Supporting Information

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SI Materials and Methods

Antibodies. Anti-Erbin (ErbB2 interacting protein) antibody (rabbit polyclonal sera) was kindly provided by J.-P.B. Anti–β-actin antibody (Sigma-Aldrich), anti-ERK, anti-pERK (phosphorylated ERK) (Santa Cruz), anti-GAPDH (Santa Cruz), anti–Soc-2 suppressor of clear homolog (Shoc2) (Biotest), anti-pRaf1 (Millipore), and anti-pAKT (Cell Signaling) were purchased. These antibodies were used for Western blots and immunoprecipitation.

Mice. All mice lines were held and propagated in a specific pathogen-free environment. *Erbin*$^{-/-}$ mice were kindly provided by Jean-Paul Borg and were generated as described before (1). *Erbin*$^{-/-}$ genotyping was performed by PCR with genomic DNA extracted from mouse tail tips. Primers used for *Erbin*$^{-/-}$ mice genotyping were as follows: Erbin sense, 5′-CTAGT TCAAG GCCAG TCTGA-3′; Erbin antisense, 5′-CAGTT AGGGT TGCTG GATTA-3′; neomycin sense, 5′-TGA ATG AAC TGC AGG ACG AG-3′; neomycin antisense, 5-ATA CTT TCT CGG CAG GAG CA-3′. For all of the experiments the same number of male and female mice was used. Mice aged 6–8 wk were used for all of the experiments. All experiments were performed in compliance with the Israeli Prevention of Cruelty to Animals Law and were approved by the Hebrew University Animal Care and Use Committee.

Administration of Isoproterenol. For the induction of cardiac hypertrophy, 6-wk-old *Erbin*$^{-/-}$ and WT mice were administered 15 mg/kg isoproterenol i.p. for 7 d.

Abdominal Aortic Constriction. Abdominal aortic constriction (AAC) was used as a pressure overload-induced cardiac hypertrophy model. Eight-week-old *Erbin*$^{-/-}$ and WT mice (20–25 g) were anesthetized with 1.5% isoflurane [2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane]. A suprarenal aortic constriction was performed using either a 25 (mild) or 28 (severe)-gauge (G) needle to establish the diameter of constriction. Sham operated animals had placement of a loosely tied ligature at the same location.

Animals were killed and the ratios between their heart weight (HW), lung weight (LW), and body weight (BW) were calculated and expressed as mg HW or LW per g BW.

Human Left Ventricular Biopsies. Left ventricular (LV) tissue was obtained from male patients undergoing cardiac transplant for end-stage heart failure. Normal non–donor-suitable human LV tissue was obtained from healthy male individuals involved in road traffic accidents. At the time of transplantation or donor harvest, whole hearts were removed after preservation and transported in cold cardioplegic solution (cardioplegia formula and Hartmann's solution) similar to the procedure described before at Imperial College, London (2). Following analysis by a cardiovascular pathologist, LV segments were cut and stored immediately in RNAlater (Ambion).

Echocardiography. Echocardiograms of *Erbin*$^{-/-}$ mice and normal littermates were carried out on mice anesthetized with 1.5% isoflurane at baseline and after 7 d of isoproterenol treatment or 10 d after mild AAC. Echocardiography was performed using a VEVO 770 equipped with a 30-MHz linear transthoracic transducer (VisualSonics). Measurements were performed in triplicate using the leading-edge convention for myocardial borders as defined by the American Society of Echocardiography (3). The following parameters were measured: left ventricular end diastolic and end systolic diameters (LVEDD and LVESD), diastolic and systolic interventricular septal diameters (IVSD and IVSS), left ventricular diastolic area (LVEDA), left ventricular systolic area (LVESA), and heart rate (HR). Shortening fraction (SF) was calculated as follows: SF (%) = (LVEDD – LVESD)/LVEDD × 100.

Histology. Formalin-fixed, paraffin-embedded tissue samples were cut into 5-μm sections, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with H&E (Sigma-Aldrich) and Masson trichrome (Sigma-Aldrich) according to the manufacturer's instructions.

To evaluate the mean diameter of LV cardiomyocytes, the shortest diameter of each cardiomyocyte was measured only in nucleated transverse sections stained with H&E. At least 50 cardiomyocytes in each left ventricle were measured using an ocular micrometer disk with a linear scale at a magnification of 400×, and the average cardiomyocyte diameter of each specimen was calculated using cellSens imaging software (Olympus). Five hearts were measured in each group.

RNA Isolation. Whole hearts were harvested and frozen in liquid nitrogen. Total RNA was extracted using EZ-RNA Total RNA Isolation Kit (Biological Industries) for quantitative RT-PCR analysis. The extracted RNA was then precipitated in ethanol and dissolved in diethylpyrocarbonate-treated water prior to analysis.

Real-Time Quantitative PCR. Total RNA was extracted from hearts of *Erbin*$^{-/-}$ and WT mice. mRNA levels of various genes were quantified by SYBR Green incorporation (SYBR Green ROX Mix; ABgene). Real-time PCR was performed on the Rotor-Gene 3000 sequence detection system (Corbett). The primers used for gene amplification for real-time PCR were as follows: β-actin sense, 5′-CCTGATCCACATCTGCTGGAA-3′; β-actin antisense, 5′-ATTGCCGACAGGATGCAGA A-3′; Erbin sense, 5′-GCATCCGCAGACATCCAGTCCA-3′; Erbin antisense, 5′-GGCTGGC CCATTTGTCCATTACT-3′; B-type natriuretic peptide (BNP) sense, 5′-CACCGCTGGGAGGTCACT-3′; BNP antisense, 5′-GTGAGGCCTTGGTCCTTCAA-3′; atrial natriuretic peptide (ANP) sense, 5′-TTCTTCCTCGTCTTGGC CTTT-3′; ANP antisense, 5′-GACCTCATCTTCTACCGGCATCT-3′.

cAMP Measurement. Six- to 8-wk-old *Erbin*$^{-/-}$ and WT mice were administered 2 mg/kg isoproterenol i.p. (Sigma-Aldrich) for 2 min. Mice were killed by CO_2 and their hearts were removed immediately and frozen using liquid nitrogen. The intracellular cAMP content was measured using a colorimetric cAMP ELISA kit (Cell Biolabs) according to the manufacturer's instructions.

Cell Culture and siRNA Targeting Erbin. HEK293T and H9c2 cells were maintained at 37 °C in DMEM (Sigma-Aldrich) supplemented with 4 mM L-glutamine, 100 units per mL penicillin, 100 μg/mL streptomycin, and 10% (vol/vol) FBS (Biological Industries). The siRNA specifically targeting Erbin (5′-CACACUGUUGUAUGAUCAACCAU-3′) was designed and chemically synthesized by Invitrogen. Scramble siRNA (Invitrogen) was used as a control. The cells were transfected with Erbin-specific siRNA or control siRNA (24 pmol per well for six-well plates) using jetPRIME reagent (Invitrogen) (4 μL per well for six-well plates) when ∼80% confluent. The cells were incubated in six-well plates for 24 h prior to analysis.

Myocardial cells from ventricle fragments of hearts of 1-d-old Sprague–Dawley rats were isolated by serial trypsinization as previously described (4). Cells were suspended in F-10 medium (Biological Industries) containing 10% (vol/vol) heat-inactivated FBS and 10% (vol/vol) horse serum (Biological Industries) and penicillin/streptomycin antibiotic solution. This medium was also used as the standard culture medium in the experiments. The cell suspensions were enriched with myocytes by preplating on tissue-culture dishes for 30 min to allow attachment of fibroblasts. The cells were plated on 60-mm Petri dishes at a density of 10^6 cells per mL. For isoproterenol treatment, cells were incubated with serum-free medium for 18 h and treated with 10 μM isoproterenol for an additional 18 h.

Immunofluorescence Analysis. H9c2 cells cultured on glass coverslips were fixed with 4% (vol/vol) paraformaldehyde (Sigma-Aldrich) in PBS for 10 min followed by Triton X-100 (Merck) permeabilization. Heart tissues were frozen with Tissue-Tek O.C.T. (Sakura) at −80 °C. Sections 4 μm thick were fixed in an acetone/methanol solution in room temperature for 20 min. Fixed cells and tissues were incubated with 20% (vol/vol) FBS/PBS with 0.1% Triton X-100 for 1 h. Anti-Erbin was incubated at 4 °C overnight (dilution 1:100). Washing by PBS was followed by incubation with secondary antibody conjugated to Alexa Fluor Cy3 (Jackson) for 1 h. After secondary incubation, coverslips were dipped rapidly in PBS with Hoechst 33258 (Sigma-Aldrich) for nuclei staining. Samples were mounted with Vectashield mounting medium (Vector). Images were taken by Zeiss LSM710 confocal microscope.

Statistics. Comparisons between mutant mice and their normal littermates were carried out by two-tailed Student t test. Exact Mann–Whitney test was performed for echocardiography measurements before and after isoproterenol treatment. BNP and ANP mRNA levels, pERK, LW:BW ratio, HW:BW ratio, SF after AAC, cardiomyocyte diameter, and treatment or lack of treatment with isoproterenol or AAC were evaluated by exact Kruskal–Wallis test. Null hypothesis was rejected at the $P < 0.05$ level for all tests. Data are reported as mean ± SEM.

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2. Movassagh M, et al. (2010) Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure. *PLoS ONE* 5(1):e8564.
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4. Hallaq H, Hasin Y, Fixler R, Eilam Y (1989) Effect of ouabain on the concentration of free cytosolic Ca++ and on contractility in cultured rat cardiac myocytes. *J Pharmacol Exp Ther* 248(2):716–721.

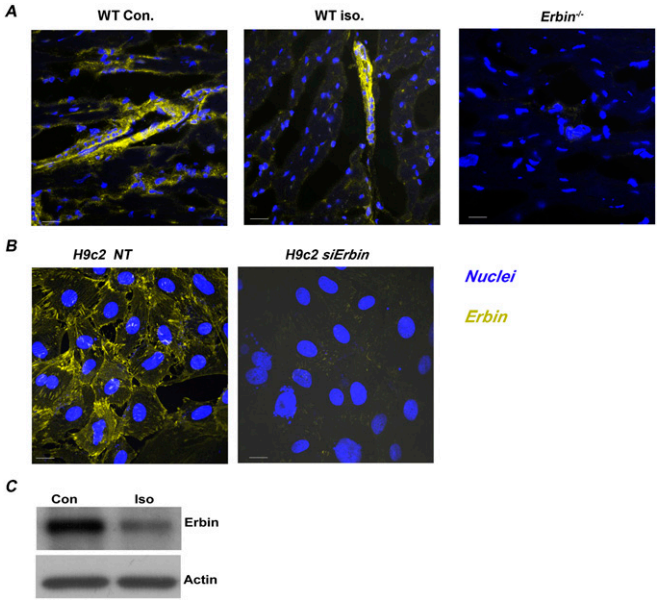

Fig. S1. (*A* and *B*) Immunofluorescence staining of endogenous Erbin (yellow) in hearts from WT mice at baseline (Con) and after treatment with isoproterenol (Iso) (*A*) and in the H9c2 cardiomyoblast cell line treated with nontargeting siRNA (NT) (*B*). The specificity of Erbin staining was verified by using *Erbin*$^{-/-}$ mice (*A*) and H9c2 cells that were treated with siRNA against Erbin for 24 h (siErbin) (*B*). (Scale bars, 20 μm.) (*C*) Erbin expression in neonatal rat cardiomyocytes that were treated either with 10 μM isoproterenol overnight or without isoproterenol (Con) in serum-free medium. One representative experiment out of three is shown.

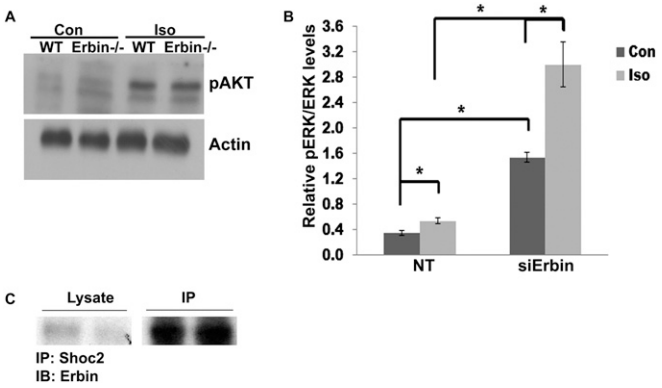

Fig. S2. (*A*) Western blot analysis of pAKT in hearts from WT and *Erbin*$^{-/-}$ mice at baseline (Con) and after treatment with isoproterenol for 7 d (Iso). One representative experiment out of four is shown. (*B*) HEK293T cells were transfected with either nontargeting siRNA or Erbin-specific siRNA for 24 h, followed by either overnight treatment with 10 μM isoproterenol or without isoproterenol in serum-free medium. Densitometry results are expressed as pERK:ERK ratio, and represent mean ± SEM ($n = 3$). *$P < 0.05$. (*C*) Coimmunoprecipitation of Erbin and Shoc2 in HEK293T cells using anti-Shoc2 antibody for immunoprecipitation (IP) and anti-Erbin antibody for immunoblotting (IB). One representative experiment out of three is shown.

Table S1. Morphometric measurement of *Erbin*$^{-/-}$ mice and their normal littermates

Parameter	WT con ($n = 5$)	WT iso ($n = 5$)	*Erbin*$^{-/-}$ con ($n = 5$)	*Erbin*$^{-/-}$ iso ($n = 7$)
HW, mg	128.7 ± 4.8	134.0 ± 4.8	121.3 ± 4.0	155.3 ± 5.0
BW, g	21.6 ± 1.1	20.8 ± 0.7	20.8 ± 1.7	20.9 ± 0.6
HW:BW, mg/g	6.0 ± 0.1	6.4 ± 0.2	5.9 ± 0.3	7.4 ± 0.1

Heart weight and body weight in *Erbin*$^{-/-}$ mice and their normal littermates at baseline and after treatment with isoproterenol. The results shown represent mean ± SEM.

Table S2. Echocardiography of untreated WT and *Erbin*$^{-/-}$ mice

Parameter	WT con ($n = 17$)	*Erbin*$^{-/-}$ Con ($n = 18$)
LVEDD, mm	3.4 ± 0.1	3.5 ± 0.1
LVESD, mm	1.8 ± 0.1	2.2 ± 0.2
SF, %	46.9 ± 1.4	39.4 ± 2.7

Cardiac function was measured by echocardiography of wild-type and *Erbin*$^{-/-}$ mice at baseline. The results shown represent mean ± SEM.

Table S3. Echocardiography of WT and *Erbin*$^{-/-}$ mice either untreated or treated with isoproterenol

Parameter	WT con ($n = 5$)	WT iso ($n = 5$)	*Erbin*$^{-/-}$ con ($n = 6$)	*Erbin*$^{-/-}$ iso ($n = 6$)
LVEDD, mm	3.2 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.2 ± 0.1
LVESD, mm	1.7 ± 0.2	2.1 ± 0.1	2.3 ± 0.4	1.5 ± 0.1
SF, %	48.2 ± 3.2	39.6 ± 3.9	34.2 ± 6.3	53.3 ± 2.7
LVEDA, mm^2	8.6 ± 0.1	7.9 ± 0.8	10.0 ± 1.2	7.1 ± 0.4
LVESA, mm^2	3.4 ± 0.7	2.9 ± 0.9	5.6 ± 1.8	1.5 ± 0.2
HR, bpm	302 ± 19	383 ± 29	344 ± 23	393 ± 17

Cardiac function was measured by echocardiography of wild-type and *Erbin*$^{-/-}$ mice at baseline and after 7 d of isoproterenol treatment. Results are expressed as mean ± SEM. bpm, beats per min.

Table S4. Morphometric measurement of *Erbin*$^{-/-}$ mice and their normal littermates

Parameter	WT sham ($n = 6$)	WT AAC ($n = 6$)	*Erbin*$^{-/-}$ sham ($n = 6$)	*Erbin*$^{-/-}$ AAC ($n = 6$)
HW, mg	113 ± 3	116 ± 8	113 ± 4	134 ± 10
BW, g	20.6 ± 0.2	22.5 ± 1.6	21.9 ± 1.4	20.9 ± 2.1
HW:BW, mg/g	5.5 ± 0.2	5.3 ± 0.1	5.2 ± 0.3	6.5 ± 0.2

Heart weight and body weight in *Erbin*$^{-/-}$ mice and their normal littermates following sham and after AAC operation. The results shown represent mean ± SEM.

Table S5. Echocardiography of WT and *Erbin*$^{-/-}$ mice at baseline and following AAC

Parameter	WT con (n = 6)	WT AAC (n = 6)	*Erbin*$^{-/-}$ con (n = 6)	*Erbin*$^{-/-}$ AAC (n = 6)
LVEDD, mm	3.5 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	3.9 ± 0.1
LVESD, mm	1.8 ± 0.2	2.2 ± 0.3	1.9 ± 0.2	2.9 ± 0.5
SF, %	46.3 ± 4.9	38.6 ± 5.9	43.4 ± 4.2	24.2 ± 2.2
HR, bpm	322.0 ± 7.0	355 ± 15	350 ± 15	356 ± 15

Cardiac function was measured by echocardiography of WT and *Erbin*$^{-/-}$ mice at baseline and after 10 d of mild (25G) abdominal aortic constriction. Results are expressed as mean ± SEM.