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Supplemental Information

A TRPC6-Dependent Pathway for Myofibroblast Transdifferentiation and Wound Healing In Vivo

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Inventory of supplemental information

1) Supplemental Figures (S1-S3).
Supplemental figure S1 is in support of data shown in Figure 1
Supplemental Figure S2 is in support of data shown in Figure 5
Supplemental Figure S3 is in support of data shown in Figure 6.

2) Extended Experimental Procedures
This section is in support of the Experimental Procedures section.

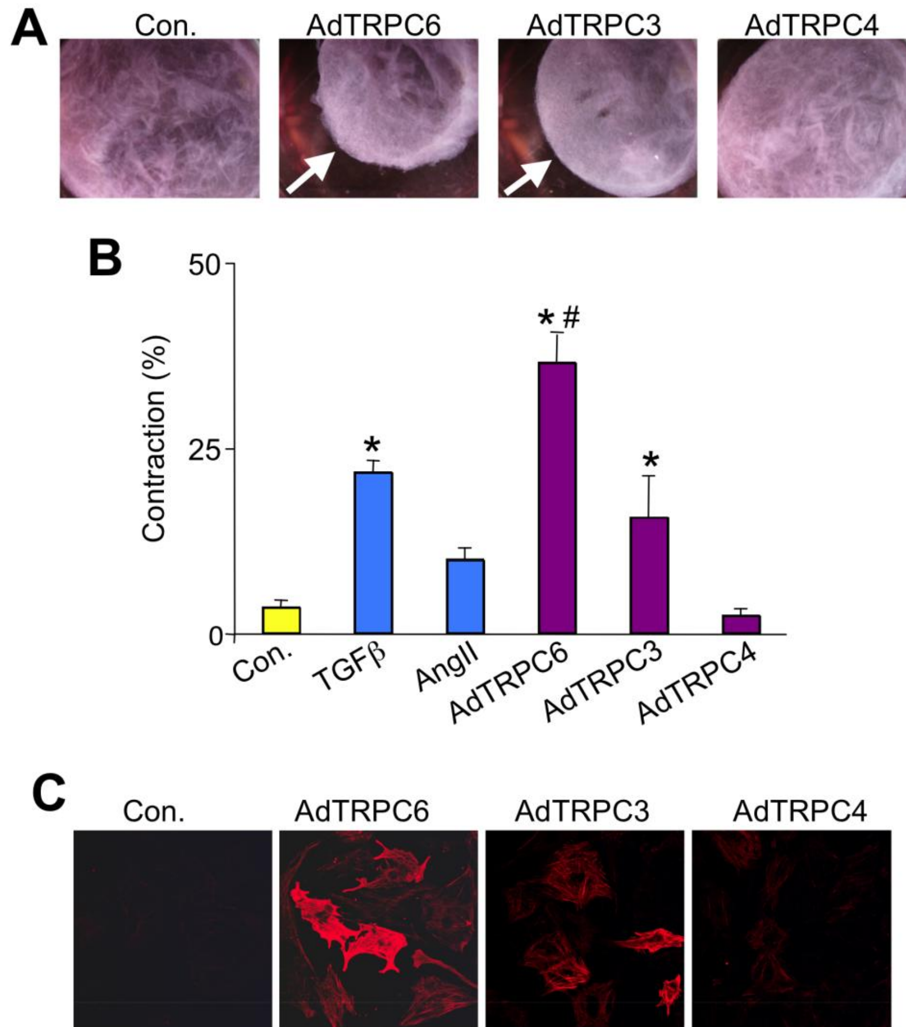

Figure S1. TRPC6 preferentially mediates myofibroblast transformation over other TRPCs (**A**) Photographs and (**B**) quantification of floating collagen gel matrices seeded with cardiac fibroblasts that have contracted after 36 hrs of TGFβ stimulation or AdTRPC6, AdTRPC3, or AdTRPC4 infection. Error bars are +/- s.e.m., ANOVA statistical test, *P<0.05 vs. control; # P<0.05 vs. TGFβ, N=3 independent experiments. Arrows in A show contraction of the gel. (**C**) Immunofluorescent staining of αSMA (red) positive stress fibers in cardiac fibroblasts infected with Adβgal (con), AdTRPC6, AdTRPC3, or AdTRPC4.

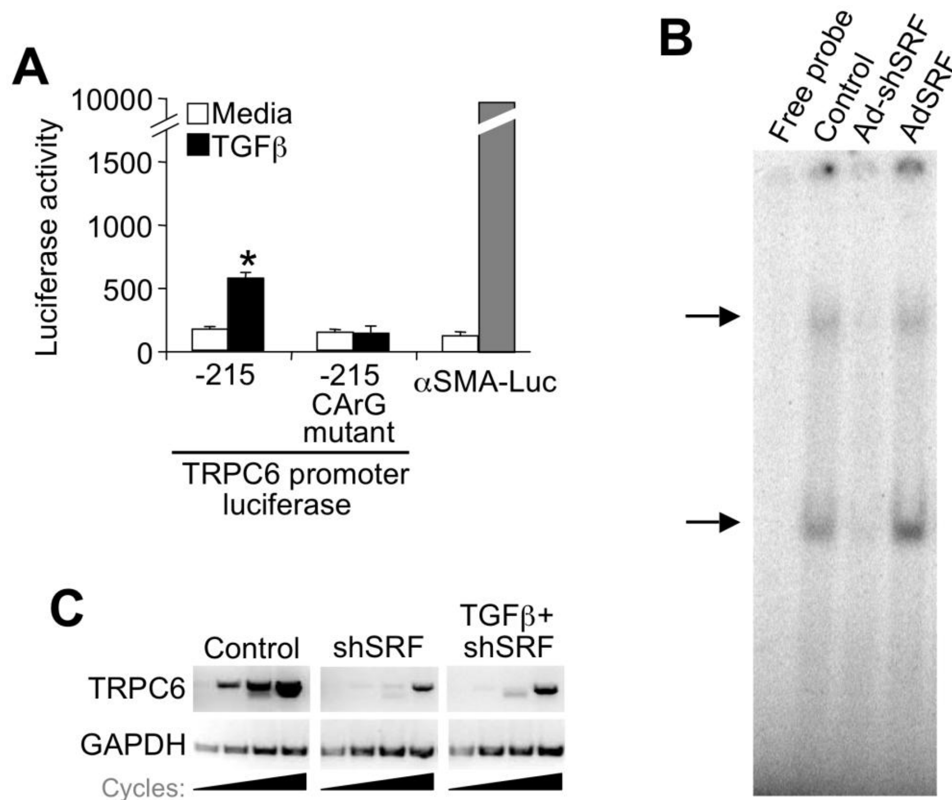

Figure S2. The first conserved consensus CArG box is a dominant SRF binding site in the murine TRPC6 promoter. (**A**) TRPC6-luciferase promoter activity in fibroblasts transfected with the indicated constructs and treated with media or TGFβ. The TRPC6 promoter construct (-215/+1) contains only the most proximal consensus CArG element, which was mutated to eliminate SRF binding in the second construct shown. Error bars are s.e.m. (**B**) Gel shift analysis of SRF binding to the proximal CArG site of the TRPC6 promoter. Whole cell extracts were prepared from cardiac fibroblasts that were adenovirally infected with shGFP (control), shSRF, or SRF, and then incubated with a radio-labeled double-stranded oligonucleotide corresponding to the proximal CArG site. The arrows show a larger and smaller complex that is SRF dependent (lower band is likely just SRF, while upper band likely contains other interacting proteins but is still SRF dependent). (**C**) Representative RT-PCR of TRPC6 mRNA in primary cardiac fibroblasts infected with the indicated adenoviruses and treated with media or TGFβ.

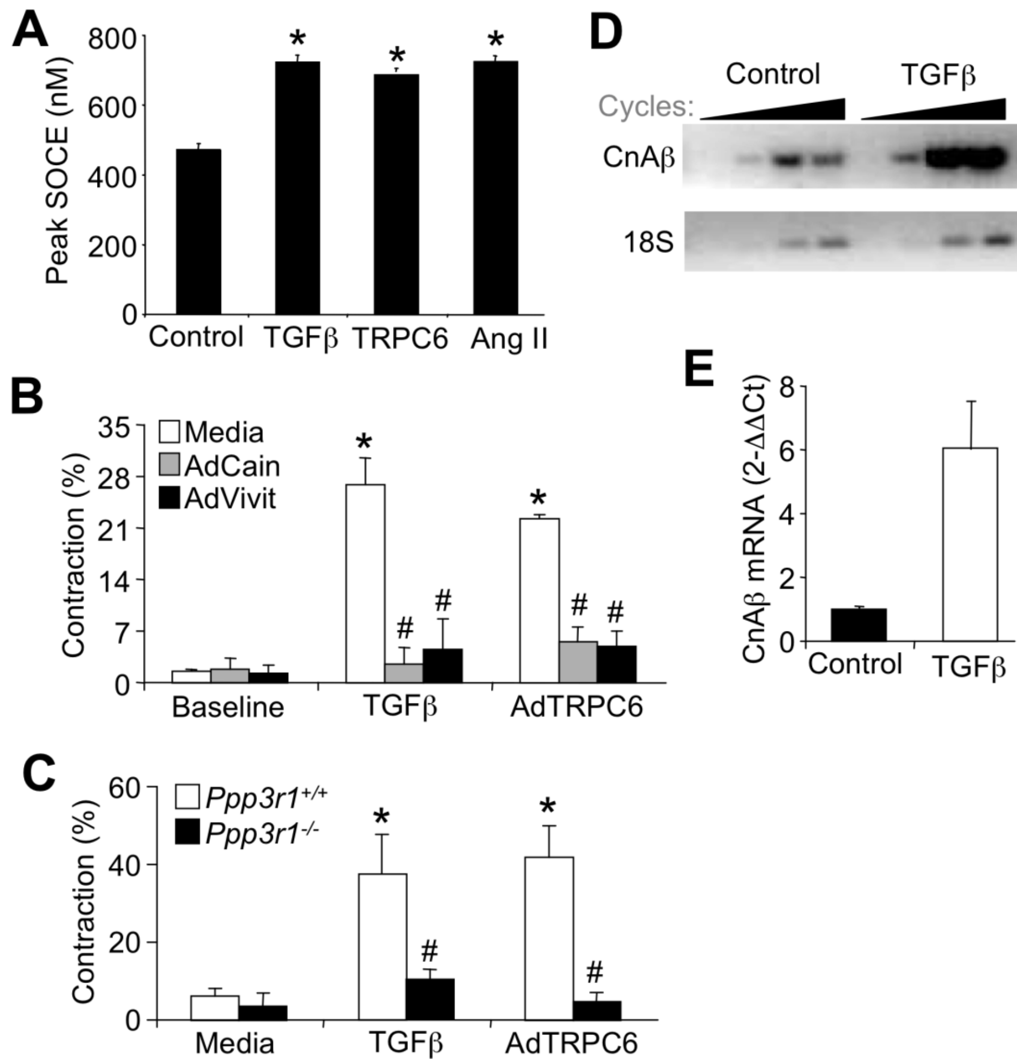

Figure S3. TGFβ enhances calcineurin calcium-signaling and calcineurin-NFAT is required for myofibroblast formation/function. (**A**) Quantification of peak $[Ca^{2+}]_i$ during SOCE from control, TGFβ treated, AngII treated and AdTRPC6 infected cardiac fibroblasts. (**B**) Quantification of collagen gel contraction with cardiac fibroblasts after 36 hrs of TGFβ stimulation or AdTRPC6 infection in the presence or absence of Cain (calcineurin inhibition) or VIVIT (NFAT inhibition) delivered by adenovirus. (**C**) Quantification of collagen gel contraction seeded with MEFs that were *Ppp3r1* Wt or null after 36 hrs of TGFβ stimulation or AdTRPC6 infection. *Ppp3r1* encodes the necessary calcineurin B1 subunit and its deletion renders all calcineurin absent. (**D**) Representative RT-PCR of calcineurin Aβ (*Ppp3cb* gene) mRNA in primary cardiac fibroblasts treated with media or TGFβ for 48 hours. (**E**) Real time PCR for calcineurin Aβ mRNA induction (*normalized to 18s RNA*) from cardiac fibroblasts that were simultaneously treated for 24 hours with TGFβ. Error bars are $\pm$ s.e.m and ANOVA statistical test and post hoc comparisons were made. *P<0.05 vs control; #P<0.05 vs media or *Ppp3r1*$^{+/+}$

Supplemental Experimental Procedures

Animal Models. Trpc6$^{-/-}$ mice were previously described (Dietrich et al., 2005). *Ppp3cb*$^{-/-}$ mice were also previously described (Bueno et al., 2002). For both dermal wound healing assays and myocardial infarction mice were 8-10 weeks of age, and studies terminated between 3-14 days after injury. All mouse protocols were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Dermal wound healing assay and topical adenoviral application. The dermal wound-healing model was developed from previously described methods in which mice were first induced and maintained on 2% isoflurane anesthesia (Fang and Mustoe, 2008; Galiano et al., 2004; Reid et al., 2004). Then, the backs were shaved, cleaned, and treated with betadine. Two 6 mm excisional wounds were created with a disposable biopsy punch (Integra Miltex) on either side of the dorsal midline (Krampert et al., 2005; Werner et al., 1994). Wounds were covered with Tegaderm adhesive strips to protect the skin from infection. Wounds were measured and photographed daily in order to assess wound retraction and health. In some experiments biopsies were made and topically treated with the following adenoviral vectors (Galeano et al., 2003; Lee et al., 2010): green fluorescent protein (GFP, internal control), TRPC6, and constitutively activated calcineurin (ΔCnA). Adenoviral vectors were soaked into the biopsy area for 15 minutes before applying the Tegaderm adhesive which held the virus in the wounded area. Wound photographs were analyzed by ImageJ software (NIH) to measure surface areas. Wound measurements were normalized back to their original size at the time of surgical biopsy. Wounds were excised with a 4 mm margin around the wound and fixed in 4% paraformaldehyde and embedded in paraffin at various time points after injury. For immunofluorescence staining antigen retrieval was performed and subsequently stained with a monoclonal mouse αSMA antibody (1:500, Sigma, Clone 1A4) as described below, and isolectin-B4 (Vector Biolabs) for identifying epithelial cells.

Myocardial infarction and echocardiography. To create myocardial infarcts (MI) 10-week old mice were subjected to permanent occlusion of the left coronary artery as previously described (Oka et al., 2007; Wilkins et al., 2004). Briefly the heart was exposed by thoracotomy and the left coronary artery was ligated with 8-0 suture. MI studies were terminated 7 days after surgery at which time hearts were perfused in cardioplegic buffer to preserve diastolic dimensions and fixed in 10% formalin, embedded in paraffin, and prepared for histological analysis. Mason's trichrome staining was used to assess scar thickness in mice surviving for 7 days. M-mode echocardiography (Hewlett Packard SONOS 5500) was performed with a 15-MHz transducer at 3 and 7 days after MI surgery to assess cardiac function

and ventricular geometry (Oka et al., 2007). For echocardiographic measurements mice were induced and maintained on 2% isoflurane anesthesia.

Fibroblast culturing. Primary cultures of cardiac fibroblasts were obtained from 1-2 day old neonatal Sprague-Dawley rats by enzymatic digestion of the left ventricle (Taigen et al., 2000). Myocytes were allowed to settle by gravity for 10 minutes and then the supernatant was collected and centrifuged for 5 minutes at 1,000g. The fibroblast pellet was resuspended and plated in DMEM supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Primary cardiac fibroblasts were used at passages 2 and 3 to ensure a more homogeneous population of fibroblasts. Primary murine dermal fibroblast cultures were obtained from 2-3 month old mice. Mice were anesthetized with sodium pentabarbitol and the dorsal skin shaved. The skin was excised and spread dermis side down for overnight incubation in 0.25% trypsin at 4°C. The skin was then minced and further digested in 0.25% collagenase type I (Worthington) for 3 hours at 37°C. The skin was further dissociated and suspended in DMEM supplemented with 10% FBS and nonessential amino acids. The suspension was centrifuged for 5 minutes at 1,000g and the pellet resuspended in DMEM+10%FBS. The centrifugation and resuspension step was repeated twice and the fibroblasts were plated. Primary murine dermal fibroblasts were used at passages 2 and 3. Simian virus 40 (SV40) transformed mouse embryonic fibroblasts (MEF) were cultured in Iscove's modified Dulbecco's Medium supplemented with 10% FBS, 2 mM glutamine, and non-essential amino acids. Primary adult human dermal fibroblasts (ATCC, PCS-201-012) were used at passages 2-5 and cultured in low serum Fibroblast Basal Media (ATCC) per the manufacturer's instructions. For most assays unless otherwise noted serum concentrations were reduced to 1% 24 hours prior to the start of the experimental protocol.

TGFβ/AngII treatments, adenoviral gene transfer and shRNA lentiviral knockdown. Application of recombinant porcine TGFβ (10 ng/ml, R&D System) or AngII (100 nM, Sigma) was used to induce myofibroblast transformation. The p38 inhibitor (SB731445) was delivered in 50 nM doses. For experiments that required adenoviral gene transfer, fibroblasts were incubated in adenovirus for 24 hrs at which time the media was changed and the cells were typically incubated for an additional 24-48 hrs. The following adenoviruses have been previously described: ΔCnA, GFP, β-galactosidase, NFATc1-GFP, NFAT(9x)-luciferase, TRPC3, TRPC4, SMAD6/7, SRF, and MEF2a (Liu et al., 2001; Sanna et al., 2005; Taigen et al., 2000; Wilkins et al., 2004; Wu et al., 2010; Xu et al., 2006). For the TRPC6 adenoviral vector, a murine TRPC6 cDNA was cloned into the pShuttle vector (AdEasy adenoviral system) and virus was made as previously described (Xu et al., 2006). For knockdown of the SRF gene rat cardiac fibroblasts were adenovirally transduced with short hairpin (sh) RNA constructs shGFP (control) or shSRF (Streb and Miano, 2005). At two days post shSRF/shGFP gene

transfer fibroblasts were adenovirally infected with AdTRPC6 or AdΔCnA or pharmacologically treated with TGFβ (10 ng/ml) or AngII (100 nM). For knockdown of the ALK5 gene (TGFβ-receptor 1, NM_009370) Mission shRNA lentiviral plasmids (Sigma, TRCN0000022479, 80, 81, 82, 83) were obtained and produced by the lentiviral vector core in the Division of Experimental Hematology & Cancer Biology at Cincinnati Children's Hospital Medical Center. Lentivirus was delivered in culture media containing polybrene (8 μg/ml) to SV40 MEFs or cardiac fibroblasts and incubated for 24 hours at which time fresh media was supplied. Knockdown was achieved at 4 days post gene transfer.

Cell based genome wide screen. Cell-based gain of function screening was performed in SV40 transformed MEFs with the mammalian genome collection (MGC) cDNA library. The MGC library contains 16,953 human and 10,344 mouse full-length cDNA clones (some overlap) that are plated 1 clone per well at a concentration of 40 ng in a 384-well format (Scripps Institute, Florida, Cell-based Screening Core). The readout for our screen was an αSMA-luciferase reporter that was derived from -893 to +51 of the αSMA promoter and cloned into a pGL3 basic luciferase vector (Promega). The αSMA-luciferase construct was generously supplied by Dr. Gerard Elberg (Elberg et al., 2008). The MGC library was cotransfected (Mirus, Trans-IT) with the αSMA-luciferase reporter construct into SV40 MEFs in media with 1% serum. Luciferase activity was measured 48 hrs post transfection. The criterion for a positive clone was induction of luciferase activity greater than or equal to the threshold achieved by TGFβ (10 ng/ml) application over the same time period within 1 well of each plate in the screen.

TRPC6 luciferase reporter and luciferase assay. Three DNA fragments, -215 to +1, -215 to +1 with a mutated CArG site, and 1187 to +1, of the murine TRPC6 promoter were individually subcloned into the *XhoI* and *HindIII* sites of the pGL3 promoter luciferase plasmid (Promega) to make TRPC6 reporter constructs. The proximal and highly conserved consensus CArG site at -123 (5' CCTTTAAAGG 3') in the TRPC6 promoter was mutated to TGTGCGAAGG (Genewiz Inc.). For luciferase assays rat cardiac fibroblasts were maintained in media + 1% serum and transiently cotransfected with a TRPC6 luciferase reporter (Mirus, Trans-IT) and either a βgal or SRF plasmid. In addition some transfected fibroblasts were also treated with TGFβ (10 ng/ml) or p38 inhibitor (50 nM). Luciferase activity was measured 48 hours post transfection.

Chromatin immunoprecipitation. Rodent cardiac fibroblasts were maintained in media + 1% serum and adenovirally transduced with either βgal or SRF and treated 24 hours later with TGFβ (10 ng/ml) or media. Chromatin immunoprecipitation was performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions. Anti-SRF (G-20, Santa Cruz), Anti-phospho-SRF (SER103, Cell

Signaling), or anti-RNA Polymerase II (Millipore) was used for immunoprecipitation. PCR was performed using TRPC6 promoter specific primer pairs that amplify at 383 bp fragment: forward 5'-GGCCAGGCTTGCTTGGAACTCAT and reverse 5'-ATGGGCATTCAAATTCCCCACCC. GAPDH primers used as a control.

Immunofluorescence and western blotting. Fibroblasts were fixed in 4% paraformaldehyde and blocked in 10% goat serum. Fibroblasts were incubated for 1.5 hrs at room temperature in primary antibody followed by secondary antibody or TO-PRO 3 iodine (Invitrogen) incubation. The following primary antibodies were used: αSMA antibody (1:1000, mouse monoclonal, Clone 1A4, Sigma), collagen I (1:100, rabbit polyclonal, Rockland), TRPC6 (1:100, rabbit polyclonal, Alamone), SRF (1:100, Santa Cruz). αSMA was detected with either Alexa 488 or Alexa 568 conjugated secondary antibody directed against mouse IgG. Collagen I and TRPC6 were detected with Alexa 488 conjugated secondary antibody directed against rabbit IgG. Secondary antibodies were diluted at 1:1000 (Invitrogen), and all antibodies were diluted in 1% goat serum + 0.5% Triton X-100/phosphate buffered saline (PBS). Immunofluorescence was visualized on a Nikon TE2000 confocal microscope. For western blotting cell lysates were collected and 40 μg of protein was subjected to 10% SDS-PAGE and transferred to PVDF membrane for immunodetection. The fibronectin ED-A splice variant was detected with a mouse monoclonal antibody directed towards the ED-A extracellular domain of fibronectin (1:1000, clone DH-1, Millipore), SRF was detected with anti-SRF (1:100, rabbit polyclonal antibody, Santa Cruz), and anti-GAPDH (1:5,000, mouse monoclonal, Fitzgerald Industries) was used as a loading control, and goat-anti-mouse alkaline-phosphatase (1:5000, Santa Cruz Biotechnology) was used as a secondary antibody.

Collagen gel contraction assay. Collagen contraction assays were performed as previously reported (Ngo et al., 2006). Fibroblasts at 70% confluence were adenovirally infected if experimentally required or grown in culture media for 24 hrs. Fibroblasts were harvested from a confluent monolayer by Trypsin-EDTA digestion (0.25%), pelleted and resuspended in DMEM with 1% serum. Fibroblasts were then seeded into collagen matrices such that each gel contained 40,000 fibroblasts and cast in 24-well plates. The collagen gels were released from the edges and floating in DMEM with 1% serum. Experimental groups were seeded in triplicate and the gels were photographed at 0, 24, 36, and 48 hours. Data reported is from the 36 hr time point. ImageJ software (NIH) was used to calculate the surface area, which are reported as values normalized to the initial size of the gel.

In vitro scratch assay. Scratch assays were performed as previously reported (Liang et al., 2007). *Trpc6* null or Wt dermal fibroblasts were plated into 6-well plates with or without adenoviral infection.

Once the plate reached confluence a scratch was made with a sterile P-200 pipette tip in the middle of each well and the plate was washed 2 times in PBS and incubated in DMEM with either 1 or 10% FBS. Photographs of the same fields were taken at 0, 12, 18, and 24 hours. Marks were made on each well to ensure the same field was photographed. Three fields were photographed for each experimental group and each group was plated in triplicate. ImageJ software (NIH) was used to calculate the wound areas at each time point and the change in area over time was used as an index of fibroblast migration.

Fibroblast proliferation assay. Fibroblast proliferation was evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) colormetric assay. Briefly, fibroblasts with or with out TRPC6 adenoviral gene transfer were seeded at high density in 96-well plates and cultured with or without serum for 24 hrs. MTT reagent was added and the assay performed per the manufactures instructions (Millipore). The OD of individual wells was measured at 550 nm after a 4-hr incubation and the actual cell number was calculated from a standard curve obtained using known amounts of cells that were plated as controls.

Real-time polymerase chain reaction (PCR).
In some experiments cardiac fibroblasts were either treated with recombinant TGFβ (10 ng/ml), AngII (100 nM), or p38 inhibitor (SB731445, 50 nM) for 12 or 24 hrs. In other experiments cardiac fibroblasts were adenovirally infected with control (β-galactosidase), SMAD6/7, SRF, shSRF or MEF2a for 48 hrs. Drug treatments to adenovirally-infected fibroblasts were delivered 24 or 48 hrs after infection so as to permit ample time for gene transcription or silencing. RNA from fibroblasts was extracted using QIA shredder homogenization and an RNeasy kit (Qiagen). RNA from wounded and uninjured skin was extracted with TRIzol Reagent and cleaned using the RNeasy (Qiagen) clean-up protocol. Reverse transcription (RT) reaction was performed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) and oligo-dt primers according to the manufacturer's instructions. Real-time PCR was performed using the Quantifast Sybrgreen PCR kit (Qiagen). Data were normalized to 18SRNA expression. The following primer sets were used: TRPC6 5'-GCTACTACCCCAGCTTCCGGGG, 5'-TGGATGGTTGAGGATTGCCTCCACA; TRPC3 5'-GGGGCCACCATGTTTGGCACAT, 5'- GCAGCACGCCGCCCAATTGT; TRPC4 5'-TTCGGGACAATTGGGCGGCGT 5'-CCACCAGGGCGGAACCATTGC; TRPC1 5'GCCCTGTACCCGAGCACGGA, 5'-GCATCTGCGGACTGACAACCGT; *Ppp3cb*$^{-/-}$ (Calcineurin Aβ) 5'- CCA CAG GGA TGT TGC CTA GTG, GTC CCG TGG TTC TCA GTG GTA; 18SRNA 5'-GTAACCCGTTGAACCCCATT, 5'-CCATCCAATCGGTAGTAGCG.

Electro mobility shift assay (EMSA).

EMSA was performed as previously described (Molkentin et al., 1993) using 20 μg of cardiac fibroblast whole cell extract from each of the indicated groups that was collected in Dignam A buffer. Binding reactions were performed with 1 μg of poly (dIdC) and incubated at room temperature for 30 minutes. The 26 bp DNA probe was designed using the murine TRPC6 promoter (-131 to -106) with the following sequence: 5'-TTTCGATC**CCTTTAAAGG**TGGGGATC-3' and labeled with γP32 and made double stranded with cold antisense to the shown sequence.

Store operated Ca^{2+} entry (SOCE). Depending on the experiments cardiac or dermal fibroblasts were seeded on 25 mm circular coverslips and incubated for 24-48 hrs in DMEM containing 1% FBS with or without various pharmacologic treatments or adenoviral gene transfer. Fibroblasts were washed in Ringer's solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES), loaded with 5 μM FURA2-AM (Invitrogen) for 20 minutes, and washed in Ringer's three times over a 20 minute period for de-esterification. Coverslips were loaded into a perfusion chamber and excited at 340/380 nm. Measurements were made at 200X using Nikon Eclipse Ti-U inverted microscope equipped with a Delta Scan dual-beam spectrofluorophotometer (Photon Technology, Birmingham, NJ). Baseline Ca^{2+} transients were measured in Ca^{2+}-Mg^{2+} free Ringer's (CMF) solution over a 3 minute period at which time CMF Ringer's containing 10 μm cyclopiazonic acid (CPA) and 200 μm EGTA (CMF+CPA) was perfused for 20 minutes to reach a steady-state and deplete intracellular Ca^{2+}. Ringer's solution replete with Ca^{2+} was added back for another 3 minutes, at which time the cells took up Ca^{2+} depending on their degree of SOCE induction. The average maximal SOCE was calculated as the change in the 340/380 ratio (ΔRatio). Fura signals were also calibrated to define exact Ca^{2+} concentrations using previously described methods (Grynkiewicz et al., 1985).

Statistical Tests. Statistical significance was determined by ANOVA and Newman-Keuls pairwise comparisons for multivariate experiments and t-test for experiments with 2 groups.

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