Supporting Information

Chen et al. 10.1073/pnas.1003833107

SI Materials and Methods

Antibodies and Reagents. The antibodies used in the present study were anti-SIRT1 from Millipore, and anti-pan-AMPKα, anti-phospho-AMPK Thr-172, anti-phospho-ACC Ser-79, anti-acetyl-p53 Lys-379/382, anti-p53, anti-PGC-1α, anti-eNOS, anti-acetylated lysine, anti-PARP-1, anti-α-tubulin, horseradish peroxide-conjugated anti-rabbit, and anti-mouse antibodies from Cell Signaling Technology. Anti-phospho-eNOS Ser-633/635, anti-phospho-eNOS Ser-1177/1179, and anti-eNOS used in immunoprecipitation were from BD Transduction Laboratory.

Cell Culture. HUVECs were cultured in medium M199 (Gibco) supplemented with 15% FBS (Omega), 3 ng/mL β-EC growth factor (Sigma), 4 U/mL heparin (Sigma), and 100 U/mL penicillin-streptomycin. MEFs and HEK293 cells were cultured in DMEM containing 10% FBS.

Adenoviral Infection, siRNA Knockdown, and Transient Transfection. Ad-AMPK-CA, a recombinant adenovirus expressing a constitutively active (CA) form of AMPKα2, was described previously (1). MEFs at 70% confluency were seeded on six-well plates, infected with Ad-null or Ad-AMPK-CA at 100 multiplicities of infection (MOI), and incubated for 24 h before further experimentation.

Transient transfection was performed with Lipofectamine RNAiMAX (Invitrogen). In brief, HUVECs at 70% confluency were transfected with AMPKα1 and AMPKα2 at 10 nM each (Qiagen, SI02622235 and SI02758595), and SIRT1 (Qiagen, SI00098434) or scramble (ctrl) siRNA, each at 20 nM, in Opti-MEM (Gibco). Four hours after transfection, the medium was changed to fresh M199, and cells were kept in culture for 48 h before shear stress experiments.

HEK293 cells were transiently transfected with 1 μg of respective DNA and 2.5 μL Lipofectamine 2000 (Invitrogen) per 10^6 cells, following a standard protocol. Twenty-four hours after transfection, cells were infected with Ad-SIRT1 or Ad-SIRT1-DN (2) for another 24 h.

Western Blot Analysis and Immunoprecipitation. Lysates from ECs, MEFs, HEK293 cells, or mouse aortas were resolved on 8% SDS/PAGE, and proteins were transferred to PVDF membrane. The immunoblotting with different antibodies followed instructions provided by various manufacturers. Immunoprecipitation for SIRT1 and eNOS was performed following the standard protocol provided by the manufacturers (Millipore for SIRT1 and BD Biosciences for eNOS).

Quantitative RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription was carried out with 3 μg of total RNA by the SuperScript II reverse transcriptase (Invitrogen). The synthesized cDNA was used to perform real-time quantitative PCR (qPCR) with the iQ SYBR Green supermix (Bio-Rad) on the iCycler real-time PCR detection system (Bio-Rad). The sequences of primer sets used were as follows: for PGC-1α, forward: GGAGCAATAAAGCGAAGA; reverse: GAGGAGTTGTGGGAGGAG; for NRF1, forward: ACTCTACAGGTCGGGGAAAA; reverse: AGTGAGACAGTGCCATCAGG; for COX4, forward: GCAGTGGCGGCAGAATGT; reverse: GGCTAAGCCCTGGATGGG.

NAD^+ Measurement and MTT Assays. The cellular level of NAD^+ and was measured as previously described (3). The cell extract was neutralized and centrifuged to collect the supernatant. Forty microliters of supernatant was added to 80 μL of an NAD^+ reaction mixture and incubated for 5 min at 37 °C. After measuring the basal absorbance at 570 nm, the reaction was initiated by adding 20 μL of alcohol dehydrogenase solution and then incubated at 37 °C for 20 min. The absorbance of the reaction mixture was determined again at 570 nm. The NAD^+ level was obtained by subtracting the basal absorbance from the second reading.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes) was used to assess mitochondrial activity with a protocol adapted from Xia et al (4). HUVECs were incubated with 150 μg/mL MTT under normal growth conditions (5% CO_2, 37 °C) for 2 h. The medium was removed, the formazan product was dissolved in DMSO, and the solution was then incubated at 37 °C for 10 min. The absorbance of formazan product from MTT was measured at 540 nm by using a spectrophotometer. After subtracting the background, the reading was normalized to total protein concentration.

NO Bioavailability Assays. The NO production from cells transfected with various plasmids was detected as the accumulated nitrite (NO_2^-), a stable breakdown product of NO, in cell culture media by using Griess reagent (Sigma). An aliquot of cell culture media was mixed with an equal volume of Griess reagent and then incubated at room temperature for 15 min. The azo dye production was analyzed by use of a spectrophotometer with absorbance set at 540 nm.

1. Foretz M, et al. (2005) Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* 54:1331–1339.
2. Luo J, et al. (2001) Negative control of p53 by Sir2α promotes cell survival under stress. *Cell* 107:137–148.
3. Jacobson EL, Jacobson MK (1976) Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells. *Arch Biochem Biophys* 175: 627–634.
4. Xia S, Laterra J (2006) Hepatocyte growth factor increases mitochondrial mass in glioblastoma cells. *Biochem Biophys Res Commun* 345:1358–1364.

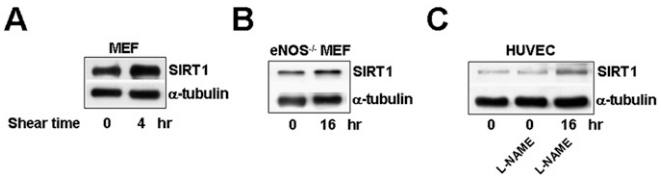

Fig. S1. Confluent monolayer of the wild-type MEF (*A*) and eNOS$^{-/-}$MEF (*B*) subjected to a laminar flow for the time as indicated or kept under static condition (time 0). In (*C*), HUVECs were treated with L-NAME (100 μM) 30 min before the exposure to the laminar flow for 16 h. In parallel static controls, HUVECs were incubated with or without L-NAME. The collected lysates were analyzed by Western blot with anti-SIRT1 and anti-α-tubulin.