Supporting Information

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SI Materials and Methods

Primary Rat Neonatal Ventricular Cardiomyocyte Culture. Hearts from 3-d postnatal Wistar rats or PINK1 mice were dissected, minced, and enzymatically isolated with collagenase II (0.5 mg/mL; Invitrogen) and pancreatin (1 mg/mL; Sigma). Preplated cardiomyocytes were held for 36 h in the presence of 25 μM arabinosylcytosine (AraC; Sigma) and 5% donor horse serum (Sigma) to inhibit noncardiomyocyte proliferation. Spontaneously beating cardiomyocytes were subjected to treatment with Ang II (A9525; Sigma), antimycin (A8674; Sigma), or JC-1 (T-3168; Sigma) in culture medium DMEM/F12 containing 3 mM Na-pyruvate, 2 mM glutamine, routine antibiotics (Gibco), 0.2% BSA (Sigma), 0.1 mM ascorbic acid, and 0.5% insulin-transferrin-selenium (Sigma).

Human Cardiac Samples. Human studies were conducted with the approval of the University Health Network Research Ethics Board (protocol 10–0703-TE). Informed written consent was obtained from patients with end-stage HF before the insertion of a left ventricular assist device. We purchased normal adult human LV lysates (ProSci).

Echocardiography. Echocardiography in anesthetized mice (2% isoflurane, 98% oxygen) was performed using a 15-MHz linear ultrasound transducer (Vivid7; GE). Body temperature was maintained at 37 °C. M-mode measurements of the LV end-diastolic diameter (LVEDD) and LV end-systolic diamter (LVESD) were made in triplicate from short-axis views at the level of the papillary muscle and averaged over three to six beats. LVEDD was measured at the time of the apparent maximal LV diastolic dimension, whereas LVESD was measured at the time of the most anterior systolic excursion of the posterior wall. LV fractional shortening (FS) was calculated as follows: FS = (LVEDD − LVESD)/LVEDD × 100%.

Antibodies. Actin (cardiac; A9357; Sigma), α-actinin (cardiac; A7811; Sigma), Atp5g2 (ARP40228; Aviva), Atp5b (A9728; Sigma), collagen I (ab292; Abcam), collagen III (ab7778; Abcam), CoxIV (ab16056; Abcam), Cytb5 (ab69801; Abcam), Drp1 (611738; BD Transduction), lamin A/C (39287; Active Motif), mitofusin 2 (M9073; Sigma), Npm1 (B0556; Sigma) Opa1 (ab42364; Abcam), PINK1 (H00065018-A01; Abnova), phosphothreonine (9381; Cell Signaling), von Willebrand factor (AB7356; Millipore), V5 (R960-25; Invitrogen), Alexa Fluor 488-conjugated WGA (W7024; Molecular Probes), appropriate Alexa Fluor-conjugated anti-IgG antibodies (Molecular Probes).

Lentiviral PINK1 Constructs. Lentiviruses (1) were obtained from Addgene (pLenti6-DEST PINK1-V5 WT, pLenti6-DEST PINK1-V5 KD). HEK293FT cells were used for homologous recombination and packaging. Lentiviral supernatants were 100-fold concentrated by PEG precipitation. Cardiomyocytes were sequentially transduced at 10 pfu/cell in the presence of 4 μg/mL polybrene (Sigma) 0, 12, and 24 h after seeding.

Heart Tissue Extracts, Organelle Fractionation, and Isolation of Mitochondria. Total LV extracts were generated with RIPA buffer (Cell Signaling). Protein content was determined with the Bradford protein assay kit (23200; Pierce) and BSA as standard. Nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Reagents Assay kit (Pierce). Isolation of mitochondria from adult mouse ventricle was performed using the Mitochondrial Isolation kit (MITOISO1; Sigma).

Determination of the Mitochondrial Membrane Potential (ΔΨm). JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). The depolarization ΔΨm occurs at early stages of cell death. This potential-sensitive emission shift from red to green is due to concentration-dependent formation of red fluorescent J aggregates, which in turn is dependent on ΔΨm. Thus, decreases in ΔΨm are measured by decreases in the intensity of emitted red fluorescence. Neonatal mouse ventricular cardiomyocytes in HBSS (without phenol red) were incubated with JC-1 (5 μg/mL) for 1–2 min and then treated with antimycin. JC-1 emission at 525/595 nm was recorded (1 reading/min for 30 min) using a fluorescence spectrophotometer (Flex Station 3; Molecular Devices). The rate between two time points (Δemission at 595 nm/min) was calculated in the most linear range of decline for JC-1 emission.

Detection of Oxidative Stress and Antioxidant Mechanisms. Ventricular specimens were snap frozen in dry ice and stored at −80 °C. Each sample was measured in duplicate. Final values were normalized by the total protein concentration for each sample. All assays were performed according to the manufacturer's instructions with minor modifications: MDA/4-HAE (Bioxytech LPO-586 kit; Oxis), GSH/GSSG (Bioxytech GSH/GSSG-412 kit; Oxis), aconitase (Bioxytech Aconitase-340 kit; Oxis), 8-OHdG (Bioxytech 8-OHdG-EIA kit; Oxis), SOD (SOD activity kit; 900–157; Assay Designs), and ATP (ATP Bioluminescence Assay kit CLS II; Roche).

Immunofluorescence Microscopy, Morphometric Analyses, and Cell Death Assays. Longitudinal or cross-sections (8-μm thickness) were stained with Masson (HT15-1KT; Sigma) for cardiac muscle. Cardiomyocyte cross-dimensions and cell size of isolated cardiomyocytes were determined by planimetry of immunofluorescence microphotographs using ImageJ 1.43 (National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/). We quantified fragmented genomic DNA in isolated cardiomyocytes and cardiac ventricular specimens by immunocytochemistry and TUNEL assay (Roche). Caspase 3 proteolytic activity in LV tissue extracts (20–30 μg total protein) was determined using the Caspase-3 Colorimetric Activity Assay kit (APT131; Millipore).

Immunoblotting and PINK1 Immunocomplex Kinase Assay. For immunoblotting, we used SDS/PAGE gels (4–12% NuPAGE; Invitrogen), PVDF membranes (iBlot; Invitrogen), horseradish-peroxidase conjugated secondary antibodies (Amersham, GE), and an enhanced chemiluminescence system (Amersham, GE). To determine PINK1-dependent kinase activities, mitochondria were lysed in cell lysis buffer (Cell Signaling). Extracts were incubated with anti-V5 antibodies (1 μL/reaction) and protein G sepharose (GE Healthcare) for 3 h at 4 °C. Negative control reactions contained either control antimouse IgG, no antibodies, or samples were omitted. Immunoprecipitates were incubated for 30 min at 30 °C in 50 μL kinase buffer (9802; Cell Signaling: 25 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4), 30 μM ATP (9804; Cell Signaling), and 3 μM histone H1 (H4524; Sigma). Reactions were stopped by addition of SDS sample buffer.

Real-Time PCR Assays. Total RNA was isolated using TRIzol (Invitrogen). We used 1 μg total RNA/reaction for first-strand cDNA synthesis using random primers (Retroscript AM1710; Ambion). Real-time PCR assays were performed using 50 ng RNA or DNA/30 μL reaction volume (QuantiFast SYBR Green PCR kit 204054; Qiagen) on a Mastercycler EP Realplex (Eppendorf). Relative quantification of gene expression levels was performed using the ΔΔCt method with normalization to GAPDH, using the data analysis module (Eppendorf). We used the following primers (Invitrogen): ANF forward 5′-CATCACCCTGGGCTTCTTCCT-3′, reverse 5′-TGGGCTCCAATCCTGTCAATC-3′; BNP forward 5′-GCGGCATGGATCTCCTGAAGG-3′, reverse 5′-CCCAGGCAGAGTCAGAAACT G-3′; α-MHC forward 5′-CCAATGAGTACCG CGTGAA-3′, reverse 5′-ACAGTCATGCCGGGATGAT-3′; β-MHC forward 5′-ATGTGCCGGACCTTGGAA-3′, reverse 5′-CCTCGGGTTAGCTGAGAGATCA-3′; GAPDH forward 5′-ATG TTCCAGTATGACTCCACTCACG-3′, reverse 5′-GAAGACACCAGTAGACTCCACGACA-3′; Sk–α-actin forward 5′-AGACACCATGTGCGACGAAG-3′, reverse 5′-CCGTCCCCAGAA TCCAACACA-3′; Cytb forward 5′-TTCTGAGGTGCCACAGTTATT-3′, reverse 5′-GAAGG A AAGGTATTAGGGCTAAA-3′; and β-actin forward 5′-GAAATCGTGCGTGACATCAAAG-3′, reverse 5′-TGTAGTTTC ATGGATGCCACAG-3′.

Transmission Electron Microscopic Analysis. LV apexes from age-matched syngenic male PINK1$^{+/+,\ +/-,\ -/-}$ mice (4 wk) were rinsed in PBS (pH 7.4) and then fixed in 3.5% glutaraldehyde/Sorensen's phosphate buffer (pH 7.4) at room temperature for 24 h. After then being washed in phosphate buffer, samples were treated with 2% osmium tetroxide for 1 h. The samples were then dehydrated in a series of aqueous ethanol solutions 60, 75, 85, 95, and 100% and placed into propylene oxide for 30 min before being infiltrated with a 1:1 followed by 1:3 propylene–epon resin mixtures for 60 min each. The LV tissues were then transferred into pure epon resin and degassed under partial vacuum to ensure infiltration. Following overnight curing at 80 °C, 100-nm-thick sections were cut and placed on copper grids, stained, and examined with a Hitachi H-7000 TEM at HV = 75 kV with a digital image acquisition system. Mitochondrial cross-sectional area, in cardiomyocytes sectioned along the longitudinal plane, was determined by planimetry of electron microphotographs using ImageJ.

Statistical Analyses. Numerical data are expressed as the means ± SEM. One-way analysis of variance (ANOVA) with Dennett's posttest was used to analyze data between groups using Graph Pad Instate (version 3.10 for Windows, Graph Pad software). Significant ANOVA values were followed by post hoc comparisons of individual means using the Turkey–Kramer method. Level of significance was $P < 0.05$.

1. Beilina A, et al. (2005) Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc Natl Acad Sci USA* 102:5703–5708.

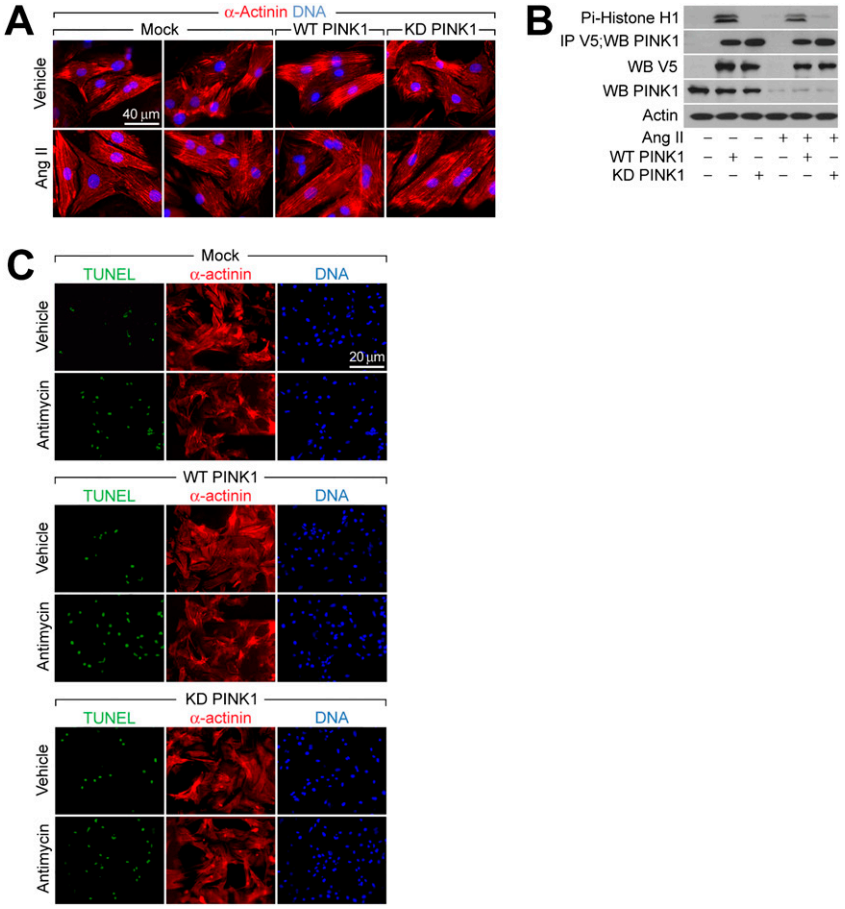

Fig. S1. (*A*) Wild-type PINK1 (WT PINK1) fails to promote cardiomyocyte hypertrophy. Mutant kinase-dead PINK1 (KD PINK1) does not inhibit cardiomyocyte hypertrophic growth. Fixed neonatal rat ventricular cardiomyocytes were stained with cardiomyocyte-specific antibodies to α-actinin. (*B*) Determination of ectopic PINK1-dependent histone H1 kinase activity in neonatal rat ventricular cardiomyocytes. Total cell lysates were subjected to immunoprecipitation (500 μg total protein/reaction) with anti-V5 tag antibodies and protein A agarose. Kinase activities were measured using histone H1 as substrate. Relative kinase activities were determined by immunoblotting using antiphosphothreonine antibodies. Protein expression was analyzed by Western blot using specific antibodies as indicated on the *Left*. One result of two independent experiments is shown. Pi, phospho; IP, immunoprecipitation; WB, Western blot. (*C*) Ectopic PINK1 does not inhibit cardiomyocyte apoptosis in vitro. Analysis of apoptosis of fixed cells was performed by in situ terminal transferase (TdT) mediated fluorescein-dUTP nick end labeling (TUNEL) immunofluorescence microscopy.

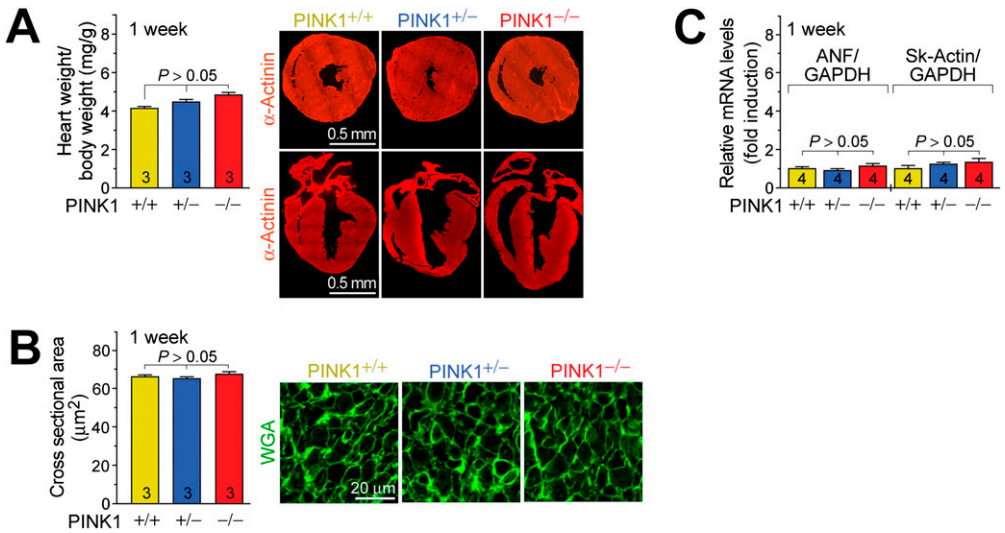

Fig. S2. PINK1 knockout mice exhibit no early cardiac phenotype. (*A*) Heart/body-weight ratios (*Left*) and anti–α-actinin immunofluorescence microscopical analysis (*Right*) of mycocardial sections derived from 7-d-old neonatal PINK1 littermates. Mean ± SEM. (*B*) Quantification of cardiomyocyte size (*Left*). WGA stain of myocardial cross-sections (*Right*). Mean ± SEM. (*C*) Quantitative RT-PCR analysis of mRNA levels of hypertrophic marker genes normalized to GAPDH mRNA. The ratio of hypertrophic markers to GAPDH in PINK1$^{+/+}$ hearts was arbitrarily set to 1. Mean ± SEM.

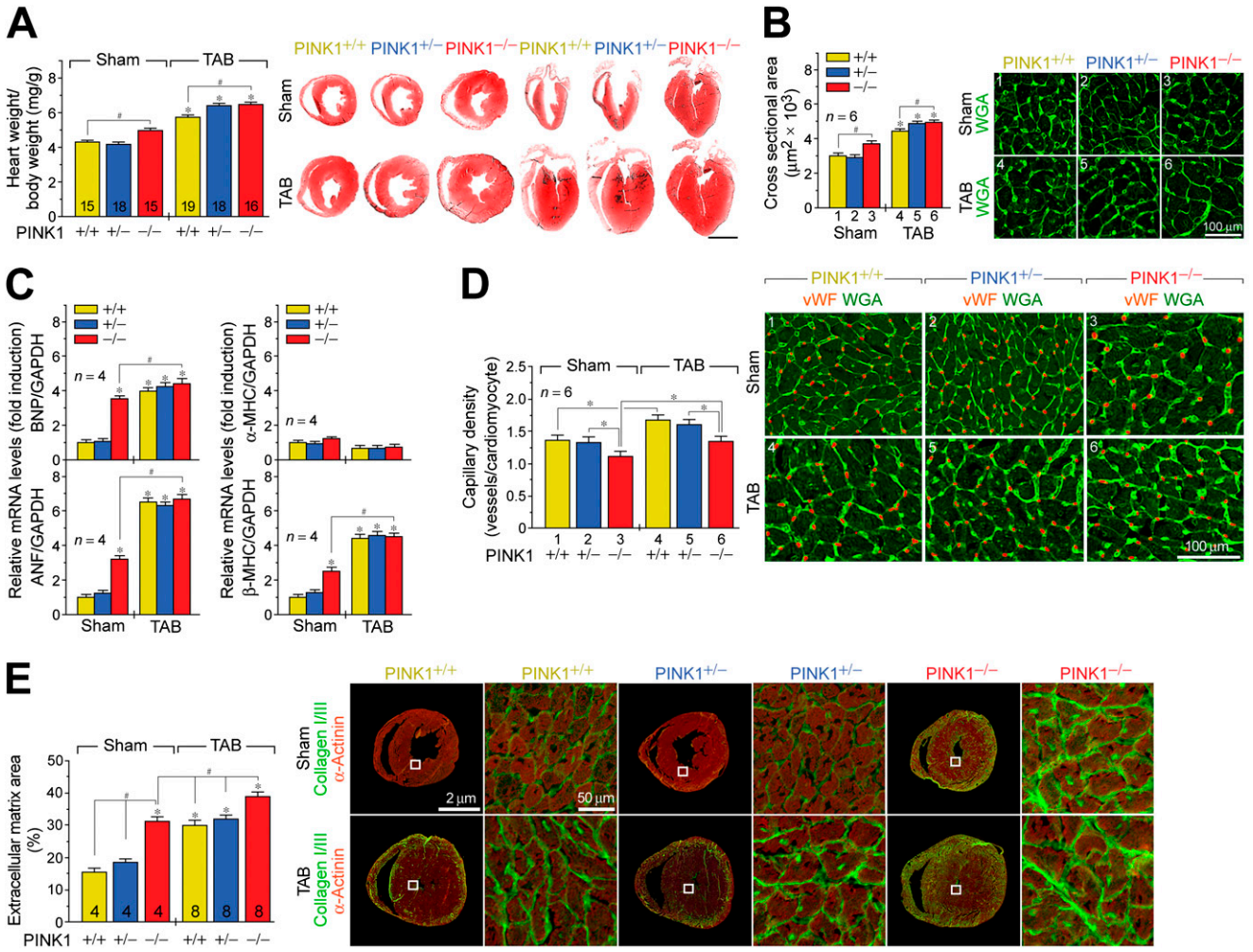

Fig. S3. (*A*) Heart/body-weight ratio (*Left*), Masson stain of myocardial cross-sectional (*Center*) and longitudinal sections (*Right*) 4 wk after TAB in 3-mo-old mice (*Upper*). Mean ± SEM. $^{*}P < 0.01$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. (*B*) Quantification of cardiomyocyte size (*Left*). WGA stain of myocardial cross-sections (*Right*). Mean ± SEM. $^{*}P < 0.05$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. (*C*) Quantitative RT-PCR analysis of mRNA levels of hypertrophic marker genes normalized to GAPDH mRNA. The ratio of hypertrophic markers to GAPDH in PINK1$^{+/+}$ sham hearts was arbitrarily set to 1. Mean ± SEM $^{*}P < 0.01$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. (*D*) Quantification of the number of microvessels/cardiomyocyte (*Left*) in hearts of 3-mo-old mice. Representative immunofluorescence micrographs of WGA and von Willebrand factor (vWF) of myocardial cross-sections (*Right*). Mean ± SEM. $^{*}P < 0.05$. (*E*) Quantification (*Left*) of extracellular matrix area by anticollagen I/III and anti–α-actinin immunofluorescence staining (*Right*) of cross-sectional LV specimen from 3-mo-old mice. Mean ± SEM. $^{*}P < 0.01$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. White box in low magnification fluoresent micrographs indicates region of interest in adjacent higher magnification. Sample size for each group is indicated inside the respective bar.

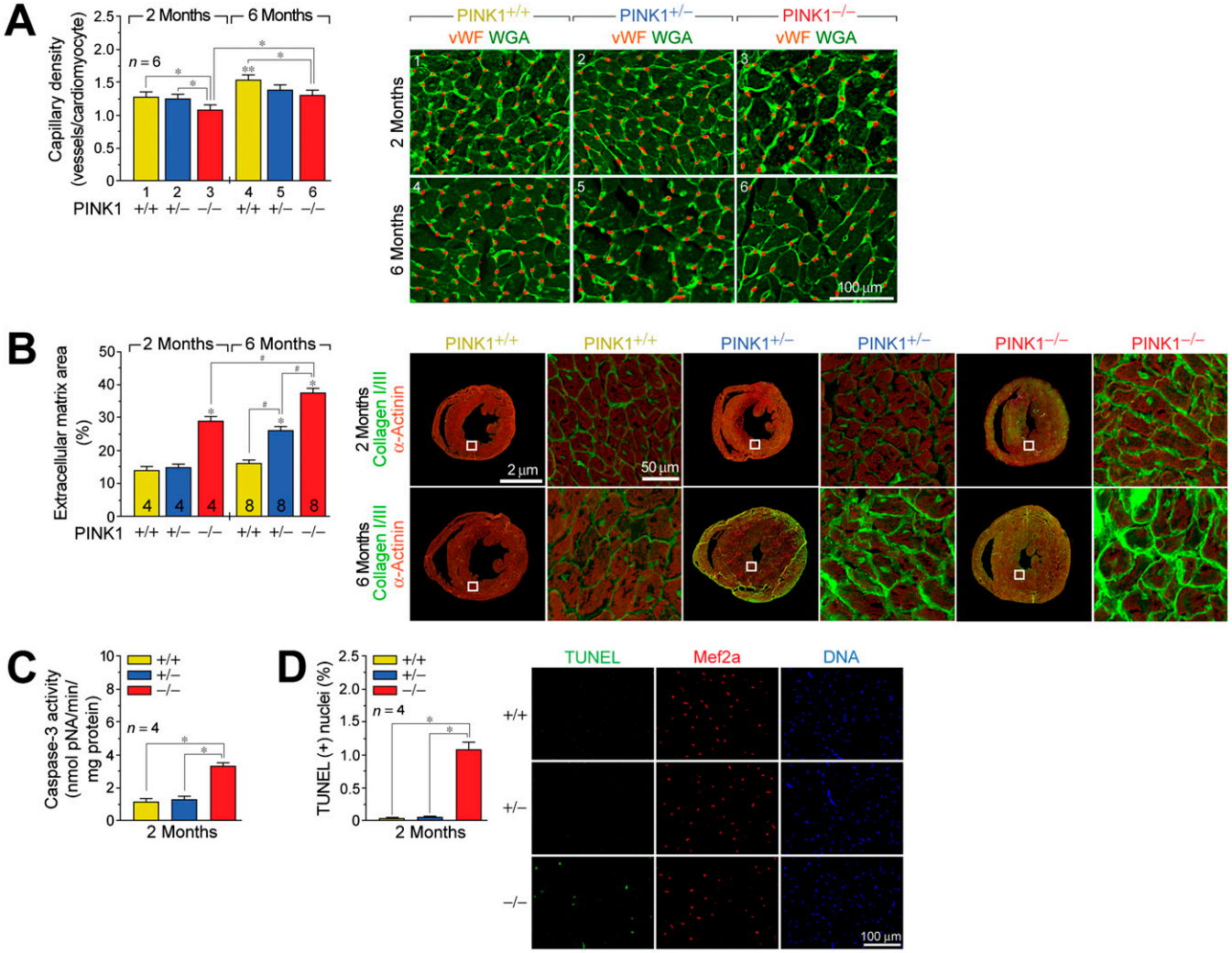

Fig. S4. PINK1-deficient mice exhibit impaired cardiac angiogenesis, increased fibrosis, and apoptosis. (*A*) Quantification of the number of microvessels/cardiomyocyte (*Left*). Representative immunofluorescence micrographs of wheat-germ agglutinin (WGA; green) and von Willebrand factor (vWF; red) of myocardial cross-sections (*Right*). Mean ± SEM. $*P < 0.05$. $**P < 0.05$ versus 2 mo. (*B*) Quantification (*Left*) of extracellular matrix area by anticollagen I/III (green) and anti–α-actinin (red) immunofluorescence staining (*Right*) of cross-sectional LV specimen. White box in low magnification fluoresence micrographs indicates region of interest in adjacent higher magnification. Mean ± SEM. $^{\#}P < 0.05$. $**P < 0.05$ versus 2 mo. (*C* and *D*) Inactivation of PINK1 induces significant cardiomyocyte apoptosis in vivo. (*C*) Determination of caspase-3 proteolytic activity in total LV free wall samples. Mean ± SEM. (*D*) Quantification of apoptosis in the LV free wall in 2-mo-old PINK1$^{-/-}$ mice (*Left*). Mean ± SEM. Apoptosis was determined by TUNEL staining (*Right*). $*P < 0.05$.

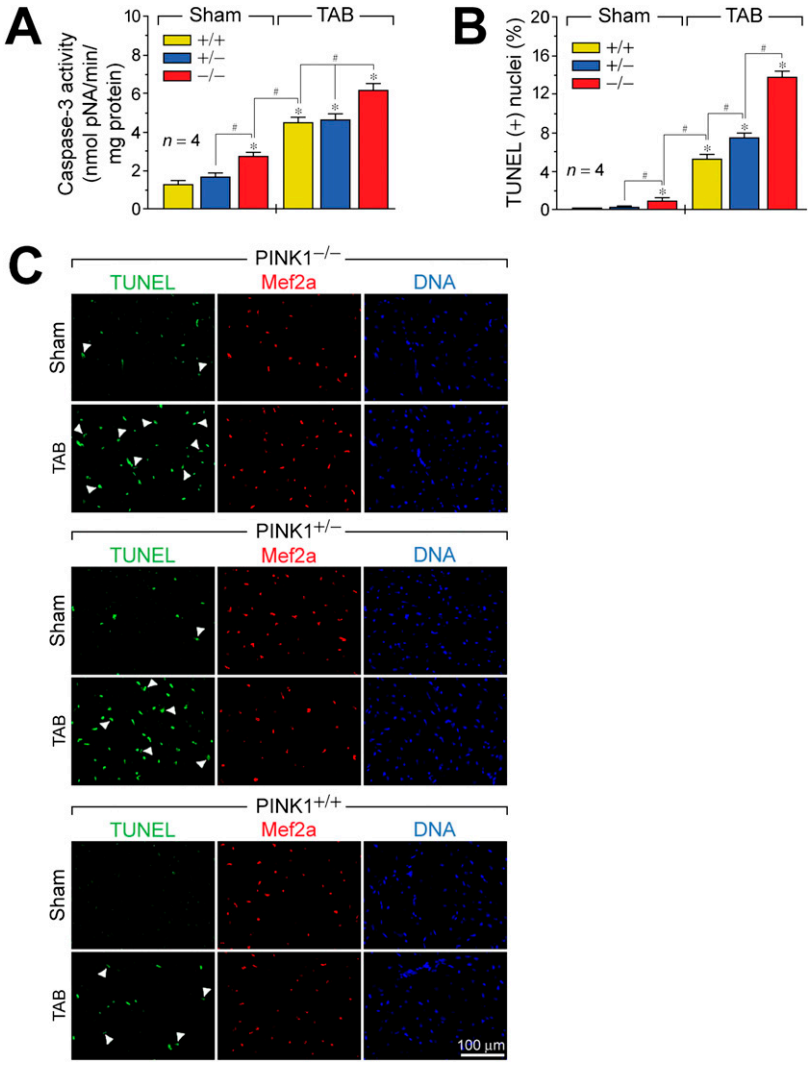

Fig. S5. Pressure overload induces exaggerated apoptosis in PINK1-deficient mice. (*A*) Determination of caspase-3 proteolytic activity in LV free wall specimen from 2-mo-old mice. Mean ± SEM $^{*}P < 0.01$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. (*B*) Quantification of apoptosis in the LV free wall. Mean ± SEM. $^{*}P < 0.01$ versus PINK1$^{+/+}$ sham. $^{\#}P < 0.05$. (*C*) Apoptosis in the LV free wall was determined by in situ TUNEL immunofluorescence histology.