Serine 105 phosphorylation of transcription factor GATA4 is necessary for stress-induced cardiac hypertrophy in vivo

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Cardiac hypertrophy is an adaptive growth process that occurs in response to stress stimulation or injury wherein multiple signal transduction pathways are induced, culminating in transcription factor activation and the reprogramming of gene expression. GATA4 is a critical transcription factor in the heart that is known to induce/regulate the hypertrophic program, in part, by receiving signals from MAPKs. Here we generated knock-in mice in which a known MAPK phosphorylation site at serine 105 (S105) in *Gata4* that augments activity was mutated to alanine. Homozygous *Gata4-S105A* mutant mice were viable as adults, although they showed a compromised stress response of the myocardium. For example, cardiac hypertrophy in response to phenylephrine agonist infusion for 2 wk was largely blunted in *Gata4-S105A* mice, as was the hypertrophic response to pressure overload at 1 and 2 wk of applied stimulation. *Gata4-S105A* mice were also more susceptible to heart failure and cardiac dilation after 2 wk of pressure overload. With respect to the upstream pathway, hearts from *Gata4-S105A* mice did not efficiently hypertrophy following direct ERK1/2 activation using an activated MEK1 transgene in vivo. Mechanistically, GATA4 mutant protein from these hearts failed to show enhanced DNA binding in response to hypertrophic stimulation. Moreover, hearts from *Gata4-S105A* mice had significant changes in the expression of hypertrophy-inducible, fetal, and remodeling-related genes.

heart failure | gene regulation | fibrosis

The heart can undergo hypertrophic growth in response to injury and various stressors associated with neurohumoral activation, increases in wall stress, and changes in volume load (1). The heart responds to these stressors by activating signaling and transcriptional pathways that ultimately result in the activation of progrowth and adaptive genes, as well as genes actively transcribed during embryonic development; this is known as the fetal gene program (2). Genes such as atrial natriuretic factor (*Nppa*), brain natriuretic peptide (*Nppb*), and skeletal α -actin (*Acta1*) become re-expressed, and there is a concomitant up-regulation of the fetal β -myosin heavy chain (*Myh7*) gene isoform. Activation of this gene program and hypertrophy of the adult myocardium, although initially adaptive, generally results in declining function and heart failure over protracted time periods (1). Several kinases, such as MAPK, protein kinases A and C, as well as the calcium-activated phosphatase calcineurin, become activated and change the phosphorylation status of downstream transcriptional regulators. For example, transcription factors such as myocyte enhancer factor 2, nuclear factor of activated T cells, and GATA4 can be directly phosphorylated or dephosphorylated to facilitate inducible hypertrophic gene expression (1–6).

The zinc finger-containing family of GATA transcription factors is divided into two groups. GATA1/2/3 are highly expressed in hematopoietic cell lineages, in which they control red cell and lymphocyte development, whereas GATA4/5/6 are expressed in

endoderm- and mesoderm-derived tissues, such as gut, gonads, heart, lungs, and liver (7). GATA factors are highly conserved, and they all bind the consensus sequence (A/T)GATA(A/G) through two zinc fingers that comprise the DNA-binding domain. GATA4 and GATA6 are both expressed in the adult heart and up-regulated in response to stress, such as pressure overload (5, 6, 8, 9). However, GATA4 appears to be more centrally involved in regulating the cardiac hypertrophic gene program through a mechanism involving phosphorylation of serine 105 (S105), which activates DNA binding and transcriptional potency (3–6, 10, 11). GATA4 is phosphorylated at S105 by ERK1/2 and p38 MAPK in cultured cardiomyocytes, although the importance of this signal transduction circuit in mediating cardiac growth in vivo is not known (3, 5). Here we demonstrate that phosphorylation of GATA4 at S105 is critical for a productive cardiac hypertrophic response to stress stimulation in adult mice.

Results

Generation of *Gata4-S105A* Knock-in Mice. We first confirmed that GATA4 was indeed phosphorylated at S105 in response to acute pressure overload stimulation in the mouse heart by using a phospho-specific antibody (Fig. 1A). We next generated knock-in mice by using standard gene targeting procedures in ES cells whereby S105 in *Gata4* was mutated to alanine (Fig. 1B). Genomic DNA from a WT mouse was extracted, sequenced, and compared against DNA from a germline heterozygous and homozygous targeted *Gata4-S105A* mouse, which showed the predicted changes in nucleotide, and consequential protein sequence (Fig. 1C). Homozygous mutant mice (hereafter referred to as S105A mut) were born alive at Mendelian frequencies and were overtly normal. Importantly, the site-directed mutation at S105 and the remaining Frt site within intron 2 did not affect expression of GATA4 protein in the heart or otherwise generate a hypomorphic allele (Fig. 1D). We also confirmed homozygous S105A mut mice no longer showed baseline phosphorylation at S105 in the heart by using a phospho-specific GATA4 antibody (Fig. 1D), although mutation of this site should preclude antibody binding in the first place. Although S105A mut mice were overtly normal, ventricular weight normalized to body weight at 8 wk of age was slightly but significantly decreased compared with control mice (Fig. 1E).

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Fig. 1. Generation of *Gata4*-S105A knock-in mice. (A) Western blotting for phospho-GATA4 (S105) from nuclear extracts of sham- or TAC-treated hearts. (B) Schematic of the targeting strategy used to create *Gata4*-S105A knock-in mice. (C) DNA sequencing traces from WT (Top), S105A heterozygous (Middle), and S105A homozygous (Bottom) mice. Arrows indicate mutated bases resulting in change of S105 to alanine, which introduced a new KpnI restriction site. (D) Western blotting of WT and homozygous knock-in mice (S105A mut) for total GATA4 protein and phospho-GATA4 (S105) from nuclear extracts. Lamin A/C was used as a loading control. (E) Ventricular to body weight ratio (VV/BW) from WT and S105A mut mice at 8 wk of age (* $P < 0.05$ vs. WT).

However, this reduction in heart weight in S105A mut mice did not result in cardiac dysfunction up to 18 mo of age (Table 1).

Phenylephrine-Induced Hypertrophy Acts Through GATA4 Phosphorylation. Although MAPK signaling has been implicated in the phosphorylation of GATA4 in cultured cells, these observations required validation in the adult heart. To this end, we acutely injected WT and S105A mut mice with phenylephrine (PE) for 2, 5, or 15 min, and then measured activation of MAPK pathways and GATA4 phosphorylation from cardiac protein extracts. ERK1/2 and p38 MAPK were both prominently activated in the heart after PE injection, with phospho-ERK1/2 in the nucleus in proximity to GATA4 (Fig. 2A). PE stimulation in vivo resulted in phosphorylation of GATA4 at S105 within 2 min, which progressively increased at 5 and 15 min. However, phosphorylation of GATA4 at S105 was lacking in S105A mut hearts after PE injection (Fig. 2A). To determine if the inability of GATA4 to be phosphorylated had a physiologic consequence in the heart, we implanted mice with osmotic minipumps to continuously infuse PE over a period of 2 wk. Importantly, at baseline, no differences were detected between WT and S105A mut mice in blood pressure, left ventricular pressure, or cardiac contractility (Table S1). The prolonged treatment with PE did not significantly change chamber dimensions or function as assessed by echocardiography. However, PE infusion did induce a prominent hypertrophic response in WT mice that was significantly blunted in S105A mut mice (Fig. 2B). As hypertrophy at the organ level was reduced, we also measured cardiomyocyte cell surface areas from histological sections and confirmed that individual cardiomyocyte size was also reduced in S105A mut mice after 2 wk of PE infusion compared with WT controls (Fig. 2C).

Results obtained in cultured cells with adenoviral-mediated overexpression of GATA4 showed that lack of phosphorylation at S105 resulted in reduced DNA binding capacity (5). Similarly, here we observed that GATA DNA binding was increased in the hearts of WT mice subjected to 15 min of PE stimulation, but not in hearts from S105A mut mice (Fig. 2D). These results suggest a mechanism whereby MAPK-mediated phosphorylation of GATA4, in vivo, enhances DNA binding activity to facilitate the hypertrophic response following PE stimulation.

GATA4 Phosphorylation Is Required for Activated ERK-Induced Hypertrophy. To more definitively examine upstream mechanisms whereby ERK1/2 MAPKs are presumably the primary effectors of GATA4, we used cardiac-specific transgenic mice expressing activated-MEK1. We have previously shown that transgenic mice expressing activated MEK1 in the heart develop prominent hypertrophy and concentric cardiac remodeling (12). Here we first crossed MEK1 transgenic mice with *Gata4-loxP* (fl) targeted mice to determine if global loss of all GATA4 protein from the heart would compromise ERK1/2-induced hypertrophy (Fig. 3A). The MEK1 transgene induced an approximately 30% increase in ventricular weight normalized to body weight in control *Gata4^{fl/fl}* mice without the β -MHC-Cre transgene. However, the MEK1-induced hypertrophy response was dramatically attenuated in *Gata4^{fl/fl}* mice containing the β -MHC-Cre transgene (*Gata4* deleted), indicating that GATA4 is necessary for MEK1-ERK1/2-regulated cardiac hypertrophy in vivo. To extend these results, we crossed the MEK1 transgene with S105A mut mice. Remarkably, MEK1-induced cardiac hypertrophy was significantly blunted in S105A mut mice compared with WT mice, indicating that S105 was critical for activating GATA4 down-

Table 1. Assessment of cardiac dimensions and function in 18-mo-old mice of indicated genotypes

Genotype	No. of mice	IVS, mm	PW, mm	LVED, mm	LVES, mm	FS, %
WT	17	0.82 ± 0.02	0.91 ± 0.02	4.28 ± 0.10	2.90 ± 0.10	32.5 ± 1.3
WT/S105A	8	0.80 ± 0.02	0.96 ± 0.02	4.35 ± 0.20	2.95 ± 0.18	32.6 ± 1.2
S105A/S105A	12	0.81 ± 0.02	0.94 ± 0.02	4.29 ± 0.09	2.81 ± 0.10	34.6 ± 1.4

FS, fractional shortening; IVS, interventricular septum; PW, posterior wall; LVED, left ventricular end-diastolic dimension; LVES, left ventricular end-systolic dimension. None of the measurements are significantly different between different groups.

Fig. 2. PE causes cardiac hypertrophy through phosphorylation of GATA4. (A) Western blotting for phosphorylated GATA4 (S105), total GATA4, and phosphorylated ERK1/2 from WT and S105A mutant cardiac lysates, and for phosphorylated and total ERK1/2 and p38 from WT and S105A mutant cytoplasmic cardiac lysates. Hearts were harvested at indicated time points after s.c. PE injection. (B) VV/BW ratio in WT and S105A mutant mice after 2 wk of continuous infusion of vehicle or PE by osmotic minipump. The number of mice analyzed is shown in the graph (* $P < 0.05$ vs. vehicle, $^{\dagger}P < 0.05$ vs. WT PE). (C) Myocyte surface area (μm^2) from cardiac histological sections of the mice shown in B. A total of 300 myocytes were counted across four or five hearts each (* $P < 0.05$ vs. vehicle, $^{\dagger}P < 0.05$ vs. WT PE). (D) Gel-shift assay shows GATA DNA binding activity from cardiac nuclear protein extracts from WT and S105A mutant mice at baseline or 15 min after s.c. PE injection. Addition of probe only and cold competitor probe show specificity of GATA binding activity.

stream of ERK1/2 signaling (Fig. 3B). Western blotting confirmed that the activated MEK1 transgene induced ERK1/2 signaling both in WT and S105A mutant hearts (threefold activation for both), but the mutant mice lacked phosphorylation of S105 (Fig. 3C). Collectively, these results further indicate that MAPK signaling through S105 in GATA4 functions as an important prohypertrophic signaling circuit in the heart.

S105A Mut Mice Develop Less Hypertrophy and Fail After Pressure Overload Stimulation. We also induced cardiac pressure overload in mice by transverse aortic constriction (TAC) to assess whether a more severe stress that activates multiple pathways simultaneously might also use GATA4. One and 2 wk of pressure overload stimulation resulted in hypertrophy of the septum and/or posterior free wall in WT mice as assessed by echocardiography (Fig. 4A and B). However, this increase was significantly blunted in S105A mutant mice. At the whole-organ level, 1 wk of pressure overload stimulation resulted in a large increase in ventricular weight normalized to body weight, yet this increase was significantly blunted in S105A mutant mice (Fig. 4C). After 2 wk of TAC, the S105A mutant mice appeared to catch up with WT mice with respect to whole-organ hypertrophy (Fig. 4C). How-

Fig. 3. Phosphorylation of GATA4 is required for activated ERK-induced cardiac hypertrophy. (A) VV/BW ratio from the indicated genotypes of mice at 8 wk of age. The number of mice analyzed is shown in the graph (* $P < 0.05$ vs. nontransgenic, $^{\dagger}P < 0.05$ vs. Gata4^{fl/fl} × MEK1 transgenic). (B) VV/BW ratio from WT and S105A mutant mice with or without the MEK1 transgene. The number of mice analyzed is shown in the graph (* $P < 0.05$ vs. nontransgenic, $^{\dagger}P < 0.05$ vs. WT × MEK1 transgenic). (C) Western blotting of protein extracts from hearts of the indicated mice for MEK1 and phospho-ERK1/2 and total ERK. Western blotting with phospho-GATA4 (S105A) antibody was performed from cardiac nuclear extracts. GAPDH was a loading control.

ever, echocardiography showed left ventricular dilation during diastole (Fig. 4D) and systole (Fig. 4E), as well as reduced fractional shortening (Fig. 4F) in the S105A mutant mice. These results at 2 wk in S105 mutant mice suggest failure with dilation, and such dilation can cause a secondary increase in heart weight that is different from compensated concentric hypertrophy. Indeed, measurement of isolated individual adult cardiomyocytes from hearts of S105A mutant mice after TAC showed lengthening and narrowing, which is a sign of eccentric remodeling with dilation (Fig. 4G–I). Hearts from S105 mutant mice subjected to 2 wk of TAC also showed enhanced cardiac fibrosis compared with WT (Fig. 4J), but no difference in cardiomyocyte death (Fig. S1). Thus, loss of GATA4 S105 phosphorylation significantly blunted the cardiac hypertrophic response to pressure overload stimulation and rendered S105A mutant mice more susceptible to dilation, decompensation, and heart failure.

Phosphorylation of S105 Is Necessary for GATA4 Transcriptional Potency. To begin to examine the molecular mechanisms whereby defective S105 phosphorylation of GATA4 might impact the heart, we performed gene array profiling. Affymetrix-based microarray analysis showed 83 up-regulated and 159 down-regulated genes in S105A mutant hearts compared with WT. One interesting pathway that was altered in S105A mutant hearts was related to ECM genes, as well as hypertrophic genes comprising the fetal gene program (*Nppa*, *Nppb*, *Myh7*, *Myh6*). The latter gene grouping was particularly germane, as many of these genes have direct GATA4 DNA-binding sites within their promoters (7). Confirmation and exact quantification of these results by real-time PCR showed baseline down-regulation of *Nppb*,

Fig. 4. Pressure overload results in blunted hypertrophy and progression to dilation in S105A mut mice. Echocardiographic assessment of interventricular septal (A) and posterior wall thicknesses (B) from sham- and TAC-operated mice measured at 1 and 2 wk after surgery (* $P < 0.05$ vs. sham, † $P < 0.05$ vs. WT TAC at 2 wk). (C) VW/BW ratio in the indicated groups of mice. The legend in A also applies to B–F (* $P < 0.05$ vs. sham, † $P < 0.05$ vs. WT TAC at 1 wk). Echocardiographic assessment of left ventricular end-diastolic (D) and end-systolic (E) chamber dimension from the indicated groups of mice (* $P < 0.05$ vs. sham, † $P < 0.05$ vs. WT TAC 2 wk). (F) Ventricular fractional shortening (FS) percentage in WT and S105A mut mice after sham operation or 1 to 2 wk after TAC surgery (* $P < 0.05$ vs. sham, † $P < 0.05$ vs. WT TAC at 2 wk). (G) Length of isolated cardiomyocytes from hearts after 10 d of TAC. (H) Width of isolated cardiomyocytes from hearts after 10 d of TAC. (I) Length-to-width ratio of isolated cardiomyocytes from hearts after 10 d of TAC. (J) Fibrosis quantification after 2 wk of TAC as a percentage of fibrotic area per section ($n = 7$ hearts; * $P < 0.05$ vs. WT TAC in G–J). The number of mice analyzed throughout is shown in the graphs in A–F. For G–J, 251 WT and 174 S105A mut cardiomyocytes were counted from three and four hearts, respectively.

Myh7, *Myh6*, *Fgf16*, *Spp1*, *Cthrc1*, *Col1a2*, *Ctgf*, *Thbs4*, *Col3a1*, *Ltpb2*, *Tgfbi*, *Timp1*, and *Fstl4*, whereas *Ephbi* and *Fgf2* were up-regulated in the hearts of S105A mut mice (Fig. 5 A–T). Overall,

these data suggest that activation of GATA4 through phosphorylation of S105 is important for GATA4 to reach its full transactivating potential as a transcription factor, likely through

Fig. 5. Phosphorylation of GATA4 is important for expression of multiple genes in the heart. Normalized TaqMan RNA expression assays for (A) *Nppa*, (B) *Nppb*, (C) *Myh7*, (D) *Myh6*, and (E) *Acta1* on RNA from WT and S105A mut hearts. Normalized SYBR green quantitative PCR for (F) *Ephbi*, (G) *Fgf2*, (H) *Fgf16*, (I) *Spp1*, (J) *Cthrc1*, (K) *Col1a2*, (L) *Ctgf*, (M) *Thbs4*, (N) *Col3a1*, (O) *Ltpb2*, (P) *Tgfbi*, (Q) *Figf*, (R) *Timp1*, (S) *Timp4*, and (T) *Fstl4* on RNA from WT and S105A mut hearts. All graphs are plotted as relative to WT. All samples were analyzed in duplicate from three hearts each.

increased affinity for DNA when phosphorylated. Moreover, activation of many of these genes was significantly blunted after pressure overload (Table S2). These changes in cardiac gene expression likely affect the ability of the heart to productively hypertrophy with stress stimulation.

Discussion

GATA4 is crucial for normal cardiac development, as *Gata4*^{-/-} embryos arrest at embryonic day 9.0 with defective heart tube morphogenesis from a lack of ventral folding (13, 14). In the adult heart, deletion of *Gata4* specifically in cardiac myocytes by using a Cre-loxP approach resulted in spontaneous heart failure with aging, and younger adult targeted mice subjected to stress stimulation failed to mount an effective hypertrophic response (15, 16). These previous results suggest that GATA4 is a crucial regulator of adaptive cardiac growth in response to pathologic and even physiologic stress stimulation. Here, we determined that phosphorylation of GATA4 at S105 is critically important in activating this transcription factor, as S105A mut mice failed to develop productive hypertrophy in response to pressure overload stimulation and neurohormonal mimicry with PE. Surprisingly, we found that phosphorylation of GATA4 is sufficient and required for activated ERK1/2-induced hypertrophy (in MEK1 transgenic mice). Finally, we showed that pressure overload requires phosphorylation of GATA4 to protect against decompensation from pressure overload, as S105A mut mice showed rapid progression of left ventricular dilation with reduced cardiac function.

Previous studies conducted in cultured cardiomyocytes showed that phosphorylation of GATA4 could augment its transcriptional potency, DNA binding activity, and increase expression of GATA4-regulated genes (3, 5, 6). GATA4 was directly phosphorylated at S105 by ERK1/2 and p38 MAPK, downstream of neuroendocrine stress signaling pathways that are known to underlie the cardiac hypertrophic response (3–5). Indeed, both ERK1/2 and p38 MAPK activity were necessary for the increase in GATA4 DNA binding that occurs in protein extracts from hearts that underwent acute wall stretching (8). GATA4 phosphorylation at S105 was also induced by treatment of cultured cardiomyocytes with hepatocyte growth factor, and this phosphorylation and subsequent increase in GATA4 DNA binding activity was abolished with MEK1–ERK1/2 signaling inhibitors (17). GATA4 is also present in cardiac fibroblasts and is activated by endothelin-1 treatment, resulting in S105 phosphorylation that is blocked with a MEK1–ERK1/2 inhibitor (18). Collectively, these studies suggest that S105 in GATA4 is directly regulated by ERK1/2 and p38 MAPK in cultured cells. However, none of these studies directly investigated if phosphorylation of GATA4 was important within the context of the adult heart or with disease. Here we showed that GATA4 phosphorylation at S105 is critical for fully activating the hypertrophic response within the context of the adult heart. Although we cannot rule out other relevant phosphorylation sites in GATA4, the fact that S105A mut mice were partially impaired in responding to the activated MEK1 transgene suggests that ERK1/2 are required mediators of GATA4 induction during hypertrophy through this specific site (although p38 is also likely partially responsible). By comparison, a limited number of knock-in mice have been generated in recent years that blocked transcription factor phosphorylation, such as of RelA, Jun, CREB, and Nkx2-1, which also showed that phosphorylation helps achieve maximal transcriptional potency (19–22).

Mechanistically, we observed down-regulation of fetal/hypertrophic-related genes already at baseline, many of which have known GATA4-binding sites in their promoters (BNP, ANF, β -MHC, and α -MHC). Furthermore, we found down-regulation of several ECM genes, such as thrombospondin 4, tissue inhibitor of metalloproteinases 4, connective tissue growth factor, and

several TGF-related genes. These changes could arise from lack of activation of GATA4 in cardiomyocytes through routine neuroendocrine inputs and G-protein–coupled receptor signaling pathways that continuously provide sensory information as to the milieu and developmental status of the organism. Another possibility is that GATA4 could be dysregulated in the cardiac fibroblast, as a recent report showed that KLF5 from cardiac fibroblasts is required for cardiac hypertrophy (23), and GATA4 is expressed in fibroblasts and phosphorylated at S105 by agonist stimulation (18, 24). Thus, the cardiac fibroblast might also contribute to the phenotype observed in S105A mut hearts, especially given the dramatic changes observed in ECM genes. Future studies will have to delineate the cardiomyocyte-specific role of GATA4 phosphorylation versus nonmyocyte effects. However, we still believe the cardiac myocyte is the main cell type whereby loss of GATA4 phosphorylation compromises the hypertrophic response, given that myocyte-specific deletion of *Gata4* (loxP-targeted mice) strongly reduces the cardiac hypertrophic response to various stress stimuli (15, 16, 25). In conclusion, we showed here that phosphorylation of GATA4 *in vivo* is required for activated ERK1/2-induced hypertrophy as well as for normal compensation to pressure overload. The likely mechanism is through increased DNA binding and thereby increased activity of GATA4, resulting in higher expression of GATA4-regulated genes.

Methods

Generation of *Gata4*-S105A Knock-in Mice. We used Red-et recombineering (Gene Bridges) to generate a targeting vector that would mutate S105 of the murine *Gata4* gene to alanine. We also introduced a restriction site at the same time for confirmation and ease of genotyping (KasI). After homologous recombination in ES cells and obtaining germline transmission of the mutant allele, the Frt-flanked neomycin selection cassette was deleted through cross-breeding with a mouse line in which Flp-recombinase is expressed in germ cells. Mice harboring the *Gata4*^{fl/fl} alleles were previously described, and we crossed these mice with previously described Cre recombinase expressing mice under control of the β -MHC promoter (16). The constitutively active MEK1 transgenic mice were previously described (12). All experiments involving mice were approved by the institutional animal care and use committee of the Cincinnati Children's Hospital Medical Center.

In Vivo Treatments and Surgeries. PE was injected s.c. (100 μ L, 15 mg/kg) and hearts were harvested at the indicated time points under 2% isoflurane inhalation anesthesia. Alzet osmotic minipumps were used for long-term treatment to continuously infuse PE at a rate of 100 mg/kg/d. TAC was performed as previously described (9). Echocardiography was performed as previously described using a Hewlett Packard SONOS 5500 instrument equipped with a 15-MHz transducer on mice that were anesthetized with 2% isoflurane inhalation (16). In vivo hemodynamic assessment of blood pressure and left ventricular function was performed as previously described (9).

Western Blotting, Gel-Shift Assay, Immunohistochemistry and Adult Cardiomyocyte Isolation. For Western blotting and gel-shift assays, hearts were first fractionated to enrich for cytoplasmic and nuclear proteins according to the manufacturer's protocol (NE-per, Pierce). Western blot analysis was performed as previously described (9). Primary antibodies included phospho p42/p44 (9101) p42/p44 (9102), phospho p38 (9211), p38 (9212), and lamin A/C (2032) from Cell Signaling Technology; phospho-GATA4 (ab5245) from Abcam; GATA4 (AF2606) from R&D Systems; and GAPDH (TRK5G4-6C5) from Fitzgerald Industries. Gel-shift assays were performed as previously described (9). Briefly, nuclear-enriched fractions of untreated and PE-stimulated WT and S105A mut hearts (25 μ g) were incubated in gel-shift buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 50 mM KCl, 12% glycerol, 1.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2 μ g/mL aprotinin, 2 μ g/mL leupeptin, and 0.7 μ g/mL pepstatin) with 0.5 μ g poly(dI-dC) and ³²P-labeled GATA-binding site from the α -MHC promoter for 20 min at room temperature followed by nondenaturing gel electrophoresis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Sections were first rehydrated, treated with antigen retrieval, and incubated with wheat germ agglutinin tetramethylrhodamine isothiocyanate-dextran to visualize membranes. Myocyte surface areas were assessed in at least three animals per

group, counting at least 75 individual myocytes per heart on five randomly taken images. Masson trichrome staining was performed on histological sections 2 wk after TAC. Fibrosis was quantified as percentage of blue area by using NIH ImageJ software in seven animals per group on five randomly taken images. Adult cardiomyocytes were isolated as previously described (9).

mRNA Microarray Analysis and Quantitative Real-Time PCR. RNA was extracted from snap-frozen hearts of 9-wk-old mice by using a Qiagen fibrous tissue RNeasy kit. mRNA was processed for hybridization on Affymetrix mouse set ST1.0 chips for gene expression profiling according to the manufacturer's protocols. Genes with significant expression differences between WT and S105A mut were selected with a Student *t* test with a *P* value cutoff of 0.05 as described previously (25). The data have been deposited into the Gene Expression Omnibus database (accession no. GSE30314). Selected gene expression differences were confirmed by real-time quantitative PCR using

SYBR green (Applied Biosystems). Fetal gene program activation was measured by using individual TaqMan assays (Applied Biosystems). Quantified mRNA was normalized and expressed as relative to control. For assessment of gene expression after TAC, we normalized to the baseline values of the same genotype and compared WT versus S105A mut mice.

Statistical Analysis. Data are represented as means $\pm$ SEM. We used a two-sample Student *t* test to compare means between groups. A *P* value lower than 0.05 was considered significant.

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