SUPPLEMENTAL MATERIAL

Detailed Methods

Adeno-Associated Virus Serotype 9 (AAV9)

To generate recombinant AAV encoding Pin1, pTRUF-CMV_{enh}MLC800 was constructed by modifying pTRUF12 (a gift from Dr. Roger Hajjar) by first removing the region encoding GFP that was downstream of the IRES. New restriction sites for Nhe1, Pme1, Xho1, and Mlu1 were inserted into the multiple cloning site. The CMV promoter was replaced with a composite promoter comprised of an 800bp fragment of the MLC2v promoter downstream of a CMV enhancer.[1] AAV9 vectors with wild-type capsids were generated by co-transfection of the helper plasmid pDG-9 (a gift from Dr. Roger Hajjar). pTRUF-CMV_{enh}MLC800-Pin1 was created by subcloning the human Pin1 cDNA from pcDNA3.1-human Pin1 (a gift from Dr. Giannino Del Sal).[2] To prepare the recombinant AAV9, HEK293T cells were grown in DMEM/F12 containing 10% FBS and penicillin/streptomycin at 37°C and 5% CO_2. HEK293T cells were plated at $8x10^6$ per T-175 flask. Twenty-four hours after plating, cultures were transfected using Polyethylenimine "Max" (MW 40,000, Polysciences, cat.# 24765) as follows: fifteen µg of helper plasmid and 5 µg of pTRUF plasmid were mixed with 1 ml of DMEM:F12 without antibiotics, and 160 µl of polyethylenimine (0.517 mg/ml), mixed for 30 sec, then incubated for 15 min at room temperature. This mixture was then mixed with 18 ml DMEM/F12 containing 2% FBS, penicillin/streptomycin, then used to replace the media on the HEK293T cultures. The cultures were then rocked intermittently for 15 min. Three days after transfection, the cells were collected from six flasks and then centrifuged at 500 x g for 10 min. The cells were resuspended in 10 ml of lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris-HCL), then subjected to three rounds of freeze-thaw, followed by treatment with Benzonase (1500U of Benzonase; Novagen) and 1 mM $MgCl_2$ at 37°C for 30 min. The cell debris was collected by centrifugation at 3,400 x g for 20 min. The supernatant containing the AAV9 virus was then purified on an iodixanol gradient comprised of the following four phases: 7.3 ml of 15%, 4.9 ml of 25%, 4 ml of 40%, and 4 ml of 60% iodixanol (Optiprep;

Sigma-Aldrich) overlayed with 10 ml of cell supernatant. The gradients were centrifuged in a 70Ti rotor (Beckman Coulter) at 69,000 rpm for 1 hour using OptiSeal Polyallomer Tubes (Beckman Coulter). The virus was collected by inserting a needle 2 mm below the 40%-60% interface and collecting 4 to 5 fractions (~4 ml) of this interface and most of the 40% layer. The fractions were analyzed for viral content and purity by analyzing 10 µl of each fraction on a 12% SDS-PAGE gel (BioRad) followed by staining with InstantBlue (Expedeon) to visualize the viral capsid proteins, VP1, VP2, and VP3. The virus was then collected from the fractions of several gradients and the buffer was exchanged with lactated Ringer's using an ultrafiltration device, Vivaspin 20, 100kDa MWCO (GE Healthcare). An aliquot of the final viral preparation was then fractionated on a 12% SDS-PAGE gel, stained with InstantBlue, and then compared with a similarly stained gel of a virus of a known titer. Alternatively, qRT-PCR was performed using a forward primer (AAGTCTCCACCCCATTGACGT) and reverse primer (AGGAGCCTGAGCTTTGATTCC), which flank the CMV_{enh}MLC800 composite promoter. A pTRUF vector containing the CMV/MLC800 promoter was used as a standard to determine copy number. pTRUF-CMV_{enh}MLC-empty was used to generate an analogous control virus. Six-week-old male C57/BL6 mice were anesthetized with 2% isoflurane. The AAV9-control or AAV9-Pin1 ($1x10^{11}$ total virus particles) was injected via the tail vein.

Transverse aortic constriction (TAC)

TAC was performed in 8-week-old male Pin1 knockout (KO) mice and 12 week-old male C57/ BL6 mice injected with AAV9. Mice were subjected isoflurane (2.0%, Abbot Laboratories). Ligation of the aorta was performed by using a 7-0 suture between the innominate and left common carotid arteries with an overlying 27-gauge needle for Pin1KO mice and 26-gauge needle for C57/ BL6 mice with AAV to produce a discrete stenosis. After ligation, the needle was withdrawn.

Echocardiography

Echocardiography was performed by using a Vevo770 High-Resolution *In Vivo*

Micro-Imaging System (VisualSonics). Mice were subjected a minimum dose of isoflurane (0.5- 0.8%, Abbot Laboratories). M-mode echocardiography of the midventricle was recorded and left ventricular (LV) end-diastolic diameter (LVDd), end-systolic diameter (LVSd) and LV posterior wall thickness (Pwd) were obtained.

Hemodynamics

Mice under chloral hydrate sedation were subjected to catheterization of the right carotid artery to enter the left ventricle. The catheter, a 1.2F PV- 4.5mm Electrode, was supplied by Scisense and data acquisition and analysis was performed using LabScribe2 software (Iworx Systems, Inc.).

Cardiomyocyte culture

Neonatal rat cardiomyocytes (NRCMs) were isolated from hearts of 1- to 2-day-old neonatal rats by trypsin digestion using standard procedures. After enzymatic digestion, cell preparations were preplated for 2hours in M199 medium (Cell-Gro) with 15% fetal bovine serum (FBS) to reduce non-myocyte contamination. NRCMs were plated in dishes pretreated with gelatin (Sigma). For cellular hypertrophy experiments in vitro NRCMs were cultured with low serum media for 24hours prior to hypertrophic stimuli. Cells were then subjected to 10% FBS or 10^{-6}M PE.

Histology

Hearts were arrested in diastole with 0.1 M $CdCl_2$, fixed in 4% paraformaldehyde through retrograde aortic perfusion, and embedded in paraffin. Heart sections were deparaffinized, and antigen was retrieved in 1mM citrate (pH 6.0) for 15min and blocked in TNB buffer (Perkin-Elmer) for 1hour. The samples were exposed to primary antibodies. Cy3- or Cy5-conjugated IgG (Jackson ImmunoResearch) was used as secondary antibodies. TO-PRO-3 iodide was used to stain for nuclei. Cross-sectional area of myocytes with a centrally located nucleus and overall circular shape was measured in the LV free walls using Image J software (NIH).

Immunohistochemistry

NRCMs cultured were fixed in 4% paraformaldehyde. NRCMs were immunostained with mouse anti-Tropomyosin (Tmyo) antibody (Ab, Sigma) and rabbit anti-GFP Ab (Invitrogen) at 4C overnight. Slides were incubated for 1hour at room temperature in the dark with Cy3-conjugated donkey anti-mouse IgG Ab and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). After secondary labeling, slides were stained with TO-PRO-3 iodide to stain for nuclei. Cross-sectional area of NRCMs was measured using Image J software (NIH). For over-expression experiments only GFP positive cells confirming transfection were analyzed.

Proximity ligation assay (PLA)

PLA was performed to manufacturers specifications. Briefly, primary antibodies were applied after 1 hour blocking in 10% horse serum and incubated at 4C overnight. The following day plus and minus PLA probes were applied and incubated at 37C for 60min. The slides were treated with ligation solution for 90min followed by 150min of amplification solution at 37C. Slides were washed three times in PBS followed by a final wash containing TO-PRO-3 iodide for 10min to stain for nuclei.

Immunoblot analysis

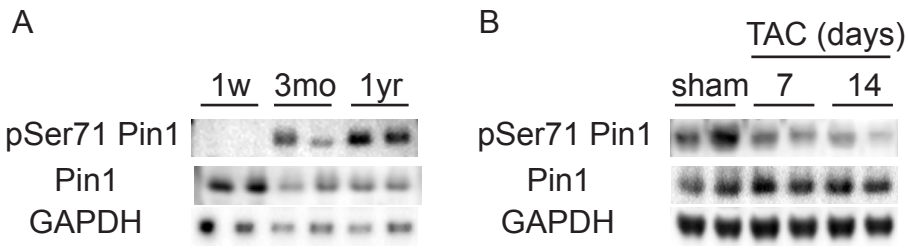
Lysates were prepared from cultured cells or cardiac tissue. Extracts were incubated at 100C for 5min and sonicated. Protein lysates were loaded onto a 4-12% NuPAGE Novex Bis-Tris Gels (Invitrogen) for electrophoresis. Separated proteins were transferred to PVDF membranes (Millipore), blocked with 1% BSA in Tri-Buffered Saline Tween-20 (TBST) for 1hour at room temperature, and exposed to primary antibodies. Alkaline phosphatase (AP), horseradish peroxidase (HRP) or Cy5-conjugated IgG (Jackson ImmunoResearch) was used as secondary antibodies. Fluorescence signal was detected using a Typhoon 9400 fluorescence scanner (Amersham Biosciences). Densitometric quantification was done by ImageJ software (NIH).

Adenovirus and siRNA

Adenovirus harboring EGFP or human Pin1 with EGFP was made using the AdEasy system (Agilent Technologies). Adenovirus vectors were prepared and concentrated as described previously.[3] NRCMs were transduced for 1hour with purified virus, and then re-fed with M199 media containing 10% FBS. On the following day, media were changed to M199 media containing 0.5% FBS. NRCMs were transfected with siRNA to Pin1 (Invitrogen) by using HiPerfect (Qiagen). 3μL siRNA and 12μL HiPerfect were diluted in 100μL low serum M199 medium. After incubation for 5min, transfection complexes were added to NRCMs.

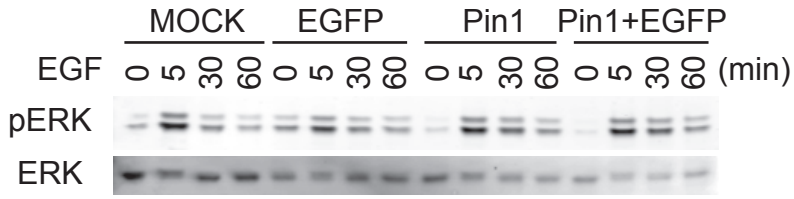
RNA isolation and Quantitative RT-PCR

Total RNA was extracted from the heart tissues or cultured cardiomyocytes using Quick-RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on all samples in duplicate using Quanti-Tect SYBR Green PCR kit (Qiagen).



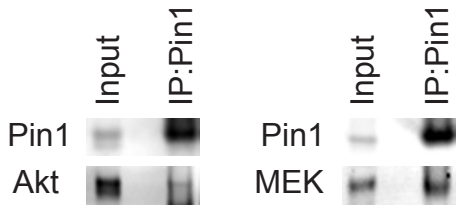
Online Figure I. Pin1 phophorylation on Ser71 increases with aging and decreases after pressure overload in the murine heart

A, Immunoblot showing phosphorylation of Pin1 on Ser71 (pSer71 Pin1) in the heart during development at 1 week (1w), 3 months (3mo), and 1 year (1yr) of age. B, Immunoblot showing pSer71 Pin1 expression in the heart 7 and 14 days after transaortic constriction (TAC).

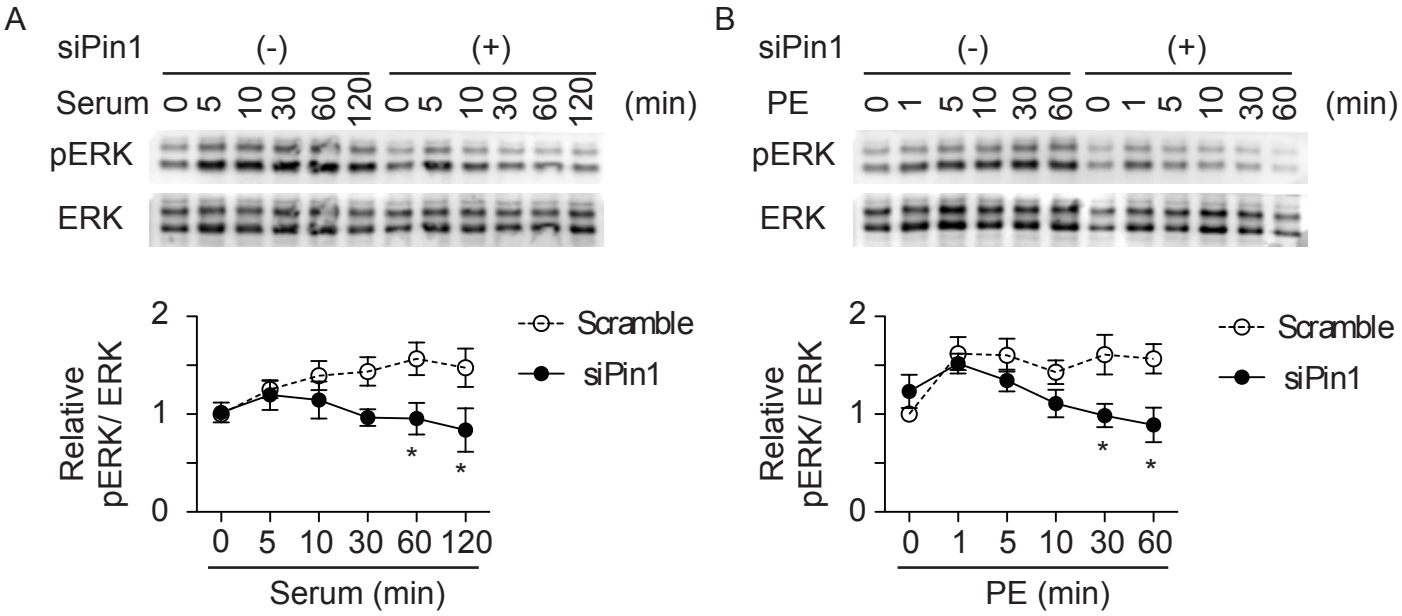


Online Figure II. Both Pin1 and Pin1 fused with EGFP prolong ERK activation in HeLa cells

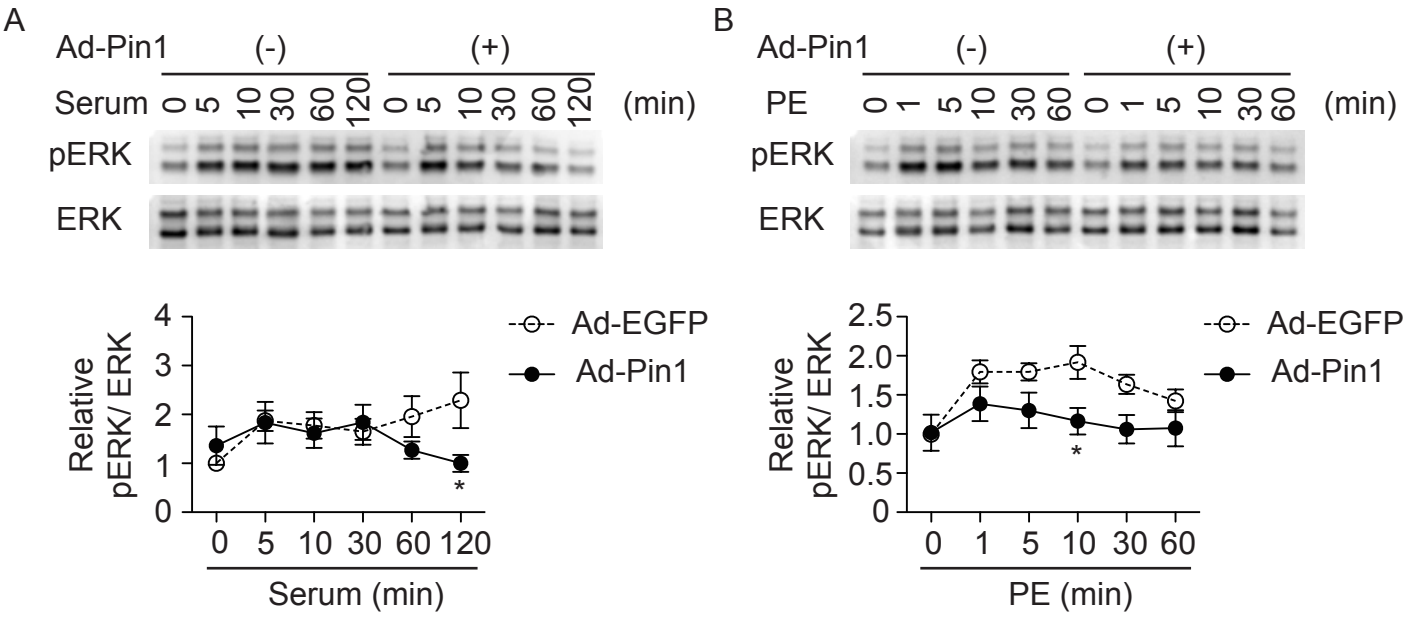
Immunoblot showing phosphorylated ERK (pERK) expression after EGF treatment in HeLa cells expressed wtih MOCK, EGFP, Pin1 or Pin1 fused with EGFP (Pin1+EGFP).



Online Figure III. Pin1 binds with Akt and MEK
Immunoblots showing binding of Pin1 to Akt (left) and to MEK (right) in immunoprecipitation assay against Pin1.



Online Figure IV. siPin1 attenuates ERK activation upon serum or phenylephrine treatment
A, B, Immunoblot showing phosphorylated ERK (pERK) expression after serum (A) or phenylephrine(PE, B) treatment. The lower panels show quantitative analysis of pERK/ ERK expression. * $p<0.05$ versus Scramble at the same time point.



Online Figure V. Pin1 over-expression inhibits ERK activation induced with serum or phenylephrine treatment

A, B, Immunoblot showing phosphorylated ERK (pERK) expression after serum (A) or phenylephrine (PE, B) treatment. The lower panels show quantitative analysis of pERK/ ERK expression. * $p<0.05$ versus EGFP at the same time point.

Online Table

Cardiac Function in Pin1 knockout mice
at 6 months of age

	WT mice	Pin1KO
n	5	3
Pwd (mm)	0.76±0.07	0.65±0.03
LVDd (mm)	3.31±0.11	3.27±007
FS (%)	40.4±3.3	45.9±3.6

Supplemental References

1. Muller OJ, Leuchs B, Pleger ST, Grimm D, Franz WM, Katus HA, Kleinschmidt JA. Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors. *Cardiovasc Res*. 2006;70:70-78
2. Rustighi A, Tiberi L, Soldano A, Napoli M, Nuciforo P, Rosato A, Kaplan F, Capobianco A, Pece S, Di Fiore PP, Del Sal G. The prolyl-isomerase pin1 is a notch1 target that enhances notch1 activation in cancer. *Nat Cell Biol*. 2009;11:133-142
3. Martina Y, Avitabile D, Piersanti S, Cherubini G, Saggio I. Different modulation of cellular transcription by adenovirus 5, deltae1/e3 adenovirus and helper-dependent vectors. *Virus Res*. 2007;130:71-84