SUPPLEMENTAL MATERIALS

Supplemental methods:

Immunohistochemistry

The heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-μm thickness. Formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated in PBS. Pretreatments included microwave antigen retrieval in a 10 mmol/L citrate buffer for 20 min. Immunohistochemical staining was performed with the ImmunoCruz staining system (streptavidin-biotin peroxidase method, Santa Cruz Biotechnology), and sections were counterstained with hematoxylin. Bcl-xL rabbit polyclonal antibody (BD Biosciences), cleaved caspase-3 rabbit polyclonal antibody (Cell Signaling Technology), 8-OHdG goat polyclonal antibody (Chemicon), FKHR (FoxO1) rabbit monoclonal antibody (Epitomics), and Troponin T mouse monoclonal antibody (Thermo scientific) were used.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from cultured cardiac myocytes with TRIzol (Invitrogen). cDNA was synthesized with the RETROscript kit (Ambion) following the manufacturer's instructions. Real time-PCR was carried out as described previously.[1] The following primer pairs were used –

FoxO1: Sense – CAGATCTACGAGTGGATGGT

Antisense – ACTTGCTGTGTAGGGACAGA

Sirt1: Sense – CTC CTG TTG ACC GAT GGA CT

Antisense – GCG TCA TAT CAT CCA GCT CA

GAPDH: Sense – GAGCTGAACGGGAAGCTCACT

Antisense – TTGTCATACCAGGAAATGAGC

Reporter Gene Assays

Reporter gene assays were conducted with a 3x insulin responsive sequence (IRS) promoter-luciferase reporter (3 x IRS-Luc) [2] as described previously [3]. To generate 3 x IRS-Luc, the two oligonucleotides

CTTCAAAATAAGTTTGTTTTGCTTCAAAATAAGTTTGTTTTGCTTCAAAATAAGTTTGTTTTGCA and

AGCTTGCAAAACAAACTTATTTTGAAGCAAAACAAACTTATTTTGAAGCAAAACAAACTTATTTTGAAGAGCT

were annealed and ligated into the SacI, HindIII sites of the pGL3-basic vector (Promega, Madison).

Fluorescence Microscopic Measurement of ROS Production

We used 5-(and-6)-chloromethyl-2′, 7′-dichloro-dihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) to detect intracellular generation of reactive oxygen species (ROS). Cardiac myocytes were loaded with 5 μmol/L CM-H2DCFDA for 15 min in the dark. After loading, cells were washed with warm HBSS without phenol red. During loading, the acetate groups on CM-H2DCFDA are removed by intracellular esterase, trapping the probe inside the myocyte. Cells loaded with CM-H2DCF were treated with chelerythrine (10 μmol/L) and analyzed by fluorescence microscopy. Production of ROS can be measured by changes in fluorescence due to intracellular production of CM-DCF (5-(and-6)-chloromethyl-2′, 7′-dichlorofluorescein) caused by oxidation of CM-H2DCF. CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Supplemental Table S1. Organ weight of cardiac specific Sirt1 knockout mouse

	Cardiac specific Sirt1 -/- (N=3)	Wild type (N=5)
Heart weight/ BW, mg/ g	**5.2±0.3**	**5.1±0.2**
LV weight/ BW, mg/ g	**3.9±0.1**	**3.8±0.1**
Lung weight/ BW, mg/ g	**5.9±0.1**	**5.9±0.2**
Liver weight/ BW, mg/ g	**52.3±3.9**	**48.5±0.9**

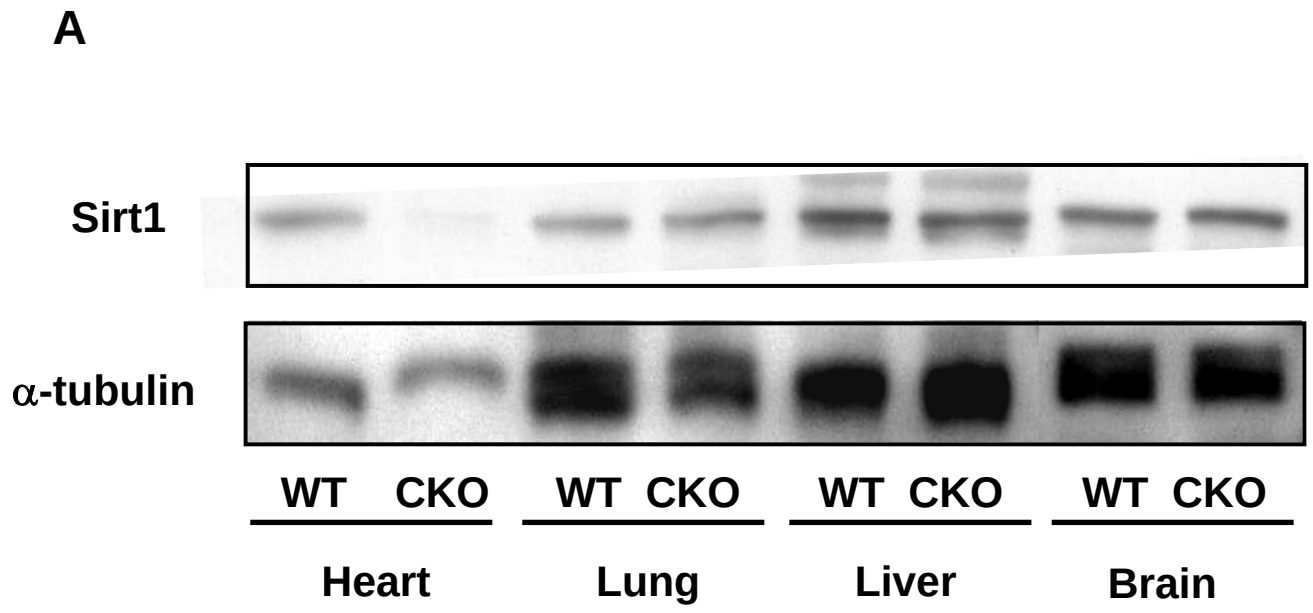
Values are mean ± SEM; BW, body weight; LV, left ventricle.

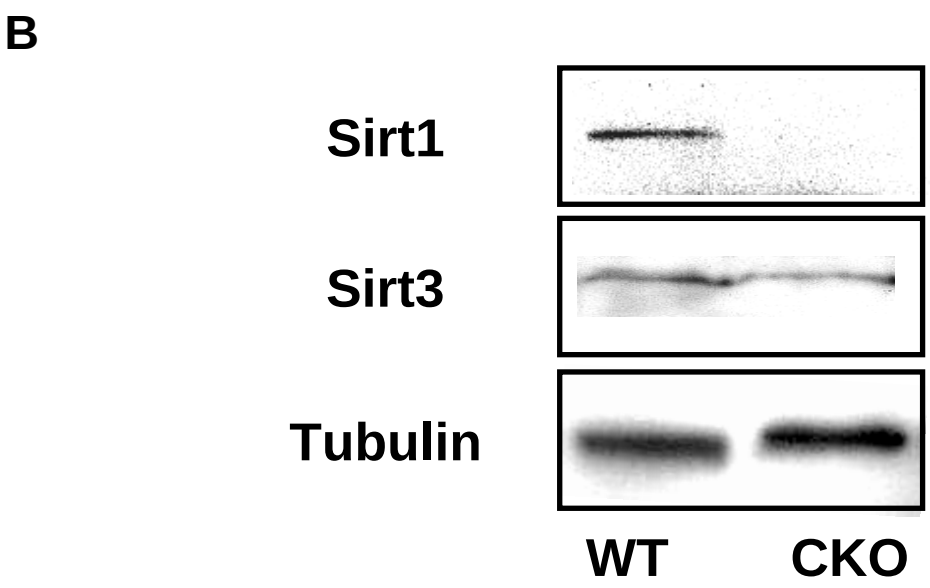
Supplemental Table S2 Baseline characteristics of Tg-Sirt1 and NTg control mice

	LVSP	LVEDP	LVDP	+ dP/dt	- dP/dt	HR
NTg	90.8±15.2	4.8±0.8	86±14.7	2620±465	1360±240	273±46
Tg	106±7.4	5.6±0.7	100.4±7.9	3160±444	1660±60	274±29

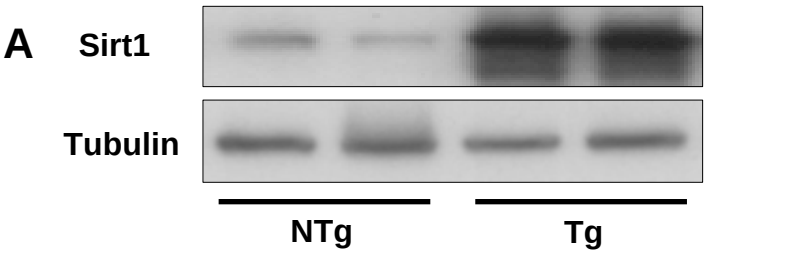
NTg, non-transgenic mice; Tg, transgenic mice with cardiac specific overexpression of Sirt1
LVSP, left ventricular systolic pressure (mmHg);
LVEDP, left ventricular end-diastolic pressure (mmHg);
LVDP, left ventricular developed pressure (mmHg); HR, heart rate (beats/min)

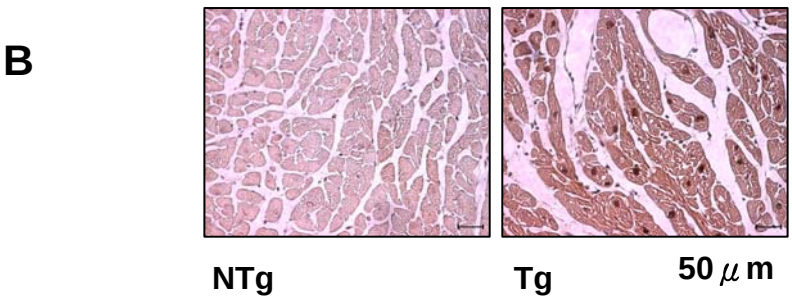
Supplemental Fig. S1



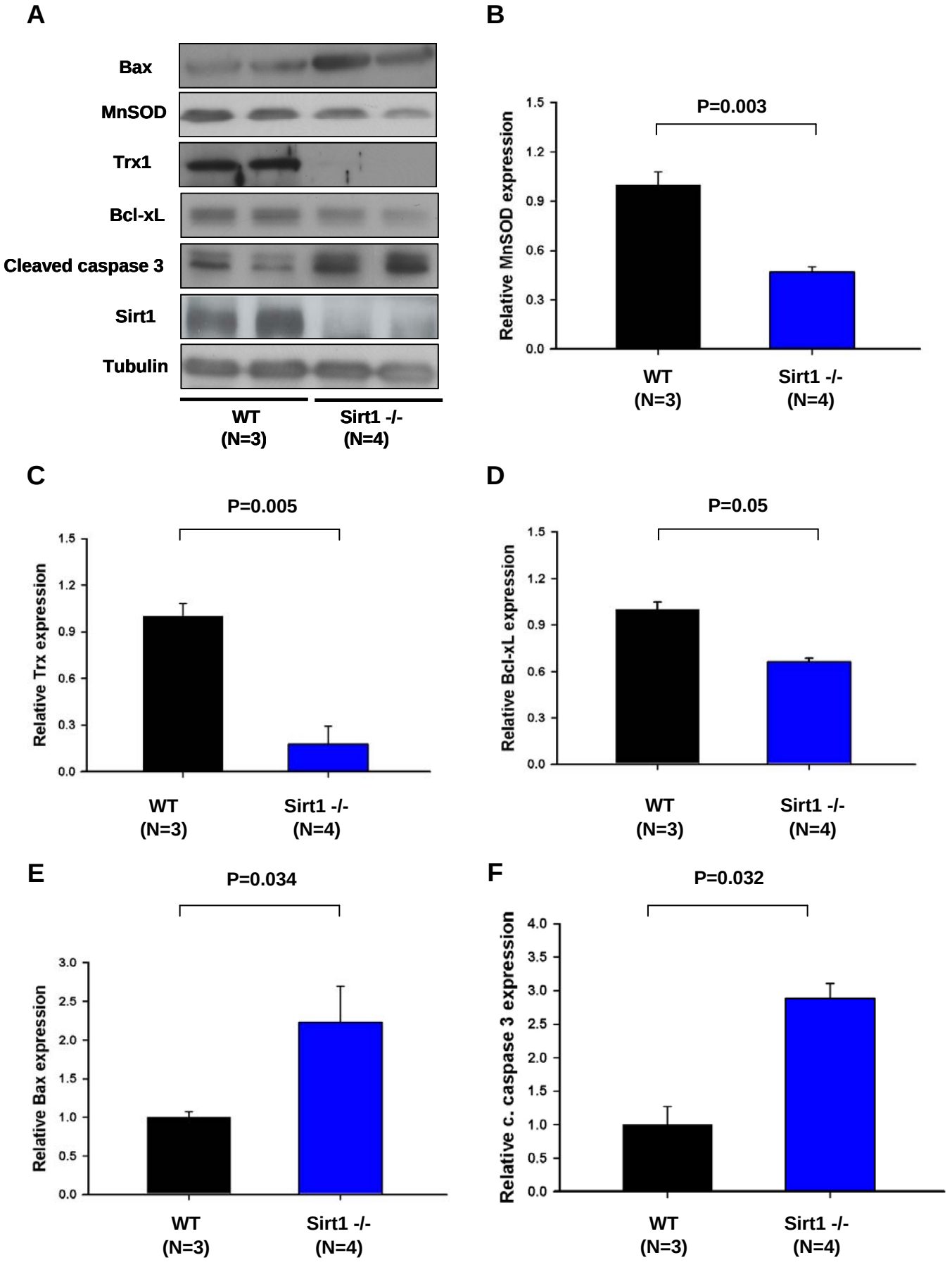


Supplemental Fig. S2





Supplemental Fig. S3



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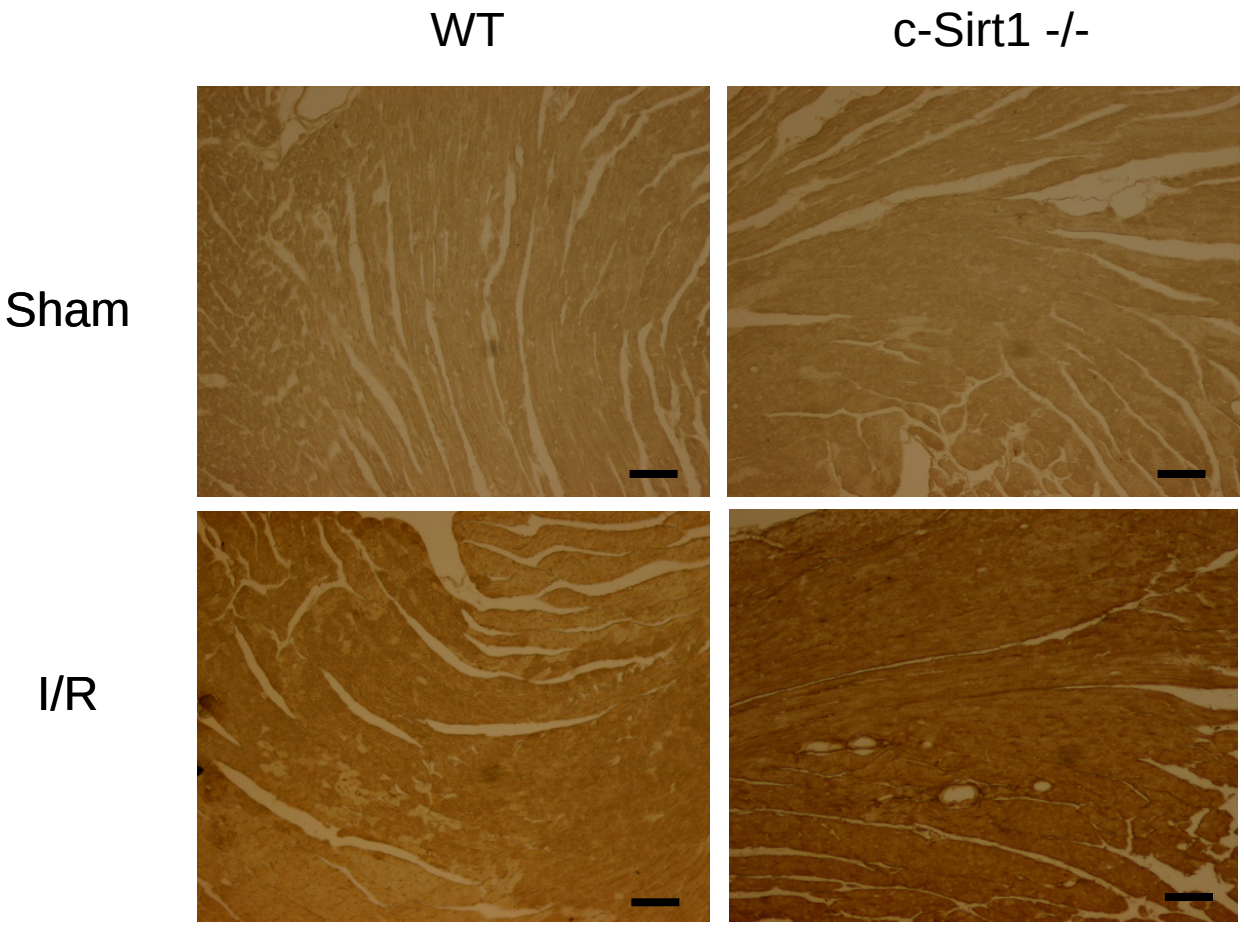
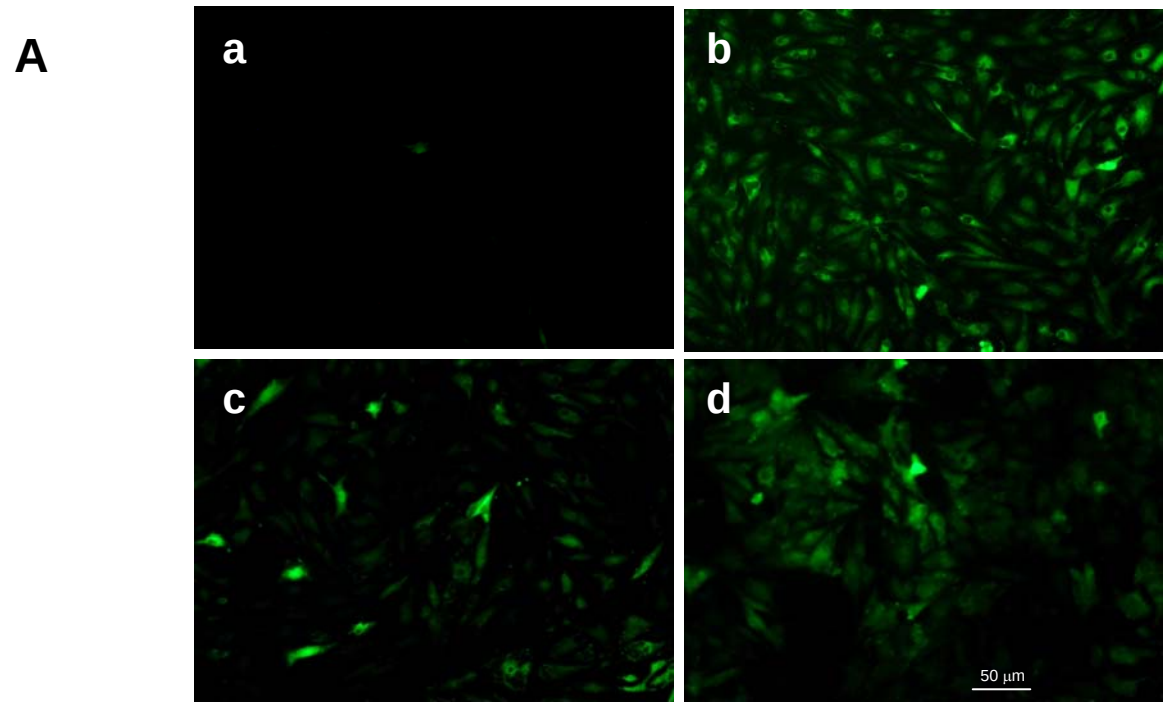


Supplemental Fig. S5

Supplemental Fig. S6



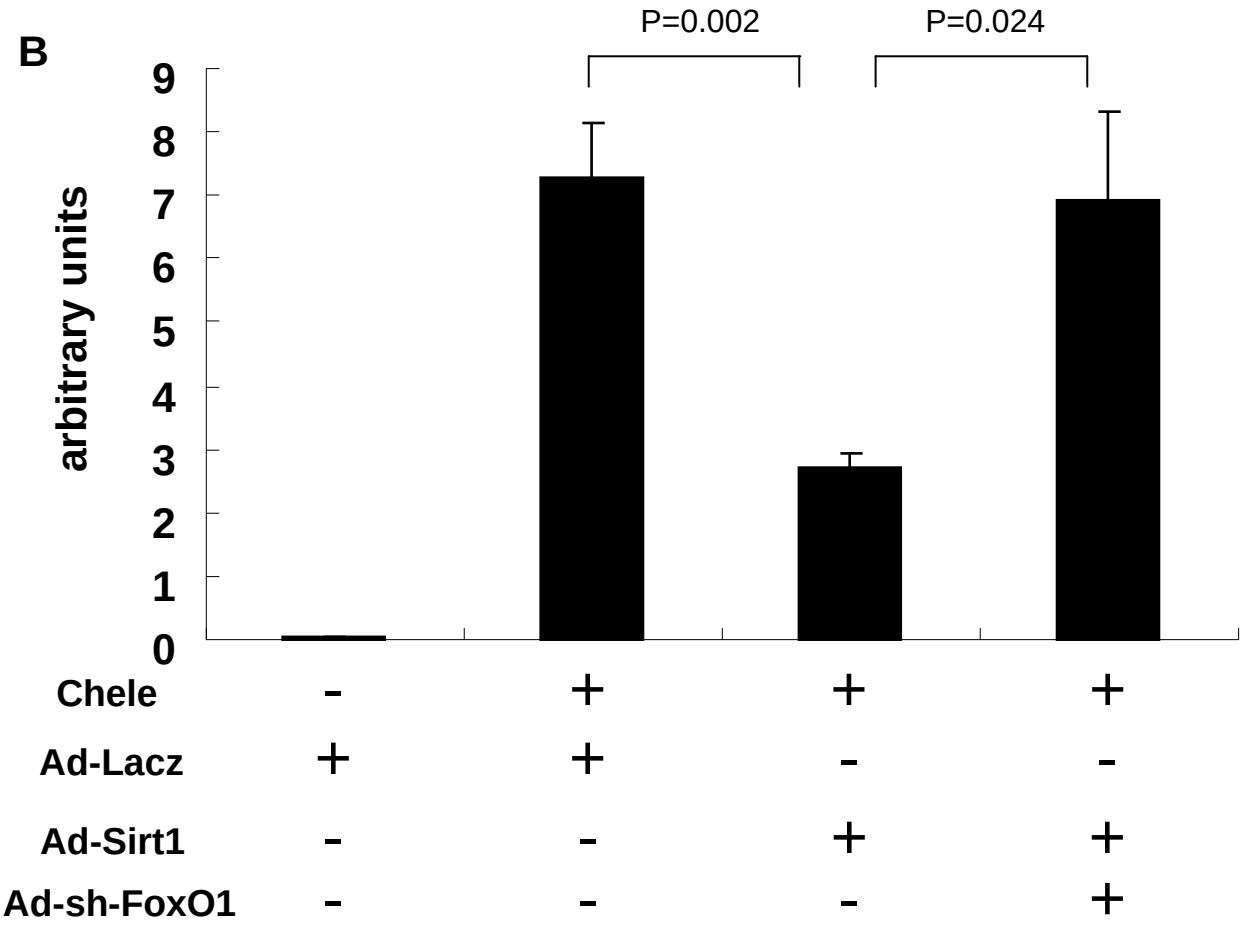


Figure legends for supplemental Figures

Supplemental Figure S1 Homogenates were prepared from indicated organs (A) and the heart (B) in wild type (WT) or cardiac specific Sirt1 -/- (CKO) mice and immunoblot analyses were conducted with anti-Sirt1, anti-Sirt3, and anti-α-tubulin antibodies.

Supplemental Figure S2 Expression of Sirt1 in Tg-Sirt1. (A) Homogenates were prepared from Tg-Sirt1 (Tg) and non-transgenic (NTg) hearts. The level of Sirt1 in the heart was evaluated by immunoblot analysis. (B) The level of Sirt1 expression in the myocardium was evaluated with immunostaining with anti-Sirt1 antibody.

Supplemental Figure S3 Heart homogenates were prepared from the ischemic area in wild type (WT) or cardiac specific Sirt1 -/- mice subjected to I/R. Expression of Bax, MnSOD, Trx1, Bcl-xL, cleaved caspase-3, Sirt1 and tubulin was evaluated by immunoblots. (A) Representative immunoblots are shown. (B-F) The quantitative and statistical analyses of the immunoblots are shown. The relative expression of MnSOD (B), Trx1 (C), Bcl-xL (D), Bax (E) and cleaved caspase-3 (c. caspase 3) (F) in Sirt1 -/- versus WT is shown. The expression level of WT mice is expressed as 1.

Supplemental Figure S4 Representative immunostaining of 8-OHdG in left ventricular myocardial sections obtained from wild type (WT) and cardiac specific Sirt1 -/- mice subjected to either sham operation or I/R.

Supplemental Figure S5 Cultured cardiac myocytes were transduced with either adenovirus harboring LacZ (Ad-LacZ) or adenovirus harboring Sirt1 (Ad-Sirt1). After forty-eight hours, the level of FoxO1 mRNA was evaluated with quantitative PCR analyses. GAPDH was used as an internal control. n=4 each.

Supplemental Figure S6 Cultured cardiac myocytes were transduced with Ad-LacZ, Ad-Sirt1 and/or Ad-sh-FoxO1 as indicated. The total amount of adenovirus was 10MOI in each sample. After forty-eight hours, myocytes were subjected to DFCDA staining as described in the Method section in the presence or absence of chelerythrine (10 μmol/L).

References

1. Hsu CP, Oka S, Shao D, Hariharan N, Sadoshima J. Nicotinamide phosphoribosyltransferase regulates cell survival through NAD+ synthesis in cardiac myocytes. *Circ Res.* 2009;105:481-491.

2. Tang ED, Nunez G, Barr FG, Guan KL. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem.* 1999;274:16741-16746.

3. Ago T, Liu T, Zhai P, Chen W, Li H, Molkentin JD, Vatner SF, Sadoshima J. A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. *Cell.* 2008;133:978-993.