The ShcA Phosphotyrosine Docking Protein Uses Distinct Mechanisms to Regulate Myocyte and Global Heart Function

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Rationale: Although tyrosine kinases (TKs) are important for cardiac function, their relevant downstream targets in the adult heart are unknown. The ShcA docking protein binds specific phosphotyrosine (pTyr) sites on activated TKs through its N-terminal pTyr-binding (PTB) and C-terminal SH2 domains and stimulates downstream pathways through motifs such as pTyr sites in its central CH1 region. Therefore, ShcA could be a potential hub for downstream TK signaling in the myocardium.

Objective: To define the role of ShcA, a TK scaffold, in the adult heart using a myocardial-specific knockout of murine ShcA (ShcA CKO) and domain knock-in models.

Methods and Results: ShcA CKO mice developed a dilated cardiomyopathy phenotype involving impaired systolic function with enhanced cardiomyocyte contractility. This uncoupling of global heart and intrinsic myocyte functions was associated with altered collagen and extracellular matrix compliance properties, suggesting disruption of mechanical coupling. In vivo dissection of ShcA signaling properties revealed that selective inactivation of the PTB domain in the myocardium had effects resembling those seen in ShcA CKO mice, whereas disruption of the SH2 domain caused a less severe cardiac phenotype. Downstream signaling through the CH1 pTyr sites was dispensable for baseline cardiac function but necessary to prevent adverse remodeling after hemodynamic overload.

Conclusions: These data demonstrate a requirement for TK-ShcA PTB domain signaling to maintain cardiac function. In addition, analysis of the SH2 domain and CH1 pTyr sites reveals that ShcA mediates pTyr signaling in the adult heart through multiple distinct signaling elements that control myocardial functions and response to stresses. (Circ Res. 2011;108:184-193.)

Key Words: adaptor protein ☐ tyrosine kinase ☐ cardiomyopathy ☐ signal transduction

Genetic analysis has established tyrosine kinase (TK) signaling as being integral to cardiac function. For example, loss of myocyte-specific signals downstream of the receptor TK ErbB2 results in a dilated cardiomyopathy, whereas myocyte-specific deletion of focal adhesion kinase, a nonreceptor TK, results in eccentric remodeling in response to hemodynamic overload and aging. The relevance of TK signaling for heart function is further supported by clinical trials in which TK inhibitors such as trastuzumab (Herceptin), which targets ErbB2, and imatinib (Gleevec), an inhibitor of kinases such as Abl and the platelet-derived growth factor receptor, have shown cardiac side effects in a subset of oncology patients. The dissection of downstream signaling networks activated by TKs in the adult heart is therefore of considerable therapeutic interest.

ShcA (also termed Shc1) is a scaffold protein for TKs that adds complexity and specificity to TK signaling. The mammalian ShcA gene encodes 3 cytosolic protein isoforms (66, 52, and 46 kDa) that bind to phosphotyrosine (pTyr)-containing motifs on activated TKs both through an N-terminal pTyr-binding (PTB) domain and a C-terminal Src homology (SH)2 domain. Once recruited to activated TKs through such pTyr recognition domains, ShcA can itself undergo tyrosine phosphorylation in the central CH1 (collagen homology 1) region, thereby stimulating the activation of specific cytoplasmic signaling pathways. Notably, phosphor-
ylation of the tyrosine residues 239/240 and 313 (mouse nomenclature) in the CH1 region creates 2 consensus pY-X-N motifs that bind the SH2 domain of the Grb2 adaptor, leading to stimulation of the Erk-MAPK (extracellular signal-regulated kinase–mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3′-kinase) pathways.7,9 Protein–protein interactions mediated by phosphorylation of the CH1 tyrosines of ShcA are important in the development of a functional monosynaptic stretch reflex circuit10 and ErbB2-induced breast cancer in the mouse.11

Germline deletion of ShcA led to profound embryonic cardiovascular defects,12 and although mitogenic signaling through the CH1 pTyr sites of ShcA represents a primary mechanism of action for the major 52/46-kDa isoforms, ShcA can use multiple mechanisms to convey signals downstream of TKs. For example, genetic analysis revealed that during heart development the 52/46 kDa isoforms can signal downstream of TK-ShcA PTB domain interactions independent of the CH1 pTyr sites.10 In addition, the p66 isoform of ShcA has a signaling role in the oxidative stress response, a function likely mediated by phosphorylation of Ser36 in the unique CH2 region at the N terminus of p66 ShcA.13,14

The observation that ShcA is essential in the developing heart led us to investigate its role in the postnatal myocardium and how it uses its various signaling domains to propagate TK signaling in the myocardium. The PTB domain of ShcA has been shown to interact with multiple TKs that are distinct from TK-ShcA SH2 domain interactions.7 These interactions, together with the ability of the CH1 region to signal through various pathways, suggest that ShcA can potentially be a hub for pTyr signaling in the myocardium. To evaluate this possibility, we conditionally excised ShcA in ventricular cardiomyocytes and used ShcA murine knock-in (KI) alleles each containing discrete point mutations that inactivate specific domains or motifs.10 The use of pTyr-binding KI mutants allowed us to investigate the functional roles of ShcA PTB and SH2 domains in coupling to upstream TKs. In addition, mice containing KI mutations of the CH1 pTyr residues allowed us to investigate downstream pathways in the postnatal myocardium.

Our results reveal that the loss of ShcA in ventricular cardiomyocytes leads to a dilated cardiomyopathy characterized by intact cardiomyocyte contractility and defects that resulted in impaired myocyte-matrix interactions. The effects of ShcA ablation in the heart were dramatically accelerated by biomechanical stress. Analysis of ShcA KI mutants indicated that the various signaling elements had distinct phenotypic profiles, with PTB domain inactivation producing similar cardiac dysfunction as found in ShcA CKO mice (myocardial-specific knockout of murine ShcA). Thus, ShcA is a critical hub protein that transmits pTyr-dependent signals to control adult myocardial function and response to biomechanical stress through a variety of distinct molecular mechanisms.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Mouse Models**

All study protocols were approved by the Animal Care Committee at the University of Toronto in accordance with animal welfare regulations. The ShcA allele series consists of the ShcA floxed allele and mutant domain KI alleles of ShcA.10 The Mlc2v Cre KI mouse (gift from Dr K. R. Chein)13 and αMHC-MerCreMer transgenic mouse (The Jackson Laboratory)16 were used to conditionally excise ShcA in the myocardium through Cre/loxP strategies. Targeting and recombination strategies were described previously.10 All mouse lines were bred in a mixed background (backcrossed with CD1 for 2 generations) and used appropriate littermate controls.

**Animal Genotyping**

Mouse tail biopsies were used for PCR amplification of the various genotypes as described previously.10,15,16 DNA sequencing from tail samples confirmed the individual mutations as reported previously.10 The excision of the ShcA floxed allele was confirmed by PCR using genomic DNA as described previously.10

**Cardiac Phenotyping**

Detailed cardiac phenotyping methods are available in the Online Data Supplement.

**Histological Analysis and Microscopy**

Hearts were processed for electron microscopy or sectioned for histological analysis as outlined in the Online Data Supplement.

**Western Blotting and Immunoprecipitation**

Whole hearts or isolated cardiomyocytes lysates were prepared as described previously10 and described in full in the Online Data Supplement.

**Zymography**

Left ventricle free wall lysates were prepared, resolved, and stained as described in the Online Data Supplement.

**RNA Isolation and Real-Time RT-PCR**

Total RNA was isolated from hearts with TRIzol and transcribed to cDNA, and targets were analyzed by quantitative RT-PCR as described in the Online Data Supplement.
Preparation of Tamoxifen

Injected tamoxifen was prepared as previously described.16 Briefly, tamoxifen citrate (Sigma) was sonicated in peanut oil (Sigma) at a concentration of 5 mg/mL, and 20 mg/kg per day was injected in the peritoneal cavity for 5 days.

Statistics

Data are presented as means±SEM. Means were compared by 2 tailed Student t test or 1-way ANOVA. *P<0.05 was considered significant.

Results

Generation of Ventricular Cardiomyocyte–Specific ShcA-Null Mice

To generate ventricular cardiomyocyte-specific ShcA-null mice, we used a Cre/loxP strategy by intercrossing mice with a ShcA floxed (ShcAflx) allele10 with mice possessing the myosin light chain (Mlc2v) Cre KI (Mlc2vflxKI) allele15 (Figure 1A and 1B). Mating of ShcAflx/Mlc2vflxKI mice and ShcAflx/Mlc2vflxKI mice yielded the expected 1:1:1:1 Mendelian ratio. As expected, ShcAflx/Mlc2vflxKI mice (designated ShcA CKO) showed selective deletion of ShcA in ventricular cardiomyocytes.15 ShcAflx/Mlc2vflxKI mice used as littermate controls (Online Table I; the Table) and were indistinguishable from ShcAflx/Mlc2vflxKI mice (n=3; 6 month left ventricle end diastolic dimension [LVEDD]: 4.00±0.11 mm and percentage fractional shortening [%FS]: 44.89±2.38; 1 year LVEDD: 4.15±0.09 mm and %FS: 44.39±2.27; 4 weeks transverse aortic constriction [TAC] LVEDD: 4.12±0.10 mm and %FS: 39.78±2.59; and 12-week percentage sarcomere shortening: 7.18±0.80%) as shown previously.4,15

Mlc2v Cre recombinase–mediated excision of ShcA, as inferred by the level of ShcA protein, was detected at a minimal level at 2 weeks of age and increased markedly over the next 10 weeks (Figure 1C), as reported previously.4,15 The residual ShcA protein at 12 weeks was likely from nonmyocyte sources (fibroblasts, smooth muscle cells, and endothelial cells), because robust loss of ShcA protein levels was observed in enzymatically isolated cardiomyocytes (Figure 1D). Lysates from spleen and lung confirmed the specificity of excision, because ShcA levels were comparable to control lysates in these tissues (Figure 1E).

ShcA Is Required for the Maintenance of Cardiac Structure and Function

Homozygous ShcA<sup>−/−</sup> mice die at embryonic day 11.5,12 whereas mice with germline ablation of the p66 ShcA allele<sup>H11001</sup> are viable to birth.12 Contractility and transient assays have a minimum of 3 hearts per group with a minimum of 15 cells. Force–Ca<sup>2+</sup> experiments have a minimum of 6 hearts per group. dSL/dT max indicates maximum first derivative of sarcomere shortening; dSL/dTmin, minimum first derivative of sarcomere shortening; TR<sub>50</sub>, time to 50% relaxation; TR<sub>80</sub>, time to 80% relaxation; F, fluorescence of calcium transient; F<sub>max</sub>, maximal force; EC<sub>50</sub>, Ca<sup>2+</sup> concentration at 50% maximal force. *P<0.05 from littermate controls at given time point.
isoform are long-lived. By contrast, although echocardiography data showed no differences in cardiac dimensions or fractional shortening at 6 weeks of age, by 12 weeks, ShcA CKO mice developed decreased fractional shortening and distended chamber morphology without evidence of concentric hypertrophy (heart weight/body weight [HW/BW] ratio: 5.30±0.25 for ShcA Con versus 5.00±0.15 for ShcA CKO [P=0.32]; n=7) (Figure 2A and 2B; Online Table I). This cardiac dysfunction combined with the absence of antecedent cardiac hypertrophy at 6 to 8 weeks of age, suggests that ShcA CKO mice enter a dilated cardiomyopathy phenotype in parallel with ShcA excision. Indeed, isolated cardiomyocytes from ShcA CKO mice at 12 weeks of age had increased cell lengths, consistent with eccentric remodeling (Figure 2D). ShcA CKO hearts also had elevations in atrial natriuretic factor and skeletal actin transcripts (Figure 2E), 2 genetic markers of cardiac pathology. Although ShcA has been reported to be critical in MAPK activation, no significant changes in phospho-Erk, indicative of Erk activation, were noted in heart lysates at 6 months (Figure 2F). This finding could be attributable to the heterogeneous cell population within the myocardium and the multiple converging signals leading to MAPK activation.

By 1 year of age, ShcA CKO mice had a further decline in cardiac function with coincident enlargement of the left ventricle chamber dimensions (Figure 2A through 2C; Online Table I) in conjunction with an increase in HW/BW ratios (Online Figure I, A). Despite severe ventricle dilation accompanied by a trend of increasing lung weight/body weight ratios (Online Figure I, A), electron microscopy revealed no evidence of myofibrillar disarray, aberrant intercalated disc structure, or mitochondrial abnormalities (Online Figure I, B). Masson trichrome staining revealed minimal myocardial interstitial fibrosis in ShcA CKO mice at 1 year of age (Online Figure I, C), despite advanced remodeling.

**Loss of ShcA Signaling Results in Enhanced Single-Myocyte Contractility**

To address whether the diluted cardiomyopathy phenotype resulting from ShcA deficiency is associated with changes in baseline cardiomyocyte function, we measured isolated single-myocyte sarcomere length shortening. Surprisingly, despite systolic function being reduced at 12 weeks of age, contractility of ShcA CKO cardiomyocytes was enhanced compared with controls, without changes in calcium transient amplitude (Online Table I). These findings suggest that the loss of ShcA enhances the calcium sensitivity of the myofilament, which was confirmed in force-calcium measurements of isolated myocytes (Online Table I). This enhanced calcium sensitivity was not associated with decreases in serine 23/24 phosphorylation of troponin I, which is a known mechanism for regulating the calcium sensitivity of contraction (Online Figure II).

To ensure the enhanced contractility was not caused by secondary compensatory mechanisms, we used the tamoxifen inducible Cre transgenic mouse (MerCreMer) driven by the αMHC promoter to acutely excise ShcA in cardiomyocytes. ShcA MCKO (ShcAfl/flx MerCreMer+/−/−) and their littermate controls, ShcA MCON (ShcAfl/flx MerCreMer+/+/+) and ShcACKO mice were injected with tamoxifen for 5 days. ShcA MCKO mice showed no evidence of chamber dilation or depressed systolic dysfunction 7 days post injection (Online Table II). However, 7 days after the tamoxifen protocol, ShcA MCKO cardiomyocytes showed elevated baseline contractility compared with controls (7.76±0.36% versus 6.42±0.34%, respectively; n=5 hearts with >25 cells, P=0.016). The single-myocyte data suggest that the changes in isolated myocyte function is a cell autonomous effect attributable to the loss of ShcA. Thus, the loss of ShcA in the myocardium leads to progressive heart dilation that is not accompanied with impaired cardiomyocyte contractility, altered myocardial ultrastructure, or exaggerated interstitial fibrosis.

**Loss of ShcA Leads to Deregulation of Extracellular Matrix Components in the Heart**

The presence of elevated single-myocyte contractility despite decreased global systolic function suggests a mechanical uncoupling within the myocardium. Therefore, we investigated whether the chamber dilation in ShcA CKO mice results from impaired extracellular matrix (ECM)–myocyte interactions. Consistent with this, force-sarcomere length measurements in papillary muscles revealed higher compliance (P<0.001) in ShcA CKO preparations compared with controls (compliance parameter [c]=0.42 versus 0.20, respectively; Figure 3A), suggesting disrupted ECM. Because sarcomere length in papillary muscles can be heterogeneous in shape resulting from shape nonuniformity, a small cohort of ultra thin trabeculae muscle preparations were also examined and gave similar results (data not shown). It is conceivable that the increased compliance of the CKO myocardium originates from cardiac remodeling induced by the loss of ShcA. However, the compliance (c=0.27) of muscles from control hearts subjected to TAC (n=5) was much less (P<0.001) than ShcA CKO hearts, despite having indistinguishable reduction in fractional shortening (36.1±1.36%) and LVEDD (4.36±0.07 mm). Consistent with the mechanical results, perimysial collagen fibers stained with picrosirius red showed decreased complexity and structural collagen content, at 12 weeks of age in ShcA CKO mice (Figure 3B and 3C). In further support of mechanical uncoupling, ShcA CKO papillary muscles also showed reduced developed tension (4.44 mN/mm² at 2.10±0.003 μm for ShcA CKO and 6.43 mN/mm² at 2.10±0.004 μm for controls; P<0.05), suggesting that poor force transmission contributes to impaired contractility in whole hearts. Although the cause of the altered ECM structure and function in ShcA CKO myocardium is unclear, matrix metalloproteinase (MMP) activity in six month old ShcA CKO hearts was elevated compared with controls (Figure 3D). Thus, despite causing enhanced cardiomyocyte contractility, ShcA excision also disrupts ECM and induces a progressive dilated cardiomyopathy.

**ShcA CKO Mice Undergo an Eccentric Remodeling Response After TAC**

To test the hypothesis that ShcA is critical for the maintenance of mechanical integrity of the heart through ECM–myocyte interactions, 8 week old ShcA CKO mice were...
subjected to biomechanical stress by TAC, before overt dilation. After 4 weeks of TAC, control mice mounted the expected concentric hypertrophy response (Figure 4A; Online Table III), accompanied by multifocal interstitial fibrosis (Figure 4B) with preserved overall heart function (Figure 4A; Online Table III). ShcA CKO mice, in contrast, quickly transitioned into congestive heart failure (HF) after the 4 weeks of TAC, characterized by severe chamber enlargement, depressed fractional shortening, minimal ventricular wall thickening, and elevated lung weights (Figure 4A and Figure 2. ShcA is required for the maintenance of cardiac function. A, Echocardiography time course of cardiac contractility as measured by percentage fractional shortening showing a decline in function of ShcA CKO hearts (n=7) over the course of 1 year compared with controls (n=5). B, Echocardiography time course of chamber morphology as measured by left ventricular end diastolic dimensions (mm) over the course of 1 year in ShcA CKO hearts (n=7) and controls (n=5). See Online Table I for complete data. C, Representative histology of transverse heart sections stained with hematoxylin/eosin at 1 year of age. Magnification, ×6.25. D, Isolated cardiomyocyte dimensions of ShcA CKO and control mice (n=3). E, Quantitative real-time RT-PCR of heart failure markers at 3 months of age (n=4). Data were normalized to GAPDH internal control and expressed as a fold change (arbitrary units [AU]) relative to control mRNA expression. ANF indicates atrial natriuretic factor; Sk Actin, skeletal actin; SERCA, sarcoplasmic reticulum ATPase pump 2A; BNP, brain natriuretic peptide. F, Representative western blot detection of pErk in heart lysates from control and ShcA CKO mice at 6 months of age (n=2). For panels A, B, D and E values are means ± SEM with *P < 0.05 compared with littermate control values for given time points.

Figure 3. The loss of ShcA leads to deregulation of the ECM components in the heart. A, Papillary passive tension, expressed as mN/mm², across increasing sarcomere lengths (μm) as described in Methods. A significant increase (P < 0.001) in ShcA CKO compliance (c=0.42) was demonstrated compared with control (c=0.20) (n=5 independent experiments per group), resulting in decreased passive tension. B, Visualization of perimysial collagen fibers stained with picrosirius red using Opti-grid structured illumination microscopy. Magnification, ×63. C, Percentage perimysial collagen area was quantified from multiple images from each heart (n=3). Values are means ± SEM with *P < 0.05 compared with littermate controls for given time point. D, Zymography of hearts from ShcA CKO and their littermate controls at 6 months (n=3). Increased activity was noted for proMMP9 and an unidentified MMP species at ~200 kDa. The positive control sample was supernatant from mouse embryonic fibroblasts stimulated with concanavalin A for 24 hours.
and isolated cardiomyocytes in the presence of MerCreMer transgene driven by the αMHC promoter compared with littermate controls, 7 days after tamoxifen injection protocol as explained in Methods. F, Representative histology of transverse sections stained with Masson trichrome of ShcA MCKO and control hearts after 4 weeks of TAC. Magnification, ×25. See Online Table III for complete echocardiography data. For A, C, and D, values are means±SEM with *P<0.05 compared with same genotype sham; **P<0.05 compared with littermate control values for the given time point. WK indicates week.

The PTB Domain of ShcA Couples to Upstream TKs to Maintain Cardiac Structure and Function
ShcA has a modular domain architecture that allows downstream signaling from TKs via several molecular mechanisms. Therefore, we used mice harboring specific ShcA KI mutations (δKI) restricted to cardiomyocytes10 as described in the Online Methods and Online Figure III.

Like the ShcA CKO mice, δPTB CKI mice demonstrated a dilated cardiomyopathy phenotype at an early age characterized by enlarged left ventricle chamber dimensions and reduced fractional shortening at 12 weeks of age (Figure 5A and 5B; Online Table I). Over the course of 1 year, ventricle dilation progressed, whereas cardiac function declined further, in conjunction with slight elevations in HW/BW ratios in δPTB CKI mice (Figure 5E). Similar to the ShcA CKO mice, Masson trichrome staining of hearts at 1 year revealed minimal areas of interstitial fibrosis (data not shown). As the δPTB CKI mice displayed robust phenotypic defects at 8 to 12 weeks of age (Figure 5A and 5B), TAC studies were not carried out. The early presentation of the dilated cardiomyopathy in the δPTB CKI mice could result from subtle gene dosage effects originating from the presence of one functionally null allele that precludes PTB coupling to upstream TKs during development. Therefore, these data establish that ShcA coupling to upstream TKs through its PTB domain is critical for maintenance of cardiac chamber dimensions and function.

In contrast to the δPTB CKI mice, at 6 months of age, δSH2 CKI mice demonstrated subtle cardiac dysfunction, and by 1 year of age, systolic function was reduced and chamber dimensions were slightly enlarged (Figure 5A and 5B; Online Table I). At one year of age, no significant difference in HW/BW ratio was noted (Figure 5E). Despite appearing normal at 8 weeks of age, δSH2 CKI mice had reduced cardiac function and blunted hypertrophy response without differences in chamber dimensions compared with controls after 4 weeks of TAC (Figure 5C and 5D; Online Table III). These results demonstrate that ShcA SH2 domain-mediated interactions play a role in hypertrophy signaling following biomechanical stress and aging.

ShcA Phosphotyrosine-Derived Signaling Is Required in Hemodynamic Overload
Whereas in vitro and in vivo studies have shown the CH1 pTyr sites are important in downstream signaling, genetic
Myocyte Contractility Requires ShcA Phosphotyrosine-Based Signaling

As the loss of ShcA in cardiomyocytes enhanced myocyte contractility, the 3 ShcA mutant allele CKI mouse lines were subjected to single-myocyte contractility assays and analysis of global calcium transients. Both the δPTB CKI and 3F CKI myocytes were hypercontractile compared with their littermate controls independent of changes in calcium handling, whereas δH2 CKI myocytes were not different from control myocytes (Figure 5F; Online Table V). These findings suggest that ShcA requires PTB domain coupling to upstream TKs and subsequent signaling through the CH1 pTyr sites to maintain homeostatic contractility.

Discussion

Our results demonstrate a requirement for the ShcA pTyr-docking protein in the postnatal myocardium. In particular, p52 and p46 ShcA are inferred to be the relevant isoforms in this respect, as the p66 ShcA deficient mouse has a protective cardiac phenotype.13 We found that TK-ShcA signaling through the PTB domain acts independently of the ShcA CH1 pTyr sites to regulate myocyte-matrix interactions, and defecits in this signaling mode precipitate a dilated cardiomyopathy phenotype. In contrast, maintenance of homeostatic myocyte contractility and appropriate responses to biomechanical stress requires ShcA to signal through the CH1 pTyr sites (Figure 6). The ability of ShcA to signal independently of the CH1 pTyr sites is consistent with previous results in embryonic heart development.10 In the postnatal myocardium, our data suggest that ShcA is a central hub for TK signaling and uses multiple biochemical mechanisms to link TK signaling to diverse aspects of cardiovascular physiology.

ShcA Regulates Myocyte–Matrix Interactions in the Myocardium

The ShcA CKO mice have enhanced cardiomyocyte contractility despite whole heart cardiac dysfunction, suggesting an...
uncoupling of global regulators of cardiac function from myocyte function. Although alterations in phosphorylation at troponin I Ser23/24 did not contribute to the enhanced calcium sensitivity found in ShcA CKO animals, ShcA has been shown to influence protein kinase C, MAPK, and PI3K pathways, as well as localization of phosphatases, all of which could potentially impact on the phosphorylation status of myofilaments. Subsequent analysis will provide further insight into TK-mediated regulation of myocyte contractility. When cardiomyocyte function is compromised, global cardiac function is expected to be reduced. However, just as importantly, blood flow change as a result of altered vascular function, disruption of normal propagation of electric signals, and uncoupling of force transmission from the myocyte along collagen struts can all contribute to impaired cardiac function, thereby precipitating heart failure. The absence of necrosis with fibrosis, arrhythmias, or sudden death in the ShcA CKO mice suggests that cardiomyopathy in these mice did not result from myocyte loss and stimulation of fibrosis. Although the activation of the MMP2 and MMP9 in ShcA-deficient mice may result from generic stimulation of a final common pathway associated with adverse cardiac remodeling, the early detection of mechanical changes in the passive tension in parallel with the excision of ShcA along collagen struts can all contribute to impaired cardiac function, thereby precipitating heart failure. The slow evolution of a HF phenotype in the SH2 CKI mice, which does not follow the time course of the loss of ShcA in the myocardium, suggests that SH2 coupling to upstream TKs has a supportive role in cardiac structure and function, distinct from PTB domain–mediated signaling. Indeed, several matrix-associated TKs have docking sites for the PTB and SH2 domains of ShcA, so that ShcA, through reciprocal signaling between cellular compartments, could potentially affect mechanical coupling through adhesion interactions, secretion of multicellular proteins, or direct remodeling of the matrix. It will be of great interest to explore more deeply the underlying mechanism through which ShcA impacts on global heart structure and function. The presence of enhanced cardiomyocyte function in the context of a dilated cardiomyopathy reiterates that HF is a mosaic syndrome and requires stratification that is based on the precipitating etiology to effectively impact on prognosis.

ShcA Directs Myocardial TK Signaling Through Recruitment of Its PTB and SH2 Domains

The ability of ShcA to impact on different aspects of cardiovascular function highlights the modular nature of the ShcA protein and its ability to direct signaling by recruitment to specific TKs through the PTB and SH2 domains. Indeed, loss of signals derived from PTB domain docking to upstream TKs phenocopies the loss of ShcA in the myocardium, whereas loss of signals involving the SH2 domain are required to augment the response to TAC and hypertrophy associated with age.

The PTB domain of ShcA has been shown to interact with multiple receptor TKs critical in cardiac function, such as VEGFR3, ErbB2, and ErbB4; in particular, loss of ErbB2 in cardiomyocytes produces a similar phenotype to ShcA in cardiac ablation studies. The loss of ShcA in other tissues also phenocopies conditional ErbB2 loss and supports the idea that ShcA is a preferential scaffold for ErbB2 signaling, especially because it contains multiple ShcA consensus binding motifs. Of clinical relevance, trastuzumab, an effective TK inhibitor of ErbB2-ErbB3 heterodimers, causes HF in a subset of patients, suggesting that ShcA could function in a pathway that is regulated by trastuzumab, potentially influencing matrix/myocyte interactions. The clinical utility of Neuregulin, the ligand of heterodimers containing ErbB2, in improving cardiac function in diseased myocardium suggests the ErbB2-ShcA signaling network could be of great interest therapeutically.

The slow evolution of a HF phenotype in the SH2 CKI mice, which does not follow the time course of the loss of ShcA in the myocardium, suggests that SH2 coupling to upstream TKs has a supportive role in cardiac structure and function, distinct from PTB domain–mediated signaling. Indeed, the SH2 domain binds the platelet-derived growth factor receptor β, focal adhesion kinase, and the epidermal growth factor receptor, suggesting a unique role for ShcA SH2-mediated signaling in response to biomechanical stress and aging.

ShcA Uses Multiple Signaling Mechanisms to Impact on Myocardial Function

Downstream signaling induced by phosphorylation of tyrosine residues in the CH1 region of ShcA appears to have evolved as a mechanism to increase signal complexity in multicellular organisms. Covalent modification of these tyrosines has shown to be critical in Ras mediated MAPK activation and PI3K activation through ShcA-Grb2 interactions. Our data demonstrate that the pTyr sites and consequent Grb2 recruitment are not required for normal cardiac function but become important in situations of mechanical stress, such as hemodynamic overload. A small percentage of 3F CKI mice (<20%) did spontaneously dilate with age, but this seems to be an isolated phenomenon and could be attributed to some unknown stress. Alternative ShcA signal-
ing mechanisms that may be important in cardiac function include adaptor binding in the CH1 region, SH3 domain containing proteins recruited to the proline rich CH1 region, or serine/threonine phosphorylation motifs within the ShcA protein.\textsuperscript{7,35,36} In addition, IQGAP links ShcA to the actin cytoskeleton through a noncanonical interaction with the ShcA PTB domain.\textsuperscript{37} Therefore, ShcA has several alternative downstream signaling mechanisms that could be used to regulate cardiac function. Thus, in the postnatal myocardium, ShcA is able to use distinct signaling pathways to impact isolated cardiomyocyte and global heart function. For example, the PTB domain is required to maintain cardiac function and is essential for suppressing the dilated cardiomyopathy phenotype. However, this finding is independent of downstream signaling from the CH1 pTyr sites. Nonetheless, the CH1 pTyr sites become important in regulating cardiomyocyte contractility and coordinating signals in response to hemodynamic stress. These data show that ShcA is a key docking protein for TK signaling in the postnatal myocardium that coordinates signaling networks underlying cardiac physiology.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Tyrosine kinase (TK) signaling is critical for heart function, yet the relevant downstream signaling pathways remain elusive.
- Scaffolding proteins mediate the formation of signaling complexes immediately proximal to TKs and thereby activate effector pathways.
- Germline ablation of murine ShcA, a scaffold protein for TKs, results in early embryonic lethality caused by cardiac defects resembling those seen in ErbB family mutant mice.

**What New Information Does This Article Contribute?**

- Selective loss of ShcA in the adult myocardium of mice results in a dilated cardiomyopathy phenotype in response to both aging and biomechanical stress.
- ShcA regulates homeostatic myocyte contractility and influences extracellular matrix integrity.
- ShcA mediates TK signaling in the adult heart through distinct phosphotyrosine recognition domains and phosphorylated binding motifs, which differentially impact on myocyte contractility and extracellular matrix properties.

Mouse genetic studies and clinical research have demonstrated the importance of TKs in the heart, particularly with respect to the ErbB2/ErbB4 receptors and their ligand neuregulin. However, the signaling pathways that mediate their cardiac effects are poorly understood. We have previously shown that the ShcA scaffold protein plays an essential role in the heart during embryonic development in mice. In this study, we report that murine ShcA is required for normal adult heart function and its response to stress. We demonstrate that ShcA is required to maintain homeostatic contractility at the level of the myofilament and for the integrity of the extracellular matrix, and that these effects are uncoupled from one another. Lastly, we show that ShcA mediates its effects through multiple distinct signaling mechanisms, based on its modular organization of phosphotyrosine recognition domains and phosphorylated tyrosine sites. It is therefore a hub for phosphotyrosine signaling in the myocardium that transmits information to cytoplasmic effector pathways. Because ShcA is a key scaffold downstream of receptor tyrosine kinases, particularly ErbB2, these findings could provide insight into cardiotoxicity induced by the therapeutic antibody trastuzumab that targets ErbB2, and signaling pathways downstream of neuregulin, an emerging cardioprotective agent.
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Supplemental Material

Detailed Methods

Animal Protocols
All mice were housed in a pathogen free facility (Department of Comparative Medicine, University of Toronto) and handled using standard protocols in accordance with animal welfare regulations.

Generation and characterization of mice with ventricular specific ShcA point mutations
We utilized mice harbouring specific ShcA KI mutations (δKI) restricted to cardiomyocytes. Specifically, mice with ShcA δKI alleles containing individual point mutations were intercrossed with ShcA δKI/Δ δKI Mlc2vKI/WT mice to give ShcA δKI/Δ Mlc2vKI/WT. In non-cardiac tissue, the KI mutant allele yields no phenotype owing to expression of the fully functional floxed allele. However, Cre recombinase excision of the floxed allele in ventricular cardiomyocytes unmasks the properties of the mutated allele (cardiac specific KI (CKI)) (Online Figure IIIA). The individual point mutations precluded either pTyr binding by the PTB domain to upstream TKs by mutating arginine 175 to glutamate (δPTB CKI; ShcA δPTB/Δ Mlc2v CreKI/WT), pTyr binding by the SH2 domain by mutation of arginine 397 to lysine (δSH2 CKI; ShcA δSH2/Δ Mlc2v CreKI/WT) or downstream signaling from the tyrosines in the CH1 region by mutating tyrosines 239/240/313 to phenylalanines (3F CKI; ShcA 3F/Δ Mlc2v CreKI/WT) (Online Figure III B and C). Mice were viable and recovered at the expected 1:4 Mendelian frequency. Controls were littermates with the genotypes ShcA δKI/Δ Mlc2v CreWT/WT, ShcA WT/Δ Mlc2v CreWT/WT or ShcA WT/Δ Mlc2v CreKI/WT.

Histological Analysis and Microscopy
Heart sections were stained with hemotoxylin and eosin, masson trichrome stain or picrosirius red and slides were scanned at 20x using the Mirax microdigital slide scanner (Carl Ziess MicroImaging). Picrosirius red with 10% PMA was used for visualization of perimysial fiber content using the Optigrid structured illumination microscopy (Qioptiq LINOS) as previously described. Using the 20x objective lens, multiple areas of the myocardium were captured and analyzed for percent area of collagen (Image J software) to infer collagen content. Only sections absent of interstitial fibrosis and perivascular fibrosis were analyzed.

Electron Microscopy
Left ventricle free wall specimens were rapidly dissected and cut into 2mm³ pieces and placed in fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH7.4. The specimens were post fixed for 1 hr in sodium phosphate buffer containing 1% osmium tetroxide and 1.25% potassium ferocyanide then dehydrated in a series of alcohol steps and embedded in Epon Araldite. Thin sections were obtained and stained with uranyl acetate and lead citrate. Random fields were scanned at various magnifications and captured for subsequent analysis.

Western blotting and Immunoprecipitation
Hearts used for biochemical analysis were excised, rinsed in cold PBS and immediately frozen in liquid nitrogen for subsequent analysis. Whole hearts or isolated cardiomyocytes were homogenized in chilled radioimmunoprecipitation buffer containing 50mM Tris pH7.4, 150 NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton, 1% NP –40, 100mM sodium fluoride, 1mM sodium orthovandadate, 1x protease cocktail containing leupeptin, aprotinin and PMSF. Lysates were clarified by centrifugation at 14,000x g for 15 minutes at 4°C. Protein Quantification was carried out following standard protocol for the BCA kit (Pierce) and proteins
were boiled in 2x sample buffer, resolved by SDS PAGE, and transferred to nitrocellulose. For immunoprecipitations, Flag M2 antibody immobilized on agarose beads (Sigma) was used to enrich for ShcA containing fraction, and beads were washed 3 times and boiled in sample buffer, resolved by SDS PAGE and transferred to nitrocellulose. Membranes were blocked in 5% BSA and incubated with the appropriate primary antibody overnight, washed and incubated with the appropriate HRP conjugated secondary antibody for 1 hr. Blots were visualized after incubation with Chemiluminesence reagents (Invitrogen). Antibodies used were: Affinity purified Shc antibody (BD Biosciences), p44/42 and pp44/42 Erk (Cell Signaling Technologies), and GAPDH (BD Biosciences).

**Zymography**
Left Ventricles free wall was rapidly dissected from hearts, washed in PBS and placed in liquid nitrogen. Heart specimens were then homogenized in RIPA buffer and clarified 2x by centrifugation at 14,000xg for 20 minutes at 4ºC. 50 μg of protein was loaded onto an 8% Tris glycin gel containing 0.5% gelatin A substrate (Sigma). Zymography sample buffer contained: 0.625M Tris pH 6.8, 10% glycerol, 2% SDS and 2% bromophenol blue. The gel was then washed 3 times for 20 minutes each at room temperature with 2.5% Triton wash. The gel was incubated at 37ºC for the desired time in incubation buffer containing 50mm Tris pH 7.4, 150mM NaCl and 5mM CaCl₂. The gel was then stained with Coomassie and destained to resolve the digested bands.

**RNA isolation and real time RT PCR**
Hearts were excised and rinsed in diethyl pyrocarbonate (DEPC) -treated PBS and quickly frozen in liquid nitrogen. Ventricle tissue was homogenized in Trizol reagent (Invitrogen) to isolate RNA, and cDNA synthesis was carried out following the Superscript II cDNA synthesis kit (Invitrogen). Real time RT PCR template detection was carried out by SYBRgreen (Applied Biosystems) and read using the Applied Biosystems 7900HT machine. Data was normalized to GAPDH internal control and expressed as fold increase or decrease from control samples.

**Echocardiography of the mouse heart**
Trans thoracic echocardiography was done as previously described. In brief, mice were anaesthetized with 2% isoflurane, shaved in the pericardial region and then brought to a semi-conscious state with 0.6% isoflurane. Mice were maintained a homeostatic conditions with the use of a heating pad and low isoflurane with a target heart rate between 450 to 650 beats per minute (bpm). Imaging was recorded on the Sequoia C256 ultrasound machine (15L8 transducer, Acuson). Mice were placed in a lateral decubitus position and both the M-mode view at the mid papillary level and the parasternal short axis view to obtain Doppler recordings in the 2 dimensional plane were preformed for visualization and assessment of left ventricular systolic function. Three independent views were obtained for each position, and analyses of 3 consecutive cardiac contractions were averaged using the Sequoia platform. Standard parameters of cardiac structure and function were assessed.

**Cardiac Catheterization of the mouse heart**
Cardiac Catheterization was done as previously described. In brief, mice for the various time points were anesthetized with inhaled isoflurane (3% induction, 1% maintenance) placed in a supine position. A 1.4 French pressure transducer catheter (Millar Instruments) was inserted into the right common carotid artery. Retrograde advancement of the catheter into the ascending aorta and subsequently through the aortic valve into the left ventricle allowed for the collection of three
independent recordings of aortic and left ventricle pressures. Measurements were recorded at a stable internal temperature of 37°C with a target heart rate above 350 bpm. Both aortic and left ventricle parameters were recorded.

**Transverse Aortic Constriction in the mouse heart**

To analyze the effect of biomechanical stress on the remodeling processes of the left ventricle in the various genetic models, a minimally invasive transverse aortic constriction procedure was done as previously described. Briefly, 25-30g male mice were anesthetized (3% isoflurane) and intubated. Once placed on a respirator and held at a stable internal temperature of 37°C, a small incision above the suprasternal notch was made to expose the aortic arch. A 6.0mm silk suture was positioned under the aorta with a blunted 27 gauge needle to set the diameter of the ligation. Sham animals underwent every aspect of the surgery with the exception of the ligation procedure. The animal was then sutured closed and allowed to recover in a heated environment.

**Dissociation of Murine Cardiac Myocytes**

Adult mice (~12 weeks) were used for dissociated myocyte studies as previously described. Briefly, mice were euthanized by cervical dislocation and the heart was rapidly removed and placed in chilled calcium free Tyrodes solution (137mM NaCl, 5.4mM KCl, 10mM HEPES, 0.5mM NaH2PO4 7H2O, 1.0mM MgCl2 6H2O and 10mM Glucose, pH 7.4(NaOH)). The aorta was then cannulated and perfused with calcium free Tyrodes solution at 37°C. Collagenase containing Tyrodes solution was then perfused (1 mg/ml, CLS2 Worthington’s) for 10-12 minutes at 37°C. The ventricular free wall was dissected free and myocytes were mechanically dispersed in high K + containing KB solution (100mM K-glutamate, 10mM K-aspartate, 2.5mM KCl, 20mM Glucose, 10mM KH2PO4, 2mM MgSO4 7H2O, 20mM Taurine, 5mM Creatine, 0.5mM EGTA, 5mM HEPES and 0.1% albumin, pH 7.2 (KOH)). Myocytes were used within 6 hours of being isolated.

**Single Myocyte Dimensions**

Using the 5x objective, fields of plated cardiomyocytes were photographed, and length and width measurements were recorded, after calibration with micrometer, using GNU image manipulation program.

**Single Myocyte Contractility**

Freshly isolated myocytes were plated on glass perfusion plate and allowed to settle for 2 minutes. Calcium containing Tyrodes (1 mM CaCl), heated to 32-4°C, was perfused for 20 minutes to ensure proper equilibration of calcium and temperature. Cells were then whole field stimulated by platinum electrodes (5-7volts, 1 Hz, 5 ms duration). Cells were allowed to equilibrate (~5 minutes) to ensure stable contraction and myocyte sarcomere length measurements were captured using the high speed video length detection (HSVL) program (Aurora Scientific, Canada). Only stable cells with no spontaneous contractions were selected for analysis. Percent Sarcomere length shortening was measured as the change in diastolic and systolic sarcomere length and normalized to the diastolic sarcomere length.

**Single Myocyte Calcium Transients**

Freshly isolated myocytes were incubated with Indo AM calcium indicator dye (Molecular Probes, final concentration 1 μM with 0.45% Pluronic Acid) for 5 minutes at room temperature. Cells were plated on a glass perfusion dish and perfused with calcium containing Tyrodes solution (1mM CaCl, 32°C) for at least 20 minutes. Cells were whole field stimulated with platinum electrodes (5-7 volts, 1 Hz, 5ms duration). The selected cell was allowed to equilibrate, and calcium transients were recorded and captured with the Felix software program. At 5 minutes, single myocyte contractility was also recorded using the HSVL program. Only cells
with stable contraction, with no spontaneous contraction, were selected for analysis. Amplitude of the calcium transient was expressed as a ratio of the fluorescence emissions (405/485nm) corrected for background.

**Single Myocyte Skinned Force- Calcium Measurements**

These experiments were done as previously described. Briefly, mice were euthanized by cervical dislocation and the heart was rapidly excised and placed in ice cold PBS solution. After excess blood was removed, ~10 mg pieces of left ventricle free wall were dissected and quickly placed in liquid nitrogen. The frozen biopsies were then kept at –80°C until they were used for mechanical isolation of single myocytes. To isolate the single myocytes, the sample was placed in relaxing solution (5.55 mM Na₂ATP, 7.11mM MgCl₂, 2.0mM EGTA, 108.01mM KCl, 8.91 mM KOH and 10.0mM Imidazol, pH 7.0) with the addition of protease inhibitor cocktail (Sigma), 10mM DTT and 0.3% Triton. On ice, the sample was homogenized, centrifugation and resuspended in relaxing solution.

Once the myocytes were isolated, a small aliquot was placed on a microscope mounting cover slip, and myocytes were selected based on size and striation uniformity. Selected myocytes were attached to a 5mN force transducer arm (Aurora Scientific, Canada) and a motor server arm by silicon adhesive (Dow Corning, ML,USA). Once the adhesive had cured, the myocyte was moved to an adjacent well containing relaxing solution (5.95mM Na₂ATP, 6.41mM MgCl₂, 10.0mM EGTA, 100.0 mM BES, 10.0mM creatine phosphate, 50.25mM potassium prop, protease inhibitor cocktail (Sigma) and 10mM DTT, pH 7.0), and the sarcomere length of the myocyte preparation was set to 2.25 μm using a spatial Fourier transform, whereby the peak power spectrum corresponded to a mean sarcomere length.

The isolated skinned myocyte alternated between relaxing solution wells and activation solution (5.95mM Na₂ATP, 6.20mM MgCl₂, 10.0mM Ca²⁺EGTA, 100.0mM BES, 10.0mM creatine phosphate and 29.98mM potassium prop, protease inhibitor cocktail (Sigma) and 10mM DTT, pH 7.0) with incremental calcium concentrations at 15°C. For each calcium concentration, the preparation was released to slack after peak isometric tension development to obtain a baseline before switching to the relaxing solution. The difference in peak tension and the baseline tension is the total force developed for the given calcium concentration. The data was then fitted using the equation:

\[ P = \frac{[Ca^{2+}]nH}{K_nH + [Ca^{2+}]K_nH} \]

Whereby \( P \) = tension, \( K \) = calcium concentration required for half-maximal activation, and \( nH \) is the Hill coefficient for cooperativity of the myofilaments. Data was analyzed as mean ± SEM.

**Dissection of mouse papillary and trabeculae muscles**

Force length experiments were done as previously described. Briefly, adult mice (10-12wks) were euthanized by cervical dislocation. The hearts were rapidly excised, cannulated and perfused with a cardioplegic Krebs solution (112.5mM NaCl, 4.7mM KCl, 1.2mM MgCl₂, 1.2mM KH₂PO₄, 25mM NaHCO₃, 12mM Glucose, containing1mM CaCl₂ and 15mM KCl, pH 7.4). The right ventricle was opened and papillary muscles or trabeculae were dissected by cutting a portion of the valve leaflet above and a small portion of the ventricular wall at the base of the muscle. Only muscles preparations free from the wall and cylindrical in shape were selected, while muscle preparations with spontaneous electrical activity were discarded. Muscles were measured manually using a stereoscopic microscope for their thickness and width to allow for calculation of cross sectional area to normalize the force measurements.

The muscle was then placed in the perfusion bath and mounted on the force transducer (5mN, Aurora Scientific, Canada). Muscles preparations were mounted horizontally with the ventricular wall piece mounted on a small wire basket connected to the force transducer by a glass tube, while the valve leaflet was connected to a hook on the motor server arm allowing for
incremental length changes. The perfusion set up allowed for constant perfusion rate and thermostatic conditions at 27°C. The preparation was field-stimulated by platinum electrodes allowing for pulse durations of 2ms, at 1 Hz with voltages a minimum of 20% over threshold. The preparation was gradually stretched to 90% of its maximal value and allowed to equilibrate for a minimum of 30 minutes. The standard Krebs salt solution (112.5mM NaCl, 4.7mM KCl, 1.2mM MgCl₂, 1.2mM KH₂PO₄, 25mM NaHCO₃, 12mM Glucose, 1.5mM CaCl₂) was used and gassed with 95% O₂ and 5% CO₂ to a pH of 7.4.

After the equilibration period of 30-45 min, the force length relation was determined. Muscle preparations were put to slack length whereby no force was detected and the baseline for the force transducer was set. Sarcomere length was measured throughout the protocol using a high speed video length (HSVL) detection system from Aurora Scientific (papillary muscles). To confirm the accuracy of the readings, a small set of trabeculae were analyzed by laser diffraction. Micromanipulators were used to increase the length of the muscle over the protocol. Small changes in length were made and muscle was allowed to equilibrate for 1 min between length changes. Length was measurements were recorded from slack to maximal developed force, twice in each muscle. Data was presented as mN/mm² and passive tension data was fitted to the exponential equation (\(y = e^{(x-x_0)/c} + a\)), whereby \(y\) is the passive tension, \(x_0\) is the average resting passive tension, \(x\) is the measured sarcomere length, \(a\) is the measurement offset, and \(c\) is the compliance constant of the myocardium. Significance was determined by the F statistical method, applied to the normalized experimental and control linear models. Developed tension (the difference between systolic and passive tension) was compared at various sarcomere lengths across the force-length curve.
Online Figure I. ShcA CKO mice undergo eccentric remodeling with preserved cyto-ultrastructure and minimal fibrosis.

(A) Gravimetric data showing increased heart weight (HW) to body weight (BW) in ShcA CKO mice compared to controls (n=5). Trend of increasing lung weight (LungW) to body weight ratios in ShcA CKO mice compared to controls (n=5). Values are means ±SEM with *p<0.05 from littermate controls. (B) Transmission electron microscope showing preserved ultrastructure and normal mitochondrial morphology at 1 year of age in ShcA CKO and littermate controls. Scale=2 μm. (C) Representative histology of masson trichome stained transverse sections showing minimal interstitial fibrosis with peri-venous fibrosis suggesting cardiac congestion in ShcA CKO hearts compared to littermate controls at 1 year of age. Magnification for upper panel 6.25x, lower panel 20x.
Online Figure II. Alterations in Troponin I phosphorylation at Serine 23/24 does not contribute to the enhanced calcium sensitivity in ShcA CKO myofilaments.

Representative western blot of Troponin I (TnI) phosphorylation at Serines 23/24 in ShcA CKO and control lysates (3 hearts were independently assessed from each group).
Online Figure III. Schematic of ShcA allele strategy to generate ventricle cardiomyocyte specific ShcA KI mutants.

(A) Breeding strategy to generate mice containing one mutant ShcA δKI allele and one ShcA floxed allele. In the presence of Mlc2v Cre, the floxed allele becomes functionally null, unmasking the properties of the individual mutant domain ShcA δKI allele. (B) Individual ShcA δKI alleles are outlined denoting the point mutations. (C) Representative western blot analysis showing reduction in protein levels of the ShcA floxed allele (3x flag) and expression of the individual mutant ShcA δKI allele (1x flag) only in the presence of Mlc2v Cre in SH2 CKI hearts compared to littermate controls.
Online Table I. Echocardiography data for timecourse studies

<table>
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<tr>
<th>12 week</th>
<th>Heart Rate (bpm)</th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>% FS</th>
<th>VCF (circ/sec)</th>
<th>Anterior Wall (mm)</th>
<th>Posterior Wall (mm)</th>
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<td>ShcA CON 12</td>
<td>500.1±1.24</td>
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<td>6 Months</td>
<td>Heart Rate (bpm)</td>
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<td>LVESD (mm)</td>
<td>% FS</td>
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LVEDD, left ventricle end diastolic dimension; LVESD, left ventricle end systolic dimension; %FS, % fractional shortening; VCF, velocity of circumferential shortening. * p<0.05 from littermate control for given time point.
Online Table II. Echocardiography data of TAC study for acute model of ShcA excision.

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<th>Parameter</th>
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<td>ShcA MCON</td>
<td>ShcA MCKO</td>
<td>ShcA MCON</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>446.7±14.56</td>
<td>473.8±11.09</td>
<td>467.8±7.07</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.06±0.09</td>
<td>3.83±0.05</td>
<td>4.16±0.11</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.29±0.09</td>
<td>2.10±0.08</td>
<td>2.67±0.12</td>
</tr>
<tr>
<td>% FS</td>
<td>43.75±1.53</td>
<td>45.33±1.67</td>
<td>36.07±1.67</td>
</tr>
<tr>
<td>VCF (circ/sec)</td>
<td>0.809±0.103</td>
<td>0.819±0.039</td>
<td>0.731±0.028</td>
</tr>
<tr>
<td>Ant wall (mm)</td>
<td>0.69±0.02</td>
<td>0.73±0.02</td>
<td>0.70±0.02</td>
</tr>
<tr>
<td>Post wall (mm)</td>
<td>0.63±0.03</td>
<td>0.61±0.02</td>
<td>0.69±0.03</td>
</tr>
</tbody>
</table>

P.I., post injection; LVEDD, left ventricular end diastolic dimension; LVESD, left end systolic dimension; %FS, % fractional shortening; VCF, velocity of circumferential shortening; Ant, anterior; post, posterior. # No significant difference was noted at 7 days post injection between ShcA $^{wt/flx}$ MerCre/Mer $^{+/wt}$ and ShcA $^{flx/flx}$ MerCreMer $^{wt/wt}$. ^ p<0.05 compared to same genotype sham; * p<0.05 compared to littermate control value for the given time point.
### Online Table III. Echocardiography and cardiac catheterization data for transverse aortic constriction studies

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate (bpm)</th>
<th>LVEDD (mm)</th>
<th>% FS</th>
<th>Anterior Wall (mm)</th>
<th>Heart Rate (bpm)</th>
<th>LVESP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>dP/dT max (mmHg/s)</th>
<th>dP/dt min (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12 week Sham</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShcA CON</td>
<td>500.1±1.24</td>
<td>4.01±0.05</td>
<td>45.4±0.18</td>
<td>0.71±0.03</td>
<td>452.1±16.2</td>
<td>105.7±3.44</td>
<td>7.49±2.11</td>
<td>8434.85±581</td>
<td>-7397.86±592</td>
</tr>
<tr>
<td>ShcA CKO</td>
<td>517.2±1.46</td>
<td>4.34±0.01*</td>
<td>39.5±0.24</td>
<td>0.67±0.04</td>
<td>465.0±12.4</td>
<td>101.6±4.40</td>
<td>5.11±1.69</td>
<td>5877.24±520*</td>
<td>-7057.63±588*</td>
</tr>
<tr>
<td>3F CON</td>
<td>517.1±11.0</td>
<td>4.00±0.06</td>
<td>46.8±0.99</td>
<td>0.79±0.04</td>
<td>446.2±25.7</td>
<td>119.7±1.22</td>
<td>10.95±1.22</td>
<td>9314.6±289</td>
<td>-8538±494</td>
</tr>
<tr>
<td>3F CKI</td>
<td>501.1±10.9</td>
<td>3.87±0.06</td>
<td>44.0±1.0</td>
<td>0.79±0.02</td>
<td>409.8±11.5</td>
<td>98.3±6.70</td>
<td>8.59±1.52</td>
<td>5769.2±535*</td>
<td>-5797.5±569*</td>
</tr>
<tr>
<td>SH2 CON</td>
<td>512.1±9.59</td>
<td>3.94±0.07</td>
<td>46.3±1.24</td>
<td>0.74±0.02</td>
<td>495.0±17.1</td>
<td>98.47±4.94</td>
<td>12.52±1.10</td>
<td>7896.4±534</td>
<td>-7161.4±618</td>
</tr>
<tr>
<td>SH2 CKI</td>
<td>502.2±11.7</td>
<td>3.78±0.04</td>
<td>45.01±2.14</td>
<td>0.74±0.03</td>
<td>469.0±26.9</td>
<td>110.7±3.35</td>
<td>9.82±1.85</td>
<td>9391.0±719</td>
<td>-8511.8±385</td>
</tr>
<tr>
<td><strong>4 week TAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ShcA CON</td>
<td>491.3±10.8</td>
<td>4.56±0.01^</td>
<td>35.2±2.59</td>
<td>91.9±0.04^</td>
<td>460.5±10.2</td>
<td>136.7±5.8^</td>
<td>21.2±1.1^</td>
<td>6664.90±354^</td>
<td>-5977.70±347^</td>
</tr>
<tr>
<td>ShcA CKO</td>
<td>525.5±6.25</td>
<td>5.80±0.02^</td>
<td>12.1±1.5^</td>
<td>63.7±0.05^</td>
<td>386.2±24.8^</td>
<td>91.5±3.0^</td>
<td>21.1±0.89^</td>
<td>3371.80±250^</td>
<td>-3041.60±191^</td>
</tr>
<tr>
<td>3F CON</td>
<td>502.9±10.3</td>
<td>4.37±0.01^</td>
<td>35.9±1.62</td>
<td>91.6±0.02^</td>
<td>468.6±16.8</td>
<td>156.6±13.27^</td>
<td>16.8±3.41</td>
<td>7418.3±692^</td>
<td>-7909.1±749</td>
</tr>
<tr>
<td>3F CKI</td>
<td>498.7±11.2</td>
<td>5.0±0±0.01^</td>
<td>18.1±1.71^</td>
<td>73.4±0.02^</td>
<td>466.1±29.8</td>
<td>129.8±11.5</td>
<td>29.8±3.79^</td>
<td>5545.8±806</td>
<td>-5124.3±562*</td>
</tr>
<tr>
<td>SH2 CON</td>
<td>530.4±11.7</td>
<td>4.35±0.01^</td>
<td>38.3±1.61</td>
<td>94.0±0.03^</td>
<td>502.3±15.9</td>
<td>172.8±14.8^</td>
<td>19.9±4.3</td>
<td>8921.1±1502</td>
<td>-8510.0±1216</td>
</tr>
<tr>
<td>SH2 CKI</td>
<td>487.3±9.22</td>
<td>4.41±0.01^</td>
<td>29.5±1.49^</td>
<td>85.0±0.02^</td>
<td>545.6±10.2</td>
<td>135.9±12.7^</td>
<td>16.8±2.6</td>
<td>7292.7±575^</td>
<td>-7194.7±829</td>
</tr>
</tbody>
</table>

LVEDD, left ventricular end diastolic dimension; %FS, % fractional shortening; LVESP, left ventricle end systolic pressure; LVEDP, left ventricle end diastolic pressure; dP/dT max, first derivative of maximal pressure development in left ventricle; dP/dT min, first derivative of minimal pressure in left ventricle. ^ p<0.05 from same genotype sham; * p<0.05 from littermate control for the given time point.
Online Table IV. Gravimetric data for ShcA CKO and control mice after TAC.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th></th>
<th>4 wk TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Con</td>
<td>ShcA CKO</td>
</tr>
<tr>
<td>BW (g)</td>
<td>30.69±0.06</td>
<td>29.80±0.79</td>
<td>29.96±0.90</td>
</tr>
<tr>
<td>Left Ventricle (mg)</td>
<td>100.0±4.1</td>
<td>103.2±10.1</td>
<td>170.0±6.3</td>
</tr>
<tr>
<td>LVW/TL (mg/mm)</td>
<td>0.611±0.025</td>
<td>0.633±0.056</td>
<td>1.028±0.035</td>
</tr>
<tr>
<td>LungW/TL (mg/mm)</td>
<td>1.14±0.032</td>
<td>1.09±0.077</td>
<td>1.56±0.115</td>
</tr>
</tbody>
</table>

BW, body weight; LVW/TL, left ventricle weight/tibial length; LungW/TL, lung weight/tibial length. ^ p<0.5 compared to same genotype sham, * p<0.05 compared to littermate control for given timepoint.
Online Table V. Single myocyte assays for ShcA mutant domain KI mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pooled</th>
<th>PTB CKI</th>
<th>3F CKI</th>
<th>SH2 CKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sarcomere shortening</td>
<td>7.17±0.24</td>
<td>9.04±0.41*</td>
<td>9.44±0.55*</td>
<td>6.72±0.26</td>
</tr>
<tr>
<td>dSL/dT max</td>
<td>2.85±0.18</td>
<td>4.26±0.25*</td>
<td>3.65±0.27*</td>
<td>2.93±0.25</td>
</tr>
<tr>
<td>dSL/dT min</td>
<td>3.89±0.20</td>
<td>5.53±0.34*</td>
<td>4.96±0.40*</td>
<td>3.92±0.22</td>
</tr>
<tr>
<td>TR50</td>
<td>0.039±0.002</td>
<td>0.035±0.002</td>
<td>0.038±0.002</td>
<td>0.034±0.003</td>
</tr>
<tr>
<td>TR80</td>
<td>0.063±0.003</td>
<td>0.056±0.003</td>
<td>0.061±0.003</td>
<td>0.055±0.004</td>
</tr>
<tr>
<td>Amplitude (F405/485nm)</td>
<td>0.316±0.011</td>
<td>0.329±0.02</td>
<td>0.281±0.014</td>
<td>0.307±0.02</td>
</tr>
</tbody>
</table>

Contractility and transient assays for ShcA mutant domain KI mice (n=3, minimum 15 cells). Pooled KI controls (n=9, minimum 45 cells). dSL/dT max, maximum first derivative of sarcomere shortening; dSL/dT min, minimum first derivative of sarcomere shortening; TR50, time to 50% relaxation; TR80, time to 80% relaxation. F, Fluorescence of calcium transient. * p<0.05 to littermate control for given time point.
References


