, cardiac-specific overexpression of active Akt prevents pressure overload–induced heart failure, part, by reducing apoptosis.39 Multiple lines of evidence thus support a cardioprotective role for Akt activation29 that however involves the phosphorylation of 2 residues for full activation29: threonine 308 in the activation loop and serine 473 in the C-terminal hydrophobic motif. One of the most surprising results of our study is that Akt phosphorylation specifically at Ser473 was largely dependent on Stim1 activity as demonstrated by the strong decrease after reduction of Stim1 expression or pharmacological inhibition of the Stim1/Orai complex. In contrast, Akt phosphorylation at Thr308, an event driven by the Pi3k/PDK1 signaling, was not affected by Stim1 silencing. Even if other candidates have been proposed, phosphorylation of Ser473 is largely supported by mTORC2.29,30 Growing evidence shows that the mTOR pathway plays a key role in the development of cardiac hypertrophy.30 Mice with inducible cardiac-specific mTOR or raptor deletion do not develop compensatory hypertrophy in response to pressure overload and rather develop massive ventricular dilation and cardiac dysfunction associated with apoptosis, autophagy, mitochondrial abnormalities, sarcomere disarray, and metabolic abnormalities,41,42 a phenotype that fits with the one we observed after Stim1 silencing. Cardiac overexpression of mTOR preserves cardiac function during pressure overload, an effect that was potentially linked to increased mTORC2 activity.40,43 mTORC2 is indeed a component of both mTORC1 and C2 complexes.

Our results indicate that cardiac abnormalities that developed after Stim1 silencing are explained by an impaired upstream activation of mTORC2. We demonstrated that Stim1 is required for proper mTORC2 kinase activity toward Akt. We then found a direct interaction between Stim1 and Rictor, a specific component of mTORC2 complex. These findings suggest that Stim1-mTORC2 interaction is critical to support mTORC2 activity and conveys prohypertrophic signaling in CMs. The data on specific role of mTORC2 in the regulation of CM growth is scarce but, in addition to our study, some evidence also argues for a cardioprotective role. Rapamycin, a drug that strongly inhibits mTORC1 but, to a lesser level, mTORC2, blunts cardiac hypertrophy development in response to pressure overload44 and improves cardiac function in mice with decompensated hypertrophy.45 It was recently reported that cardiac-specific mTORC2 disruption through Rictor deletion leads to impaired cardiac growth and response to pressure overload.
overload, cardiac dilation, and cardiac dysfunction, features that we similarly found after Stim1 silencing.

To the best of our knowledge, a connection between STIM1 activation and mTORC2/Akt signaling was not previously established. Intriguingly, modifications in Akt Ser473 phosphorylation were observed in skeletal muscle from Stim1-deficient mice and in arterial smooth muscle cells after Orai3 knockdown, thus asking whether the STIM1/Akt coupling is specifically observed in muscular cells. We and others have previously shown that, in CMs, and in smooth muscle cells, as well, STIM1 coimmunoprecipitates with Orai1 and 3 channels under resting conditions. STIM1 might thus represent a large protein complex that can adapt to different stresses to interact with a diversity of complexes. In response to hypertrophic or proliferative stimulus, a large recruitment of Orai3 to STIM1 and Orai1 occurs, thus allowing for a store-independent entry. This contrasts with the hypothetical model in intracellular signaling pathways.

Altogether, these findings support a novel model (Figure 7C) whereby STIM1 activation, notably in response to hypertrophic stimulation, is critical to tune Akt kinase activity through activation of mTORC2, which ultimately results in repression of the antihypertrophic activity of GSK-3β.

Acknowledgments

We acknowledge the support of the NHLBI Gene Therapy Resource Program (GTRP) for the production of recombinant AA V9s. Author contributions: Drs Hulot, Hajjar, and Bénard designed the study. Dr Bénard conducted the animal and biochemical experiments with the help of Drs Oh, Cachex, Lee, and Nonnenmacher, D. S., Matasic, Dr Kho. Dr Bénard and E. Kohlbrenner generated rAAV9s. Drs Hulot and Bénard drafted the manuscript with critical revision from Drs Hajjar and Pavoine.

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Disclosures

None.

References


Cardiac hypertrophy is a compensatory response to increased mechanical load or to neurohormonal stimulation that reduces wall stress by increasing wall thickness. Although it represents an initial salutary adaptation to stress, chronic hypertrophic remodeling involves maladaptive changes in cardiac function over the long term. Different molecules and signaling circuits have been shown to regulate hypertrophic growth but the upstream events at the level of sarcolemma that initiate the cardiac hypertrophic responses remain largely unknown. Here, we provide evidence that stromal interaction molecule 1, previously reported as a critical regulator of cardiomyocyte growth, activates in response to hypertrophic stimulation to tune Akt kinase activity through a direct regulation of mammalian target of rapamycin (mTOR) complex 2 activity. Growing evidence shows that the mTOR pathway plays a key role in the development of cardiac hypertrophy, and we report a direct interaction between stromal interaction molecule 1 and Rictor, a specific component of mTOR complex 2. This ultimately results in repression of the antihypertrophic molecule GSK-3β, thus promoting cardiac hypertrophic growth. Reciprocally, in the absence of stromal interaction molecule 1, the heart is unable to develop or to sustain preestablished cardiac hypertrophy, which rapidly leads to left ventricular dilation and dysfunction. These mechanistic insights show a critical connection between stromal interaction molecule 1 activation and mTOR complex 2/Akt signaling to support cardiac hypertrophic response to stress and avoid rapid transition to heart failure.
Cardiac Stim1 Silencing Impairs Adaptive Hypertrophy and Promotes Heart Failure Through Inactivation of mTORC2/Akt Signaling
Ludovic Bénard, Jae Gyun Oh, Marine Cacheux, Ahyoung Lee, Mathieu Nonnenmacher, Daniel S. Matasic, Erik Kohlbrenner, Changwon Kho, Catherine Pavoine, Roger J. Hajjar and Jean-Sébastien Hulot

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SUPPLEMENTAL MATERIAL

Cardiac Stim1 silencing impairs adaptive hypertrophy and promotes heart failure through inactivation of mTORC2/Akt

Running title: Bénard et al., Role of STIM1 in the transition to heart failure

Ludovic Bénard, PhD¹, Jae Gyun Oh, PhD¹, Marine Cacheux, PhD¹, Ahyoung Lee, PhD¹, Mathieu Nonnenmacher, PhD¹, Daniel S. Matasic, BS, MBA¹, Erik Kohlbrenner, BS¹, ChangWon Kho, PhD¹, Catherine Pavoine, PhD², Roger J. Hajjar, MD¹, Jean-Sébastien Hulot, MD, PhD¹,².

Inventory of Supplemental Material

Supplemental Figures (S1-S3) and legends
Supplemental Table (S1)
Supplemental Figure 1. Cardiac-targeted RNA interference approach in mice.
A, Sequence of shRNA targeting Stim1 and map of vector. B, Schematic timeline to study Stim1 silencing efficiency after AAV9 infection in Wild Type mice. C, Time-course analysis of Stim1 silencing assessed by Western blot. Left, Western blot. Right, quantification of Western blot. n=2 animals per group.
Supplemental Figure 2. Characterization of cardiac function of mice at the time of randomisation in HF experiment. Cardiac function assessed by ultrasound 3 weeks after TAC and before randomization. A, Intra Ventricular Septum thickness. B, Left Ventricular Internal Diameter. C, Fractional Shortening. d = in diastole. n≥10 mice per group. * p<0.05, ** p<0.01, *** p<0.001.
Supplemental Figure 3. Foxo3 and PRAS40 activation by Akt is not modified by Stim1 silencing. Left, Western blot analysis of phosphorylation of the Akt substrates Foxo3, PRAS40 on isolated cardiac myocytes from control and AAV9.shStim1 infected mice. Right, quantification of Western blots. n=3 per group.
### Supplemental table

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**Table S1. Kinases included in Human Phospho-array.** List of 45 kinase phosphorylation sites tested by the kit. Kinases were sorted according to the intensity of signal compared to p53 S392 (highest signal) in control condition. Variations of phosphorylation in percentage compared to control after TG or YM
treatment. Quantification was performed for kinases presenting with at least 5% of p53<sup>s392</sup> phosphorylation. n=2 per kinase tested.