SUPPLEMENTARY MATERIAL

SIRT2 Acts as a Cardioprotective Deacetylase in Pathological Cardiac Hypertrophy

Running Title: *Tang et al.; SIRT2 prevents cardiac hypertrophy*

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Supplementary Methods

Transgenic Mice

Transgenic mice with cardiomyocyte-specific SIRT2 overexpression were generated as previously described.[1] Briefly, a full-length human *SIRT2* (NM_012237.3) cDNA was subcloned into the pBluescript II vector under the control of a mouse *myosin heavy chain alpha* (*α-Mhc)* promoter. Then the pBSII-*αMHC-SIRT2* plasmids were cut with the restriction endonuclease *Not I* and the DNA construct was microinjected into C57BL/6J mouse zygotes. Transgenic mice were genotyped by polymerase chain reaction (PCR) analysis using genomic DNA from mouse tail and specific primers:
Forward: 5'-GTTTCTTCTCCTGTATGCAGTC-3';
Reverse: 5'-CCTCTTCTGAGATGAGTTTTTGTTC-3'.

Echocardiography Analysis

Echocardiography was performed using a VisualSonics Vevo 770 ultrasound biomicroscope (VisualSonics Inc) with a 15-MHz linear array ultrasound transducer. The left ventricular (LV) was assessed in both the parasternal long-axis and short-axis views at a frame rate of 120 Hz. End-systole or end-diastole was defined as the phase obtained with the smallest or largest LV area, respectively. Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the papillary muscle level. LV fractional shortening (FS) was calculated by ([LVEDD- LVESD]/LVEDD).

Plasmid and Recombinant Adenoviral Vectors

To obtain a vector overexpressing human SIRT2, the *SIRT2* (NM_012237.3) expression vector was constructed by ligating corresponding full-length cDNA into a pcDNA4 expression vector (Thermo, #V102020). The pcDNA3.3-LKB1-Flag plasmid is a kind gift from Dr. Cai-Sheng Lin of Xiamen University (Xiamen, China). The mutation of LKB1 at lysine 48 (LKB1K48R) was generated by PCR using PrimeSTAR® Max DNA Polymerase (TaKaRa, #R045A) and the specific mutated primers (Forward: 5'-CCCCCATCAGGTACCTGCCGATGAGCTTG-3'; Reverse: 5'-CAAGCTCATCGGCAGGTACCTGATGGGGG-3') designed using the QuickChange Primer Design (http://www.agilent.com). The pcDNA3.1-AMPKα1 and pcDNA3.1-AMPKα2 plasmids are kind gifts from Dr. Hengyi Xiao of Sichuan University (Chengdu, China).

The AdEasy Vector kit (Quantum Biotechnologies) was used to generate replication-defective adenoviral vectors expressing *SIRT2* (Ad-*SIRT2*), expressing *Lkb1* shRNA (Ad-sh*Lkb1*) or a control construct (Ad-Ctrl or Ad-shCtrl) as previously described.[1] Briefly, the *SIRT2* cDNA or *Lkb1* shRNA was cloned into the pAdTrack-CMV or pAdTrack-basic plasmid, and the pAdTrack-*SIRT2* or pAdTrack-sh*Lkb1* plasmid was obtained. Next, the pAdTrack-*SIRT2* or pAdTrack-sh*Lkb1* plasmid was linearized using the restriction endonuclease *Pme I*, and the linearized product was transformed into pAdEasy-1 electrocompetent BJ5183 cells. The clones with successful homologous recombination were picked and the plasmids were extracted and linearized with the restriction endonuclease *Pac I.* The linearized product was transfected into HEK293A cells to produce adenovirus expressing human SIRT2 or *shLkb1*. The sequence of shRNA targeting rat *Lkb1* mRNA (NM_001108069.1) is as follow:
5'-GGTTTCAAGGTGGACATCTGG-3'.

Cell Culture, Transfection and Drug Treatment

HEK293T, HEK293A, and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo, #11965-084) supplemented with 10% fetal bovine serum (FBS; Gibco, #10099-141) and

penicillin (100 U/mL)/streptomycin (100 μg/mL) (Thermo, #15140-122). Transfections were performed using VigoFect (Vigorous, #T001) according to the manufacturer's instructions.

Neonatal rat cardiomyocytes (NRCMs) were isolated from 1- to 3-day old Sprague-Dawley (SD) rats as described before.[1] Mouse cardiomyocytes were isolated from adult male mice. The isolation method is the same to NRCM isolation. NRCMs were grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL)/streptomycin (100 μg/mL) and 100 μM 5-bromodeoxyuridine (Sigma, #B5002) for 48 hours, and then infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 20 particles per cell. To induce hypertrophy, NRCMs were cultured in serum-free DMEM for 24 hours and treated with Ang II (Sigma, #A9525; 1 μM) for 48 hours. For other experiments, NRCMs were also treated with SU6656 (Selleck, #S7774; 5 μM), AGK2 (Sigma, #A8231; 10 μM), Compound C (Abcam, #ab120843; 10 μM), STO-609 (Santa Cruz Biotechnology, #sc-202820; 20 μM) for indicated time period as shown in figure legends.

Immunofluorescence Analysis

For immunofluorescence analysis in NRCMs, cultured NRCMs were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, blocked with 3% BSA solution for 1 hour and incubated with anti-α-actinin antibody (Sigma, #A7811; 1:200), anti-LKB1 antibody (Santa Cruz Biotechnology, #sc-32245; 1:200) overnight at 4°C. Then, the cells were washed and stained with fluorescence-conjugated secondary antibodies (Invitrogen; Alexa Fluor 594; #R37115) and Hoechst (Sigma, #861405). Immunofluorescence was analyzed with confocal microscopy FV1000MPE (Olympus Corporation), and the surface areas were measured using Image-Pro Plus 6.0 software.

Immunoprecipitation

Cells or heart tissues were lysed with immunoprecipitation (IP) buffer (Beyotime Biotechnology, #P0013) supplied with protease inhibitor cocktail (Roche, #04693159001) at 4°C. After being sonicated and centrifuged at 13,000g for 20 minutes, the lysates were precleared and incubated with the indicated primary antibodies overnight at 4°C, then incubated with Protein A/G PLUS-Agarose (Santa Cruz, #sc-2003) or ANTI-FLAG® M2 Affinity Gel (Sigma, #A2220) for 3 hours. The beads were then washed with IP buffer for five times, and the samples were harvested.

Western Blotting

Protein was extracted with RIPA lysis buffer (Beyotime Biotechnology, #P0013B) supplemented with protease inhibitor cocktail (Roche, #04693159001) and phosphatase inhibitor (Roche, #PHOSS-RO). Homogenates were sonicated and centrifuged at 4°C for 15 minutes, and the supernatants were used for western blotting. 20-50 μg of protein were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After being blocked with 5% fat-free milk in TBST, the membranes were incubated with individual primary antibodies overnight at 4°C. Subsequently, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO; #ZB2301, #ZB2305), and exposed to Pierce ECL Western Blotting Substrate (Thermo, #32106) for detection of protein expression. Antibodies to SIRT2 (#S8447), Tubulin (#T2200), and acetylated Tubulin (#T7451) were purchased from Sigma. Antibodies to GAPDH (#sc-47724), human SIRT2 (#sc-135794), LKB1 (#sc-32245), c-Src (#sc-8056), CPS1 (#ab3682), FKBP51 (#sc-271547) were purchased from Santa Cruz Biotechnology. Antibodies to AMPKα (#5831), p-AMPKα (#4188), LKB1 (#3047), p-LKB1 (#3482), ACC (#3676), p-ACC (#11818), acetylated lysine (#9441), mTOR (#2983), p-mTOR (#5536), Raptor (#2280), p-Raptor (#2083), IGF1R (#9750), p-IGF1R (#3918), Histone H3 (#9715), acetylated H3K27

(#8173), p-c-Src (#2101) were purchased from Cell Signaling Technology. Antibodies to MCD (#15265-1-AP) was purchased from Proteintech.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells and tissues was extracted using TRIzol reagent (Invitrogen, #15596026), and the first-stand cDNA was synthesized using First Strand cDNA Synthesis kit (New England Biolabs, #M0277). qRT-PCR was performed with SYBR Green Master Mix (TaKaRa, #R820A) to examine the relative mRNA levels of indicated genes. Sequences for qRT-PCR primers are shown below:
mouse *Anp* forward: 5'-TCTTCCTCGTCTTGGCCTTT-3', reverse: 5'- CCAGGTGGTCTAGCAGGTTC-3'; mouse *Bnp* forward: 5'-TGGGAGGTCACTCCTATCCT-3', reverse: 5'-GGCCATTTCCTCCGACTTT-3'; mouse *β-Mhc* forward: 5'-CGGACCTTGGAAGACCAGAT-3', reverse: 5'-GACAGCTCCCCATTCTCTGT-3'; mouse *Acta1* forward: 5'-CCAAAGCTAACCGGGAGAAG-3', reverse: 5'-GACAGCACCGCCTGGATAG-3'; mouse *IL-6* forward: 5'-CTGCAAGAGACTTCCATCCAG-3', reverse: 5'-AGTGGTATAGACAGGTCTGTTGG-3'; mouse *Rcan1.4* forward: 5'-TTGTGTGGCAAACGATGATGT-3', reverse: 5'-CCCAGGAACTCGGTCTTGT-3'; mouse *Ctgf* forward: 5'-CTTCTGCAGACTGGAGAAGC-3', reverse: 5'-CAGCCAGAAAGCTCAAACTTG-3'; mouse *Col1a1* forward: 5'-CTGGCGGTTCAGGTCCAAT-3', reverse: 5'-TTCCAGGCAATCCACGAGC-3'; mouse *Col3a1* forward: 5'-TGAATGGTGGTTTTCAGTTCAG-3', reverse: 5'-GATCCCATCAGCTTCAGAGACT-3'; mouse *Sirt2* forward: 5'-GCGGGTATCCCTGACTTCC-3', reverse: 5'-CGTGTCTATGTTCTGCGTGTAG-3'; mouse *Gapdh* forward: 5'-AGGTCGGTGTGAACGGATTTG-3', reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; rat *Anp* forward: 5'-GAAGATGCCGGTAGAAGATGAG-3', reverse: 5'-AGAGCCCTCAGTTTGCTTTTC-3'; rat *Bnp* forward: 5'-GGTGCTGCCCCAGATGATT-3', reverse: 5'-CTGGAGACTGGCTAGGACTTC-3'; rat *β-Mhc* forward: 5'-GCCCCAAATGCAGCCAT-3', reverse: 5'-CGCTCAGTCATGGCGGAT-3'; rat *Gapdh* forward: 5'-TGACAACTCCCTCAAGATTGTCA-3'; reverse: 5'-GGCATGGACTGTGGTCATGA-3'.

Measurement of the Enzymatic Activity of SIRT2 Protein

The SIRT2 protein was purified with immunoprecipitation from heart tissues of WT mice infused with saline or Ang II (1.3 mg/kg/day) for four weeks. The enzymatic activity of the SIRT2 protein was performed with the FLUOR DE LYS® SIRT2 fluorometric drug discovery assay kit (Enzo Life Sciences, #BML-AK556-0001) according to the manufacturer's instructions.

Measurement of Circulating Ang II Serum Level

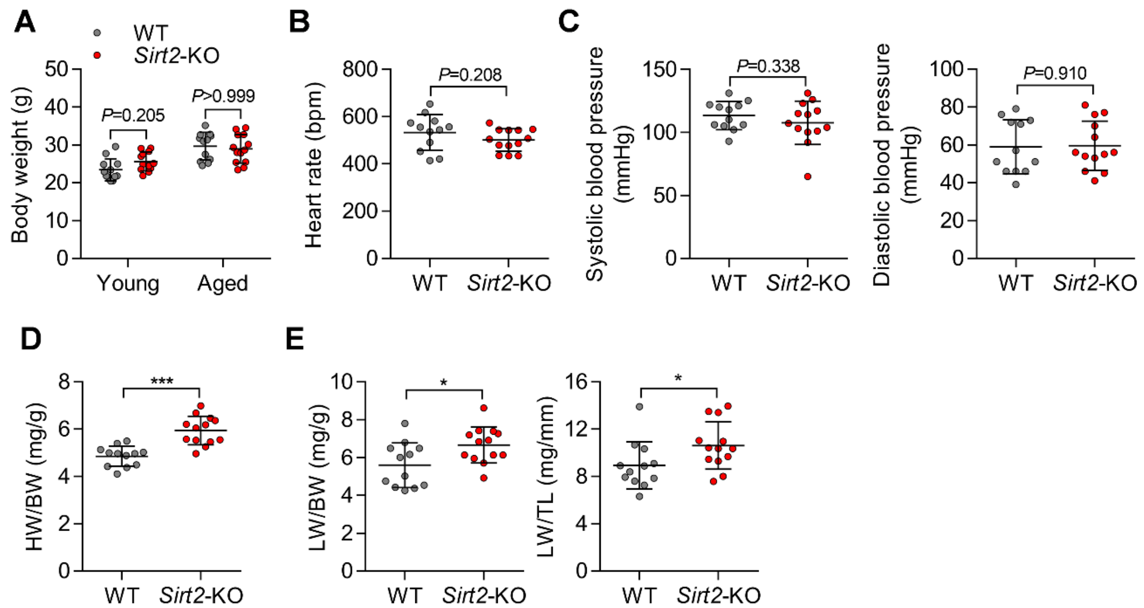
The serum of mice was collected and the measurement of circulating Ang II serum level was performed with the Angiotensin II ELISA kit (Enzo Life Sciences, #ADI-900-204) according to the manufacturer's instructions.

Supplementary Figures and Figure legends

Supplementary Figure 1

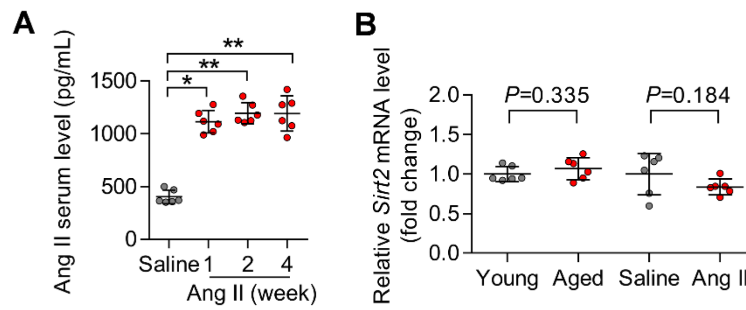
Supplementary Figure 1 Validation of *Sirt2*-knockout in mice. (**A**) qRT-PCR results showing the *Sirt2* mRNA levels in the hearts of wide-type (WT, n=6) and *Sirt2*-KO mice (n=6). (**B**) Western blotting showing the SIRT2 protein levels in the hearts (left) or isolated cardiomyocytes (right) of WT and *Sirt2*-KO mice.

Supplementary Figure 2



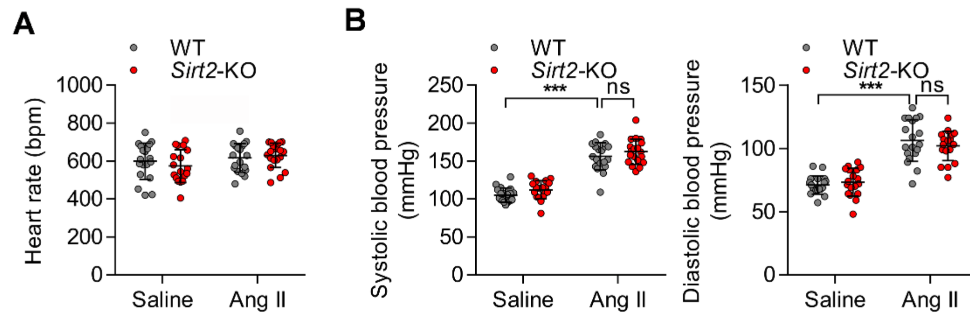
Supplementary Figure 2 Body weights and cardiac and hemodynamic features of aged WT and *Sirt2*-KO mice. (**A**) Body weight of WT and *Sirt2*-KO mice at 4 months (young) and 24 months (aged). n=12~13. (**B**) Heart rates of aged WT and *Sirt2*-KO mice (n=12~13). (**C**) Systolic and diastolic blood pressures of aged WT and *Sirt2*-KO mice (n=12~13). (**D**) Heart weight-to-body weight (HW/BW) ratios of aged WT and *Sirt2*-KO mice (n=12~13; ****P*<0.001). (**E**) Lung weight-to-body weight (LW/BW) or lung weight-to-tibia length (LW/TL) ratios of aged WT and *Sirt2*-KO mice (n=12~13; **P*<0.05).

Supplementary Figure 3



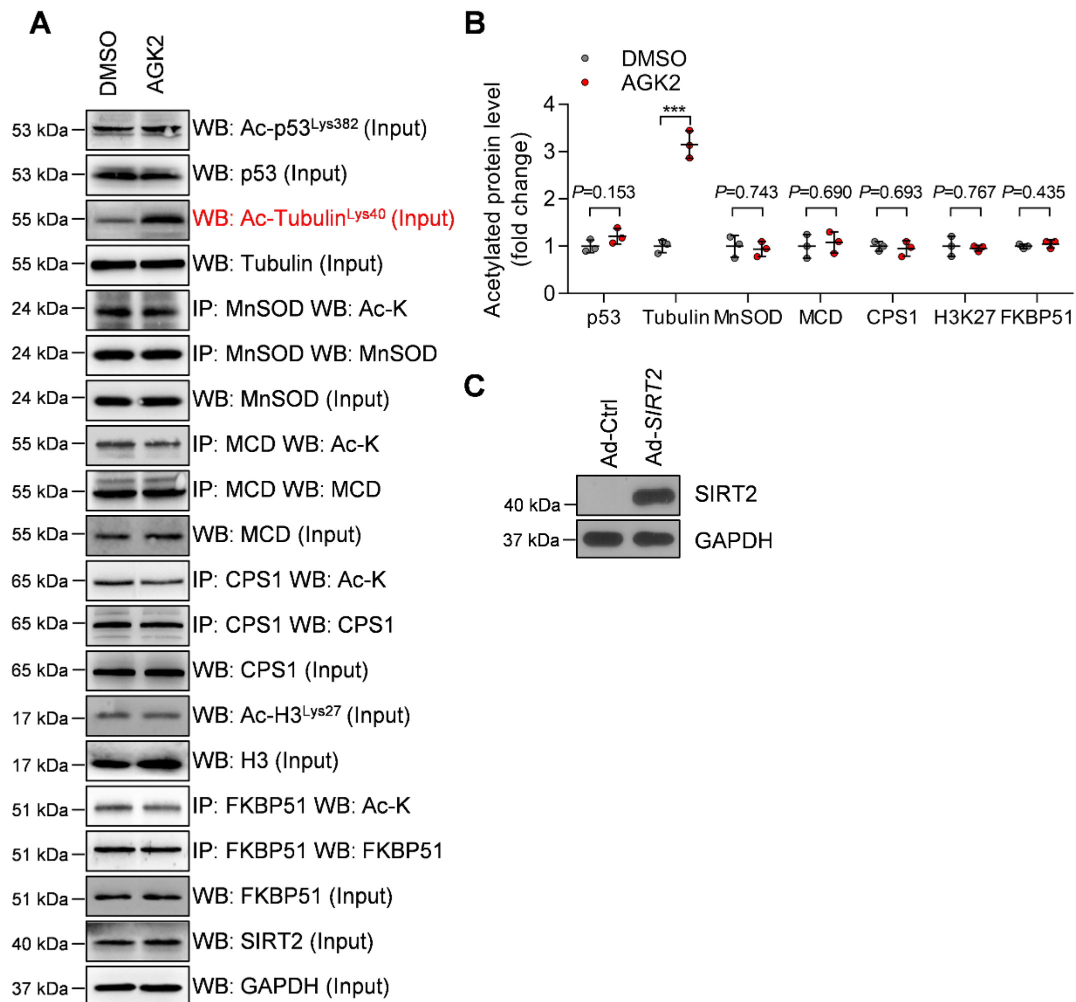
Supplementary Figure 3 Circulating Ang II level and *Sirt2* mRNA level in hypertrophic hearts. (**A**) Circulating Ang II serum level. The serum Ang II level was examined in mice treated with saline or 1/2/4 weeks of Ang II infusion with minipump (n=6; *P<0.05, **P<0.01). (**B**) qRT-PCR results showing the relative mRNA levels of *Sirt2* in mouse cardiac hypertrophy induced by aging and Ang II (n=6).

Supplementary Figure 4



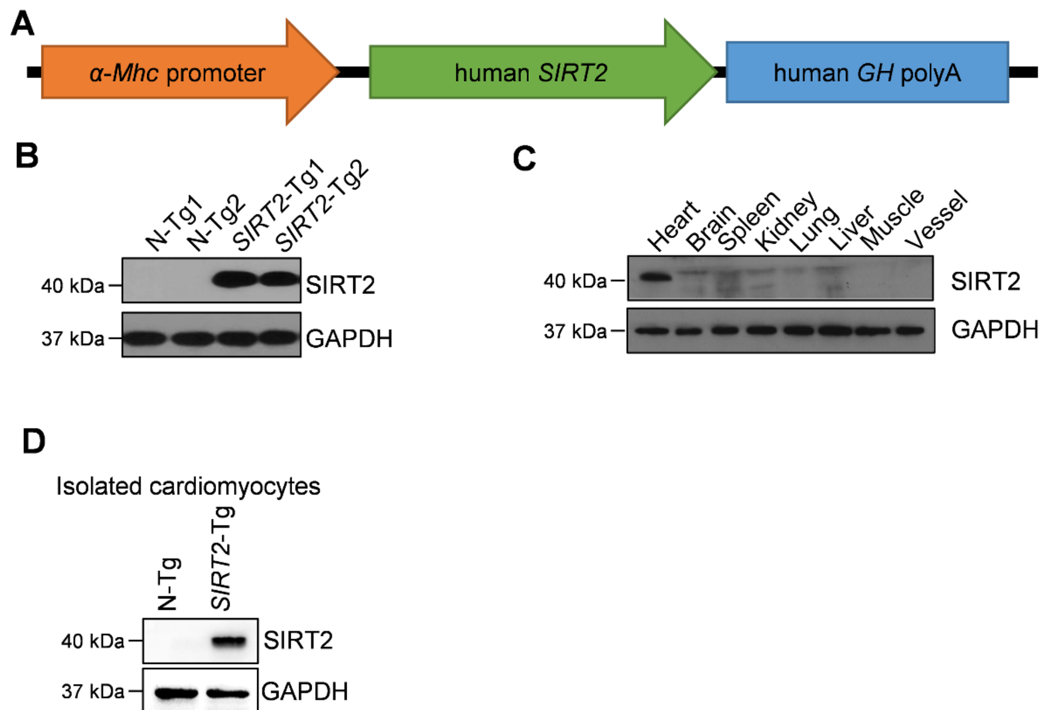
Supplementary Figure 4 Heart rates and blood pressures of the hearts of WT and *Sirt2*-KO mice infused with saline or Ang II for four weeks. (**A**) Heart rates in WT and *Sirt2*-KO mice infused with saline or Ang II (n=18~19). (**B**) Systolic and diastolic blood pressures in WT and *Sirt2*-KO mice infused with saline or Ang II (n=18~19; *** *P*<0.001, ns: not significant).

Supplementary Figure 5



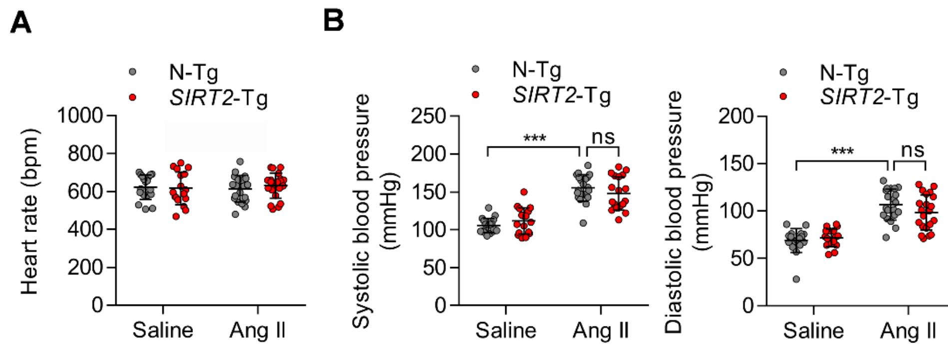
Supplementary Figure 5 SIRT2 inhibition and overexpression in neonatal rat cardiomyocytes (NRCMs). (**A**) AGK2 inhibition of SIRT2. NRCMs were treated with AGK2 (10 μM) for 24 hours, then immunoprecipitation and western blotting were performed to analyze the acetylated level of p53 (SIRT1 substrate), Tubulin (SIRT2 substrate), MnSOD (SIRT3 substrate), MCD (SIRT4 substrate), CPS1 (SIRT5 substrate), H3K27 (SIRT6 substrate) and FKBP51 (SIRT7 substrate). MnSOD: manganese superoxide dismutase; MCD: malonyl-CoA decarboxylase; CPS1: carbamoyl phosphate synthetase 1; FKBP51: FK506-binding protein 51. (**B**) Quantification of acetylated proteins in (A). ****P*<0.001. (**C**) Western blotting showing SIRT2 overexpression in NRCMs. NRCMs were infected with adenovirus carrying human *SIRT2* (Ad-*SIRT2*) or control adenovirus (Ad-Ctrl) for 48 hours, then SIRT2 protein levels were analyzed.

Supplementary Figure 6



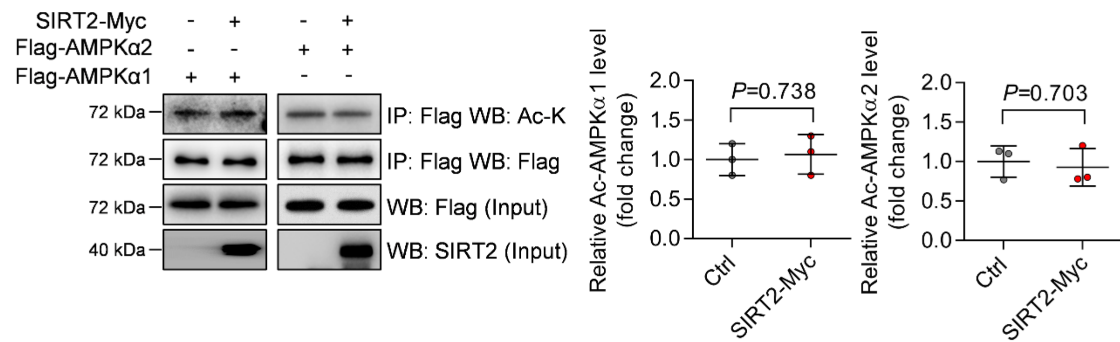
Supplementary Figure 6 Generation of cardiomyocyte-specific *SIRT2* transgenic mice. (**A**) A schematic diagram depicting the construction of *SIRT2*-Tg mouse lines. *α-Mhc*: mouse *myosin heavy chain alpha*; *GH: growth hormone*. (**B**) Western blotting showing human SIRT2 expression levels in heart samples from two lines of *SIRT2*-Tg mice and their non-transgenic (N-Tg) littermates. (**C**) Western blotting analysis of SIRT2 expression in the indicated tissues from *SIRT2*-Tg1 mice. (**D**) Western blotting analysis of SIRT2 expression in isolated cardiomyocytes from *SIRT2*-Tg1 mice.

Supplementary Figure 7



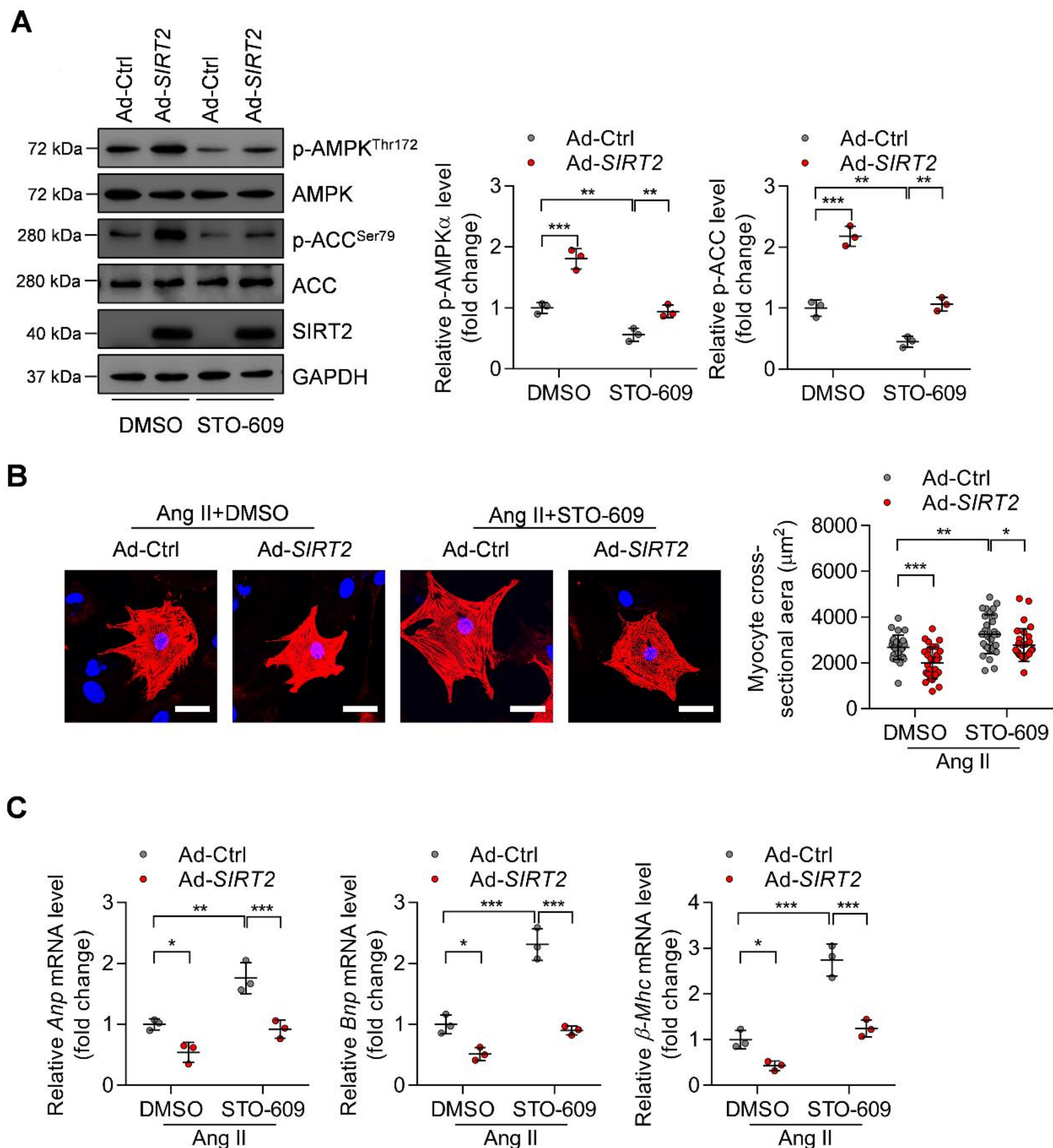
Supplementary Figure 7 Heart rates and blood pressures of the hearts of N-Tg and *SIRT2*-Tg mice infused with saline or Ang II. (**A**) Heart rates in N-Tg and *SIRT2*-Tg infused with saline or Ang II (n=17~21). (**B**) Systolic and diastolic blood pressures in N-Tg and *SIRT2*-Tg mice infused with saline or Ang II (n=17~21; *** P<0.001; ns: not significant).

Supplementary Figure 8



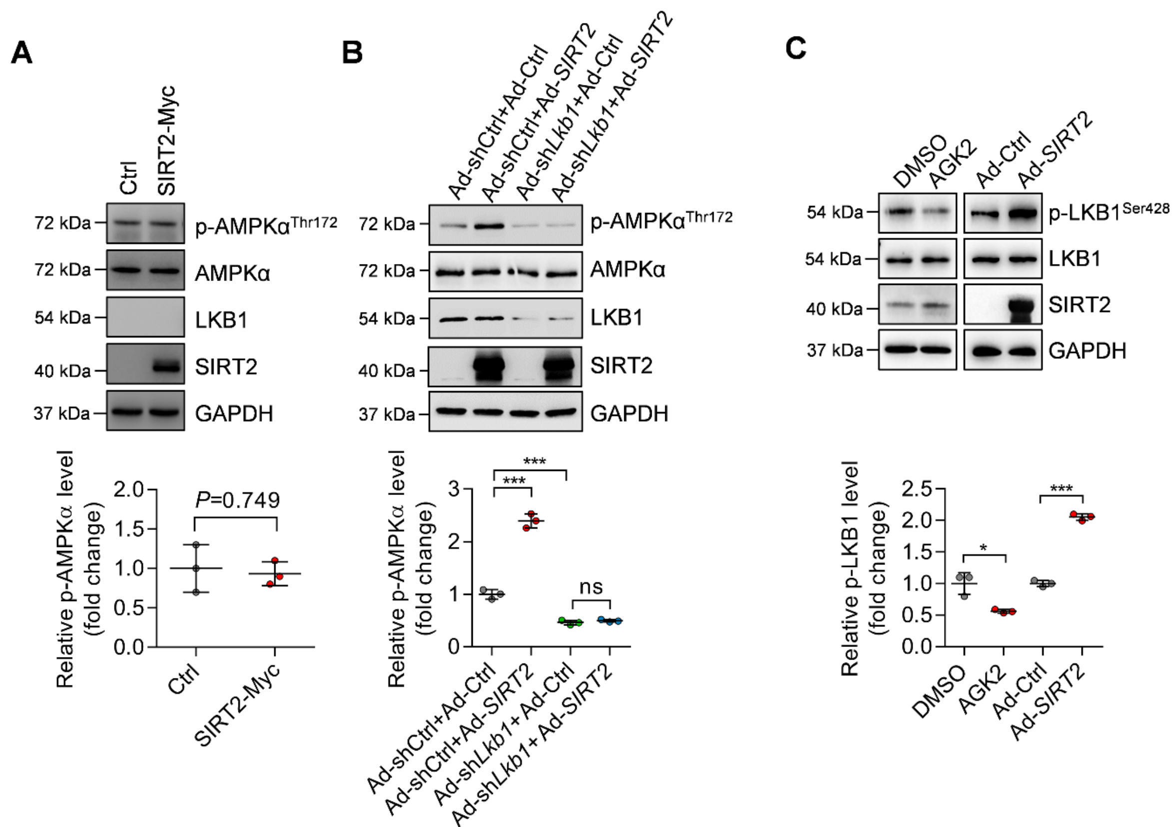
Supplementary Figure 8 Effects of SIRT2 on the acetylation of AMPKα1 and AMPKα2. Flag-AMPKα1 or Flag-AMPKα2 expression vectors were co-transfected with/without the SIRT2-Myc expression vector into HEK293T cells, and the cells were cultured for 48 hours. Then, immunoprecipitation was performed with anti-Flag-M2 antibody, followed by western blotting with the indicated antibodies.

Supplementary Figure 9



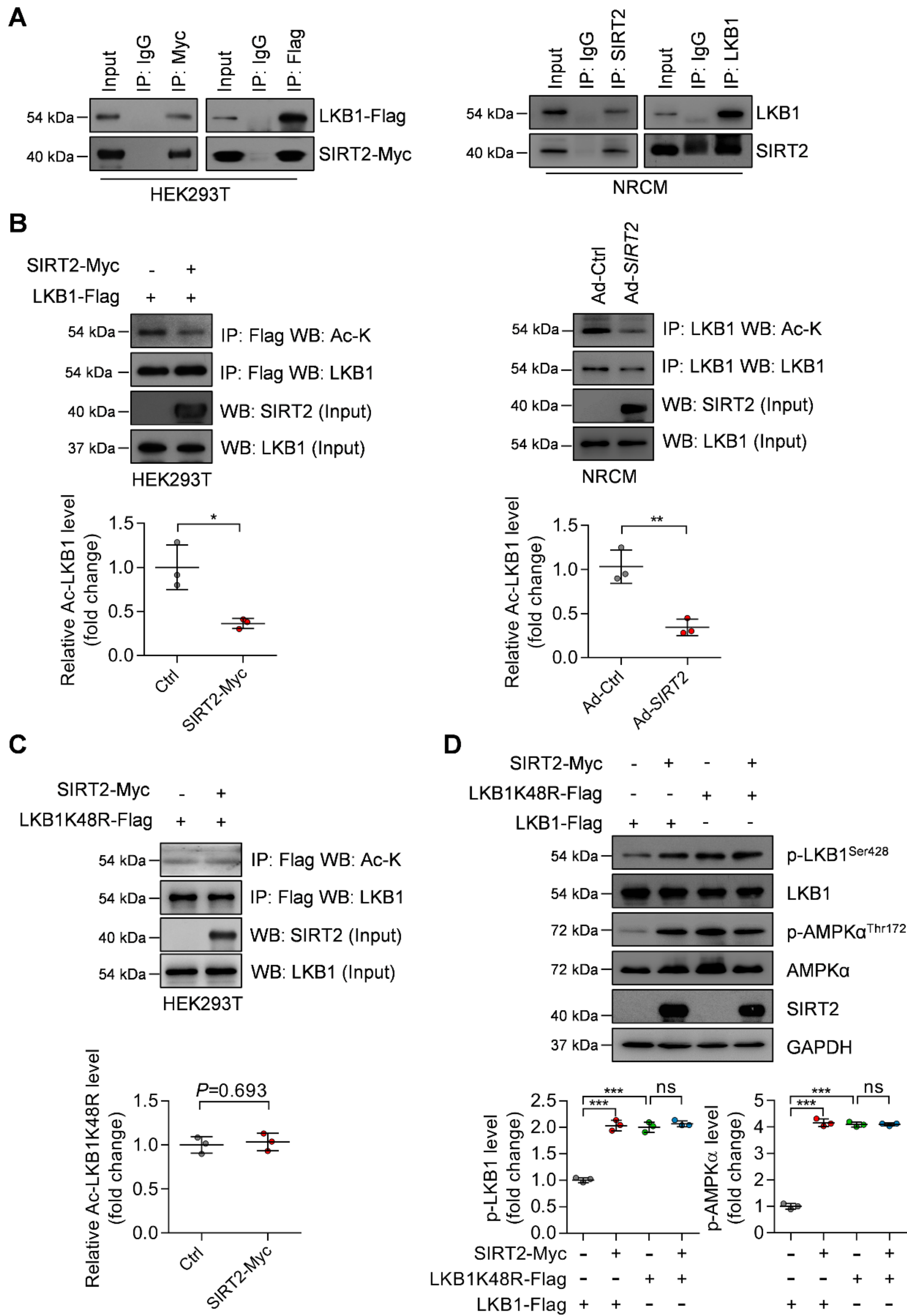
Supplementary Figure 9 CaMKKβ does not contribute to the effect of SIRT2 on AMPK and hypertrophy. (**A**) Representative western blotting showing the phosphorylation of AMPK at Thr172 and the phosphorylation of ACC at Ser79 in NRCMs. NRCMs were infected with the indicated adenovirus for 24 hours and then treated with Ang II (1 μM) for additional 48 hours with/without the presence of the CaMKKβ inhibitor STO-609 (20 μM). Then, AMPK phosphorylation and ACC phosphorylation levels were analyzed by western blotting (***P*<0.01, ****P*<0.001). (**B**) Left: Representative immunofluorescence results showing the cell surface area of NRCMs. NRCMs were treated as described in (A). α-Actinin staining was performed to identify NRCM cells. Representative images are shown. Scale bar= 30 μm. Right: Quantitative results of cardiomyocyte size. Thirty cardiomyocytes are included in each group (**P*<0.05, ***P*<0.01, ****P*<0.001). (**C**) qRT-PCR results showing the relative expression of hypertrophic genes. NRCMs were treated as described in (A) and RNA was subjected to qRT-PCR to determine the level of hypertrophic genes (**P*<0.05, ***P*<0.01, ****P*<0.001, ns: not significant).

Supplementary Figure 10



Supplementary Figure 10 LKB1 is involved in SIRT2-mediated AMPK activation. (**A**) Representative western blotting showing the effects of SIRT2 on the phosphorylation of AMPK at Thr172 in HeLa cells. HeLa cells were transfected with a SIRT2-expressing plasmid for 48 hours, and then western blotting was performed with the indicated antibodies. (**B**) Representative western blotting showing LKB1 is involved in SIRT2-mediated activation of AMPK. NRCMs were infected with indicated adenovirus for 24 hours. Then western blotting was performed with indicated antibodies (****P*<0.001, ns: not significant). (**C**) Representative western blotting showing the effects of SIRT2 on the phosphorylation of LKB1 at Ser428 in NRCMs. NRCMs were treated with the SIRT2 inhibitor AGK2 (10 μM) or DMSO for 24 hours, or NRCMs infected with Ad-*SIRT2* or Ad-Ctrl for 24 hours. Then, western blotting was performed with the indicated antibodies (**P*<0.01, ****P*<0.001).

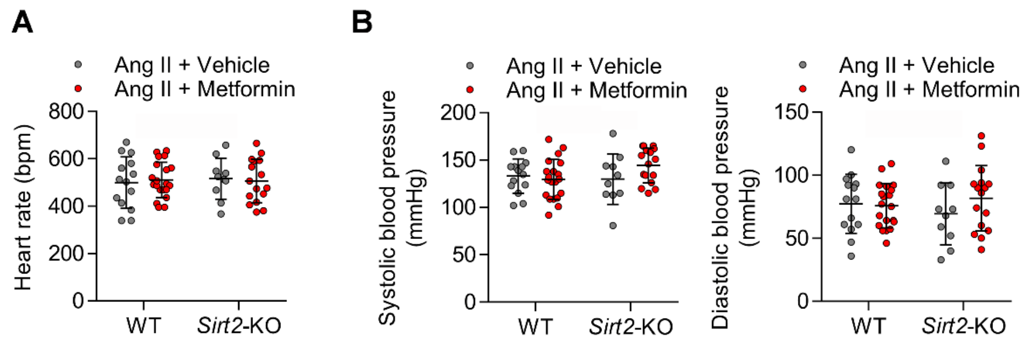
Supplementary Figure 11



Supplementary Figure 11 SIRT2 interacts with LKB1 and deacetylates it at K48. (**A**) Representative immunoprecipitation and western blotting results showing the interaction of SIRT2 with LKB1 in HEK293T cells (Left) and NRCMs (Right). Left: LKB1-Flag was co-overexpressed with SIRT2-Myc in HEK293T cells for 48 hours, then LKB1 or SIRT2 was purified by immunoprecipitation with anti-

Flag-M2 or anti-Myc antibodies, followed by western blotting with the indicated antibodies. Right: Endogenous LKB1 or SIRT2 was purified by immunoprecipitation from NRCMs with anti-LKB1 or anti-SIRT2 antibodies, followed by western blotting with the indicated antibodies. (**B**) Representative immunoprecipitation, western blotting and quantitative results showing LKB1 acetylation in HEK293T cells (Left) and NRCMs (Right). Left: LKB1-Flag was co-overexpressed with/without SIRT2-Myc in HEK293T cells for 48 hours, then LKB1 was purified by immunoprecipitation with an anti-Flag-M2 antibody, followed by western blotting with the indicated antibodies. Right: Endogenous LKB1 was purified by immunoprecipitation from NRCMs with anti-LKB1 antibodies, followed by western blotting with the indicated antibodies (*P<0.05, **P<0.01). (**C**) Representative immunoprecipitation, western blotting and quantitative results showing acetylation of Flag-tagged LKB1 with the lysine 48 (K48) to arginine 48 (R48) mutation (LKB1K48R-Flag). LKB1K48R-Flag was co-overexpressed with/without SIRT2-Myc in HEK293T cells for 48 hours. The LKB1K48R-Flag protein was purified with an anti-Flag-M2 antibody, and then western blotting was performed with the indicated antibodies. (**D**) Representative western blotting and quantitative results showing the effect of the K48R mutation on LKB1 and AMPK phosphorylation. WT LKB1-Flag or LKB1K48R-Flag was co-overexpressed with SIRT2-Myc for 48 hours, and then western blotting was performed with the indicated antibodies (***P<0.01, ns: not significant).

Supplementary Figure 12



Supplementary Figure 12 Effects of metformin on heart rates and blood pressure. (**A**) The heart rates of WT and *Sirt2*-KO mice treated with Ang II and metformin (n=15~17). (**B**) Systolic and diastolic blood pressure of WT and *Sirt2*-KO mice treated with Ang II and metformin (n=15~17).

Supplementary Reference

1. Luo YX, Tang X, An XZ, Xie XM, Chen XF, Zhao X, Hao DL, Chen HZ and Liu DP. Sirt4 accelerates Ang II-induced pathological cardiac hypertrophy by inhibiting manganese superoxide dismutase activity. *Eur Heart J*. 2017;38:1389–1398.