

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;108:00-00.)

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,^{1,2} whereas myocyte-specific deletion of focal adhesion kinase, a nonreceptor TK, results in eccentric remodeling in response to hemodynamic overload³ and aging.⁴ 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.⁵ The dissection of downstream signaling

networks activated by TKs in the adult heart is therefore of considerable therapeutic interest.⁶

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.^{7,8} 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-

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Non-standard Abbreviations and Acronyms

CH1	collagen homology 1
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
HF	heart failure
HW/BW	heart weight/body weight
KI	knock in
LVEDD	left ventricle end diastolic dimension
MAPK	mitogen-activated protein kinase
MHC	myosin heavy chain
MMP	matrix metalloproteinase
PI3K	phosphatidylinositol 3'-kinase
PTB	phosphotyrosine binding
Shc	Src homology domain-containing protein
TK	tyrosine kinase
SH2	Src homology 2
TAC	transverse aortic constriction
TK	tyrosine kinase
VEGF	vascular endothelial growth factor

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

evaluate 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-dependant 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 at <http://circres.ahajournals.org>.

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)¹⁵ and α MHC-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.

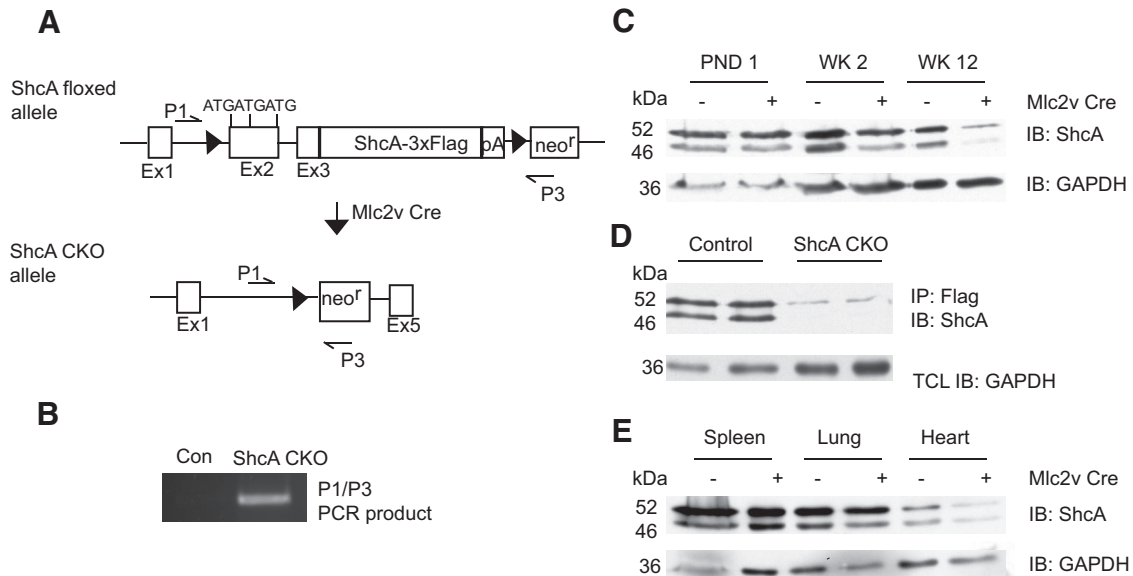


Figure 1. Ventricular cardiomyocyte-specific deletion of ShcA. **A**, Schematics of ShcA floxed and deleted (CKO) alleles. **B**, Representative PCR of genomic DNA showing recombination of the floxed allele in the heart of ShcA CKO mice only. **C**, Time line of ShcA excision in the heart as seen by representative western blot of protein levels at the time points indicated. PND indicates postnatal day; WK, week. **D**, Lysates of partially purified cardiomyocytes from control and ShcA CKO mice ($n=2$). **E**, Mlc2v Cre is specific to cardiomyocytes as lysates prepared from spleen and lung showed no reduction in ShcA protein levels in the presence or absence of Mlc2v Cre. For all Western blots, a minimum of 2 hearts were independently assessed from each group.

Preparation of Tamoxifen

Injected tamoxifen was prepared as previous 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 \pm SEM. Means were compared by 2 tailed Student *t* test or 1-way ANOVA. $P \leq 0.05$ 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 ($ShcA^{flx}$) allele¹⁰ with mice possessing the myosin light chain (Mlc)2v Cre KI ($Mlc2v^{KI/wt}$) allele¹⁵ (Figure 1A and 1B). Mating of $ShcA^{flx/flx} Mlc2v^{wt/wt}$ and $ShcA^{flx/wt} Mlc2v^{KI/wt}$ mice yielded the expected 1:1:1:1 Mendelian ratio. As expected, $ShcA^{flx/flx} Mlc2v^{KI/wt}$ mice (designated ShcA CKO) showed selective deletion of ShcA in ventricular cardiomyocytes.¹⁵ $ShcA^{flx/flx} Mlc2v^{wt/wt}$ mice were used as littermate controls (Online Table I; the Table) and were indistinguishable from $ShcA^{flx/wt} Mlc2v^{KI/wt}$ mice ($n \geq 3$; 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: $7.18 \pm 0.80\%$) 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 $ShcA^{-/-}$ mice die at embryonic day 11.5,¹² whereas mice with germline ablation of the p66 ShcA

Table. Single-Myocyte Assays

Assay and Parameter	Control	ShcA CKO
Contractility		
Sarcomere shortening (%)	6.90 ± 0.58	$8.85 \pm 0.30^*$
dSL/dT max ($\mu\text{m}/\text{sec}$)	3.57 ± 0.16	$4.93 \pm 0.34^*$
dSL/dT min ($\mu\text{m}/\text{sec}$)	2.65 ± 0.22	$3.73 \pm 0.31^*$
TR ₅₀ (sec)	0.032 ± 0.005	0.033 ± 0.002
TR ₈₀ (sec)	0.052 ± 0.006	0.064 ± 0.005
Ca²⁺ transients		
Amplitude (F405/485 nm)	0.323 ± 0.018	0.335 ± 0.019
Force Ca²⁺ curve		
F _{max} (mN/mm ²)	18.04 ± 0.55	19.81 ± 1.015
EC ₅₀ ($\mu\text{moles}/\text{L}$)	2.46 ± 0.08	$2.18 \pm 0.13^*$
Hill coefficient	3.57 ± 0.34	3.74 ± 0.64

Contractility and transient assays have a minimum of 3 hearts per group with a minimum of 15 cells. Force-Ca²⁺ 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; TR₅₀, time to 50% relaxation; TR₈₀, time to 80% relaxation; F, fluorescence of calcium transient; F_{max}, maximal force; EC₅₀, Ca²⁺ concentration at 50% maximal force. * $P < 0.05$ from littermate controls at given time point.

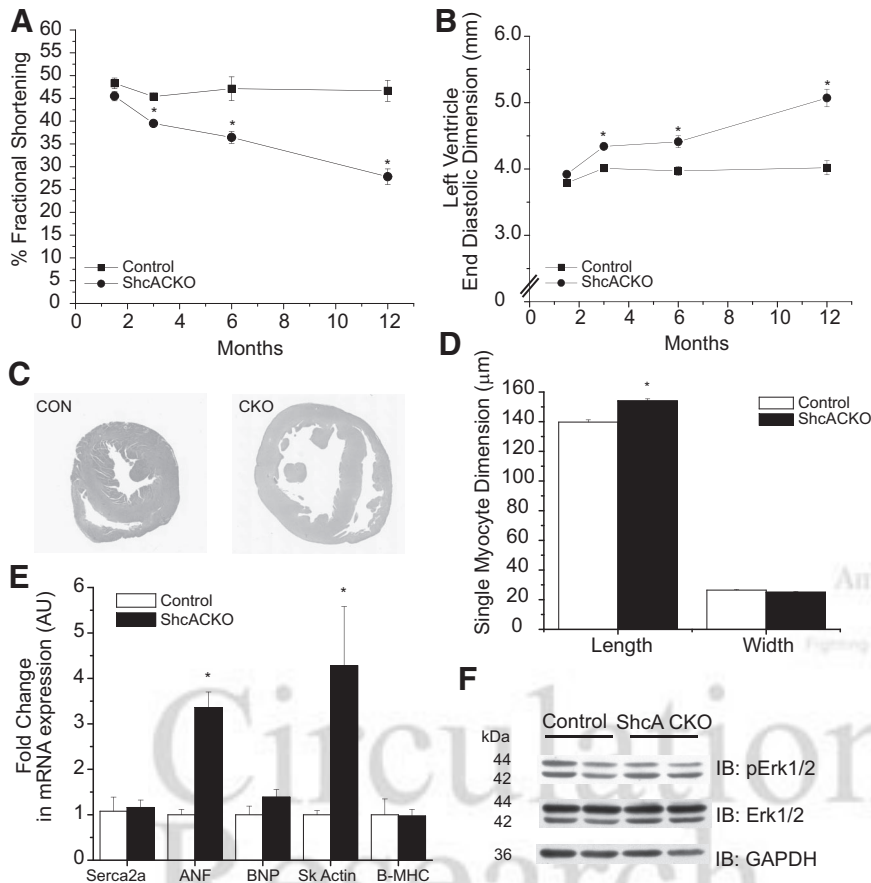


Figure 2. ShcA is required for the maintenance of cardiac function. **A**, Echocardiography time course of cardiac contractility as measured by percentage fractional shortening showing a decline in function of ShcA CKO hearts ($n \geq 7$) over the course of 1 year compared with controls ($n \geq 5$). **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 ($n \geq 7$) and controls ($n \geq 5$). See Online Table I for complete data. **C**, Representative histology of transverse heart sections stained with hematoxylin/eosin at 1 year of age. Magnification, $\times 6.25$. **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 \pm SEM with $*P < 0.05$ compared with littermate control values for given time points.

isoform are long-lived.¹⁴ By contrast, although echocardiography data showed no differences in cardiac dimensions or fractional shortening (Online Table I) 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.¹⁷

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 accom-

panied 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 dilated 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,¹⁸ 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 to regulate the calcium sensitivity of contraction (Online Figure II).¹⁸

To ensure the enhanced contractility was not caused by secondary compensatory mechanisms, we used the tamoxifen inducible Cre transgenic mouse (MerCreMer) driven by the α MHC promoter to acutely excise ShcA in cardiomyocytes.¹⁶

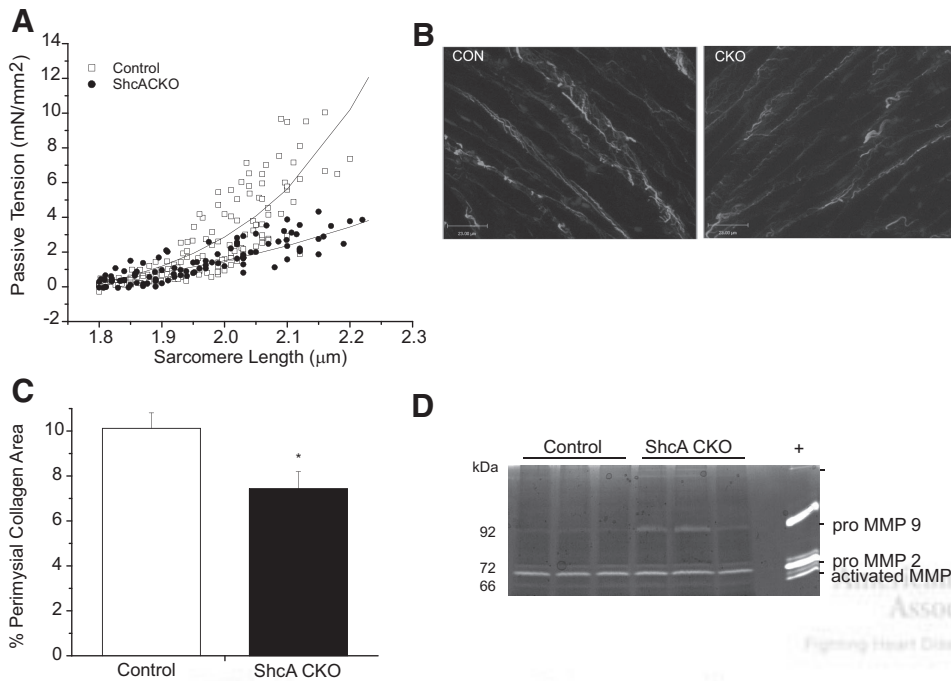


Figure 3. The loss of ShcA leads to deregulation of the ECM components in the heart. **A**, Papillary passive tension, expressed as mN/mm^2 , across increasing sarcomere lengths (μm) as described in Methods. A significant increase ($P < 0.001$) in ShcA CKO compliance ($c = 0.42$) was demonstrated compared with control ($c = 0.20$) ($n \geq 5$ 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, $\times 63$. **C**, Percentage perimysial collagen area was quantified from multiple images from each heart ($n = 3$). Values are means \pm 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.

ShcA MCKO ($\text{ShcA}^{flx/flx}$ $\text{MerCreMer}^{+/wt}$) and their littermate controls, ShcA MCON ($\text{ShcA}^{flx/flx}$ $\text{MerCreMer}^{wt/wt}$ and $\text{ShcA}^{flx/wt}$ $\text{MerCreMer}^{+/wt}$), 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 ($7.76 \pm 0.36\%$ versus $6.42 \pm 0.34\%$, 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.^{19,20} 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 conceiv-

able 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 ($n = 3$) was much less ($P < 0.001$) than ShcA CKO hearts, despite having indistinguishable reduction in fractional shortening ($36.1 \pm 1.36\%$) 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 ($4.44 \text{ mN}/\text{mm}^2$ at $2.10 \pm 0.003 \mu\text{m}$ for ShcA CKO and $6.43 \text{ mN}/\text{mm}^2$ at $2.10 \pm 0.004 \mu\text{m}$ 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;

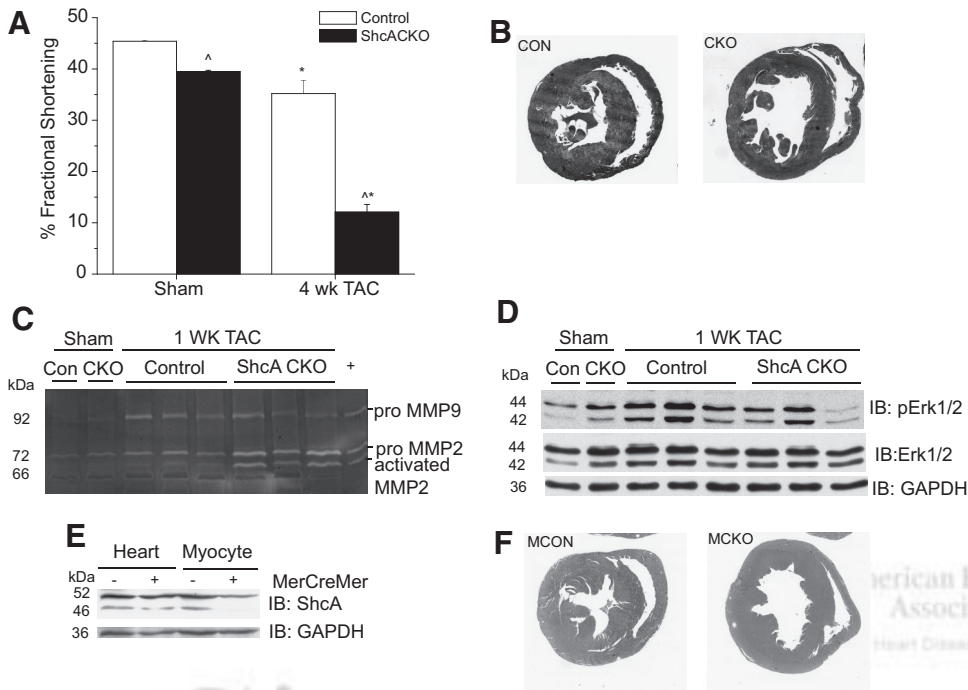


Figure 4. ShcA CKO mice undergo an eccentric remodeling response after TAC. **A**, Echocardiography data show a decline in ShcA CKO cardiac function after TAC, as demonstrated by percentage fractional shortening of ShcA CKO and control mice ($n \geq 5$). See Online Table III for complete echocardiography data. **B**, Representative histology of transverse heart sections stained with Masson trichrome of ShcA CKO and control hearts after TAC. Magnification, $\times 6.25$. **C**, Zymography of ShcA CKO and control hearts ($n=3$) after a 4-week TAC protocol. The positive control (+) sample was supernatant from mouse embryonic fibroblasts stimulated by concanavalin A for 24 hours. **D**, Western blot analysis of ShcA CKO and control hearts for pERK activation after TAC ($n=3$). **E**, Representative Western blot showing loss of ShcA protein in heart

and isolated cardiomyocytes in the presence of MerCreMer transgene driven by the α MHC 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, $\times 6.25$. See Online Table II for complete echocardiography study. For **A**, **C**, and **D**, values are means \pm SEM with $^{\wedge}P < 0.05$ compared with same genotype sham; $^{*}P < 0.05$ compared with littermate control values for the given time point. WK indicates week.

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 4B; Online Tables III and IV). Despite marked chamber enlargement and impaired cardiac function, ShcA CKO mice had a similar level of interstitial fibrosis compared with control animals after TAC (Figure 4B). These changes in ShcA CKO hearts were accompanied by elevation of MMP2 activity at 1 week TAC and subtle depressions in pERK signaling (Figure 4C and 4D).

To confirm these results, we subjected ShcA MCKO and their littermate controls to TAC, 7 days post tamoxifen injection ($n \geq 5$) (Figure 4E). After 4 weeks of TAC, ShcA MCKO showed dilated chamber dimensions with decreased systolic function compared with ShcA MCON mice (Figure 4F; Online Table II). Interestingly, anterior wall thickness (Online Table II) and HW/BW ratios (5.63 ± 0.35 for ShcA MCKO versus 4.82 ± 0.14 for controls ($P=0.07$); $n=5$) were similar between the 2 animal models. These results suggest that ShcA is necessary for preventing ventricular dilation.

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 (δ KI) restricted to cardiomyocytes¹⁰ as described in the Online Methods and Online Figure III.

Like the ShcA CKO mice, δ PTB 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 δ PTB 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 δ PTB 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 δ PTB 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 δ PTB CKI mice, at 6 months of age, δ SH2 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, δ SH2 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

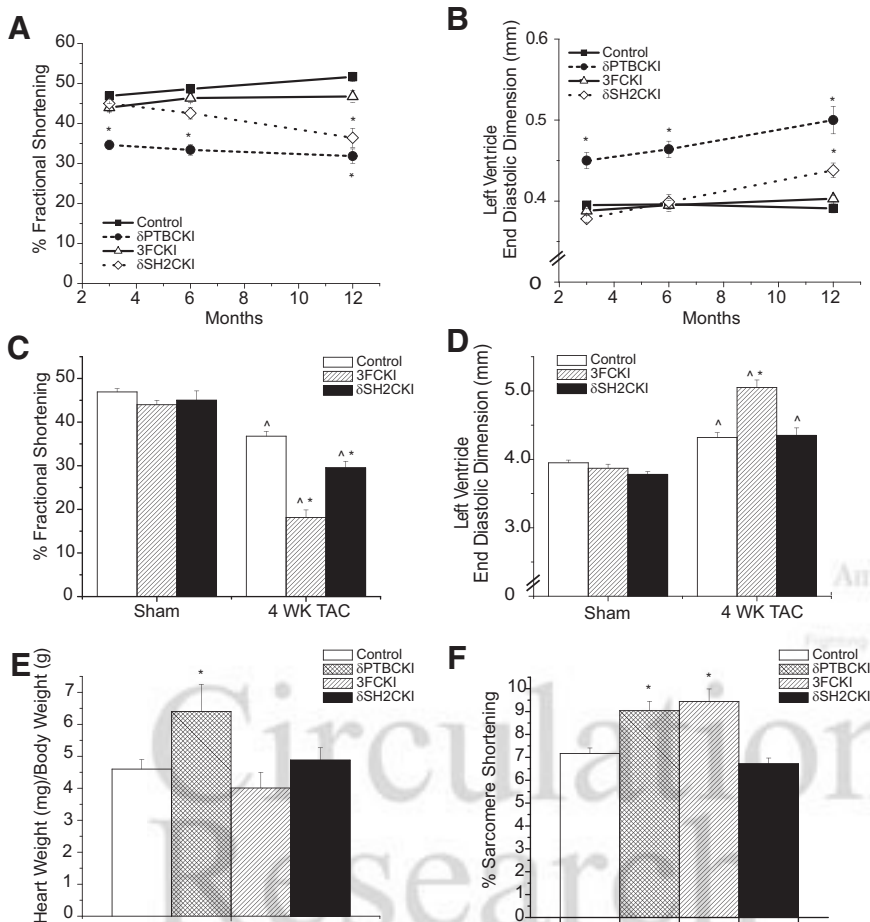


Figure 5. TK-ShcA signaling is required to maintain cardiac structure and function, whereas downstream signaling from the CH1 pTyr sites is necessary after hemodynamic overload. **A**, Echocardiography data showing percentage fractional shortening of the mutant ShcA allele series over the course of 1 year ($n \geq 5$). See Online Table I for complete study. **B**, Echocardiography data showing LVEDDs (mm) of the mutant ShcA allele series over the course of 1 year ($n \geq 5$). See Online Table I for complete study. **C**, Echocardiography data showing percentage fractional shortening of the mutant ShcA allele series after 4 weeks of TAC ($n \geq 5$). See Online Table III for complete study. **D**, Echocardiography data showing LVEDDs (mm) of the mutant ShcA allele series after 4 weeks of TAC ($n \geq 5$). See Online Table III for complete study. In **A** through **E**, individual KI controls were not significantly different and pooled for graphing purposes. Individual echocardiography data are found in Online Table III. **E**, Heart weight/body weight ratios for 1-year-old mice as indicated ($n > 4$). **F**, Contractility of isolated single myocytes ($n = 3$, > 15 cells for each group). Individual KI controls were pooled ($n = 9$, > 45 cells). Complete parameters found in Online Table V. For all panels, values are means \pm SEM. $\wedge P < 0.05$ compared with same genotype control shams; $*P < 0.05$ compared with littermate controls at given time points.

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 analysis has indicated that phosphorylation of tyrosines 239/240 and 313 is dispensable for embryonic heart development.¹⁰ Consistent with this finding, phospho-null 3F CKI mice have normal heart function and morphology at 1 year of age (Figure 5A, 5B, and 5E; Online Table I). However, 3F CKI mice subjected to TAC at 2 to 3 months rapidly advanced into HF, as demonstrated by dilated ventricle chambers coupled with reduced cardiac function (Figure 5C and 5D; Online Table III). These data suggest that the tyrosine phosphorylation sites in the CH1 region of ShcA are dispensable in maintaining normal heart function, but are required for mounting appropriate responses to biomechanical stress. Thus, our findings suggest that the maintenance of normal cardiac function does not require CH1-pTyr site dependant recruitment SH2 domain proteins such as the Grb2 adaptor.⁷

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 δ PTB CKI and 3F CKI myocytes were hypercontractile compared with their littermate controls independent of changes in calcium handling, whereas δ SH2 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 defects 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 pTyr sites in the CH1 region (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 mecha-

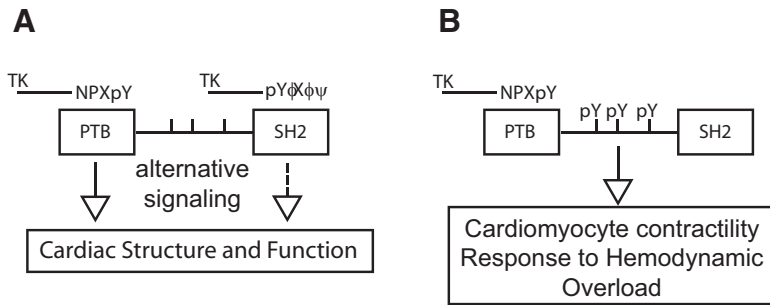


Figure 6. Model of ShcA signaling within the postnatal myocardium. **A**, ShcA uses the PTB domain to dock to activated TKs critical in maintaining cardiac structure and function, whereas the SH2 domain is required to augment cardiac function. ShcA uses an alternative mode of signaling to maintain cardiac function, because mutation of the CH1 pTyr sites did not elicit a dilated cardiomyopathy phenotype. **B**, ShcA pTyr signaling downstream of the CH1 region is required for appropriating signals after hemodynamic overload and signaling that regulates single-myocyte contractility.

nisms 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,⁷ all of which could potentially impact on the phosphorylation status of myofilaments.²¹ 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.²² However, just as importantly, blood flow change as a result of altered vascular function,²³ disruption of normal propagation of electric signals,²⁴ and uncoupling of force transmission from the myocyte along collagen struts¹⁹ 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,¹⁹ the early detection of mechanical changes in the passive tension in parallel with the excision of ShcA suggests disruption of pathways involved in myocyte/myocyte or myocyte/matrix coupling. Indeed, several matrix-associated TKs have docking sites for the PTB and SH2 domains of ShcA,⁷ so that ShcA, through reciprocal signaling between cellular compartments,^{10,11} could potentially affect mechanical coupling through adhesion interactions, secretion of multicellular proteins, or direct remodeling of the matrix.^{19,25} 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.²⁶

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 ErbB4⁷; in particular, loss of ErbB2 in cardiomyocytes produces a similar phenotype to ShcA in cardiac ablation studies.^{1,2} The loss of ShcA in other tissues also phenocopies conditional ErbB2 loss^{10,27} and supports the idea that ShcA is a preferential scaffold for ErbB2 signaling, especially because it contains multiple ShcA consensus binding motifs.^{28,29} Of clinical relevance, trastuzumab, an effective TK inhibitor of ErbB2–ErbB3 heterodimers, causes HF in a subset of patients,³⁰ suggesting that ShcA could function in a pathway that is regulated by trastuzumab, potentially influencing matrix/myocyte interactions. The clinical utility of Neuregulin,³¹ the ligand of heterodimers containing ErbB2,³² 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,^{7,33} 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.³⁴ Covalent modification of these tyrosines has shown to be critical in Ras mediated MAPK activation and PI3K activation through ShcA–Grb2 interactions.⁷ 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 signaling mechanisms that may be important in cardiac function include adaptin 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.^{7,35,36} In addition, IQGAP links ShcA to the actin cytoskeleton through a noncanonical interaction with the ShcA PTB domain.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.

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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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