Supplementary Data

Materials and Methods

qPCR analysis

The primers were designed in the laboratory and tested in a gradient temperature PCR for optimal amplification temperature and absence of double peaks. Before we run our assays, we run a test qPCR with serially diluted DNA to make sure we have no secondary amplifications and the amplification curve follows the predicted exponential. We analyze the data using the ΔΔCt method. We run triplicates for each DNA sample. For each one of the triplicates, we first we correct for loading using the 18S signal as reference for each one of the samples, then we choose a reference sample and calculate the ΔΔCt and then calculate the $2^{\Delta\Delta Ct}$. Finally, we calculate mean and standard deviation for the triplicates. This procedure is followed for every experiment; all experiments were carried out at least thrice. A final analysis of all the experiments included determination of statistical significance.

Western blot

Protein samples were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto Hybond-P membranes (Amersham) using a semi-dry transfer system from Bio-Rad. Proteins were then and analyzed by Western blotting. Membranes were first equilibrated in blocking solution (TBST) with 5% BSA for over 1 h. Membranes were then incubated in the same solution first with specific primary antibodies (2 h-o/n) and subsequently for 30 min with correspondign secondary antibodies (Amersham) linked to HRP. ECL, Amersham reagents were used for chemi-luminescence detection (ECL, Amersham).

Chromatin immunoprecipitation

Cells were first crosslinked with 1% formaldehyde, and lysed (lysis buffer: 50 m*M* Tris HCl pH8.0, 150 m*M* NaCl, 5 m*M* EDTA, 1% Triton × −100, 0.5% NP-40). The resulting chromatin preparation was sheared by sonication and incubated overnight at 4°C with antibodies against specific proteins, and then with Dynabeads® A/G for 2 h. The magnetic beads were then collected, and washed extensively. To isolate the IP DNA, the crosslinks were reversed (6 h, 65°C), the protein was digested with proteinase K, and the RNA degraded with RNAse A. The nucleic acid fraction was extracted with phenol:chlorophorm and ethanol precipitated using glycogen as carrier. The resulting DNA preparations were analyzed by qPCR with specific primers.

Antibodies used: SirT1 (Bethyl laboratories), H4K16Ac (Millipore), RNAP II (monoclonal antibody 8WG15 from COVANCE), CTD-S2P-RNAP II (monoclonal antibody H5 from COVANCE).

Oligonucleotides used

sod2 (-200)
Forward: 5′-GAGCCGTACCATCTCTTTCG-3′
Reverse: 5′-CTCGGCACTGTGGTCGG-3′
Sod2 (ATG)
Forward: 5′-AGTTGGAAGCGGCGCGCGCA-3′
Reverse: 5′-GCCGCGCTGACCTGAGACGA-3′
catalase (-300)
Forward: 5′-AGAGCTGAAAGCATGTTAACAG-3′
Reverse: 5′-CTGCAAACGCCCGATTAACTA-3′
Catalase (ATG)
Forward: 5′-GAAGTCGCCTATTTCAGCTG-3′
Reverse: 5′-GCTCCTTCCAGTGTTTCATC-3′
prx3 (-1400)
Forward 5′-TCCCACATGCCGTAACTAAGAGT-3′
Reverse 5′-ACTGGGTTGGTGGGTGTGGA-3′
prx3 (-1100)
Forward 5′-CAAAGTACCGTGCTCACCAC-3′
Reverse 5′-CACCAATAAGCCAAGTGGGTC-3′
prx3 (-800)
Forward 5′-GACCCACTTGGCTTATTGGT-3′
Reverse 5′-CAACTAAGACTTGGTGCAATC-3′
prx3 (-148)
Forward 5′-TCTTGCCACCCTGGATTGGA-3′
Reverse 5′-ACG TGG AAG AGC TAC GAC CT-3′
prx3 (ATG)
Forward 5′-AGGTCGTAGCTCTTCCACGT-3′
Reverse 5′-ACCGAAGCCCGAAACAACCT-3′
prx3 (+250)
Forward 5′-GGGCAGCGTCTAAGGCAGGC-3′
Reverse 5′-AACCGGGACCAGGGGACAGG-3′
prx3 (+500)
Forward 5′-GGGTTTCCGTGAGCATCCCCT-3′
Reverse 5′-GTTTGCTCAATTTCAGGGTTCTGC-3′

Supplementary Table S1. Western Blot Quantitative Analysis

MEFs	*0.5% FBS/10% FBS SirT1$^{+/+}$ Mean (±SD)*	*0.5% FBS/10% FBS SirT1$^{-/-}$ Mean (±SD)*	*SirT1$^{-/-}$/SirT1$^{+/+}$*
MnSOD	1.06 (±0.18)	0.77 (±0.01)	0.73 (±0.10)
Catalase	0.98 (±0.33)	0.73 (±0.11)	0.75 (±0.09)
Prx3	1.17 (±0.03)	0.7 (±0.11)	0.6 (±0.11)
TR2	0.85 (±0.11)	0.73 (±0.02)	0.86 (±0.10)
PGC-1alpha	1.40 (±0.04)	1.04 (±0.03)	0.74 (±0.03)

Response to serum deprivation of MEF SirT1$^{+/+}$ and SirT1$^{-/-}$
FBS, fetal bovine serum.

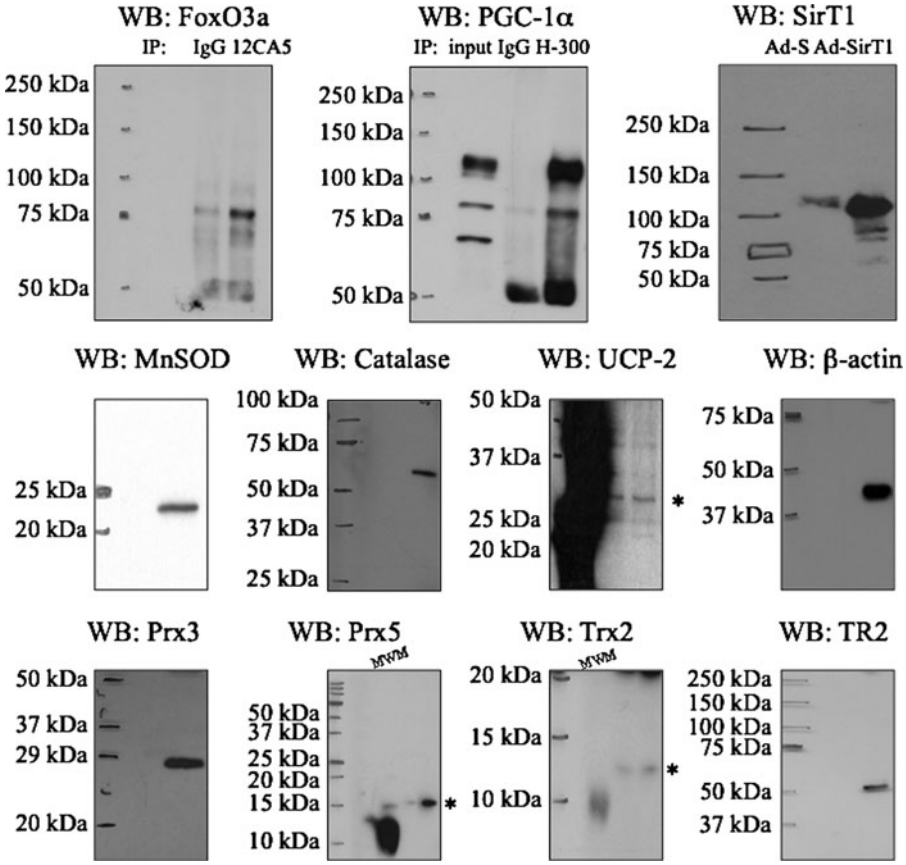
prx3 (+1700)
Forward 5′-AGGAACAAGGCCAGCGGAGG-3′
Reverse 5′-AGA AGC AGC AGG CCT AAG GGC-3′
prx5 (-300)
Forward 5′-TGGCTGAGCTAAACCTCAG-3′
Reverse 5′-CGAGTTGCCGCTGTTTCGA-3′
prx5 (ATG)
Forward 5′-TCTTCCGCAGGATTGCGTC-3′
Reverse 5′-TGTCGTTGACGCACCCTCA-3′
ucp-2 (-2800)
Forward 5′-CTGAAAGAGCAATGTGTGATAC-3′
Reverse 5′-CATTAGCGTATTCAGTCCTTG-3′
ucp-2 (-2500)
Forward 5′-GCTCTACAGGACACATAGTATG-3′
Reverse 5′-CTCCTTTAGACTGGACTCTTAC-3′
ucp-2 (-2200)
Forward 5′-AGGCCCCAATGGGACAGTGA-3′
Reverse 5′-TCGCAGTCTCAGGGACAGTCC-3′
ucp-2 (-500)
Forward 5′-TGGGCTGGTGAGCTCTGAGA-3′
Reverse 5′-AGAGGGCAGGCAGATGAGGG-3′
ucp-2 (ATG)
Forward 5′-GTTCGGCTGCTGATGGACCT-3′
Reverse 5′-GTAGGGGGCACATCTGTGGC-3′
ucp-2 (+250)
Forward 5′-CAAGGAGAAAGGCAGGGGCC-3′
Reverse 5′-CGGAGGCGAAGCTCATCTGG-3′
ucp-2 (+700)
Forward 5′-AGCAGGAGAGGCTGAGGCTT-3′
Reverse 5′-GGGTGAACGTGGCATGCTGA-3′
pgc-1α (-300)
Forward 5′-TCATTCAGGAGCTGG ATGGC-3′
Reverse 5′-TCACCCAAACCCAAGCCCT-3′
pgc-1α (ATG)
Forward 5′-AGCGTTACTTCACTGAGGCA-3′
Reverse 5′-ACAGTCCCCAGTCACATGA-3′
FoxO3a promoter
Forward 5′-ACCTAGCCCAGGAGAGACCT-3′
Reverse 5′-AGCAGGCAGACCTGGAGAC-3′

Adenoviral vectors and infections

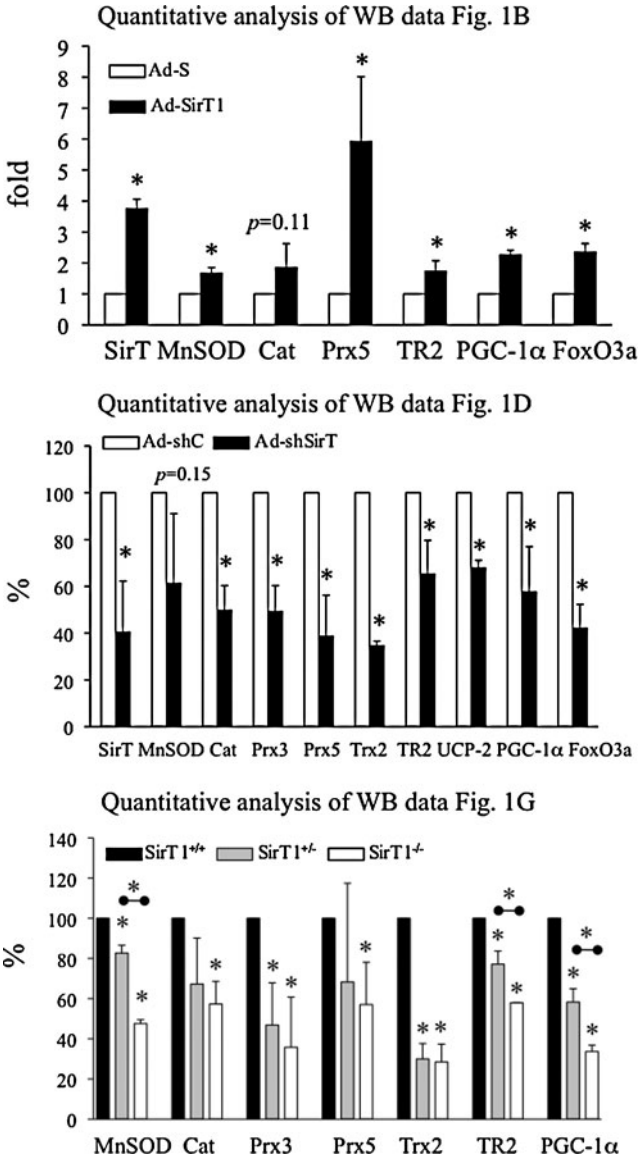
Oligonucleotides used to generate Ad-shPGC1α and Ad-shSirt1were, for shPGC-1α, sense 5′-tcgagttcatggagcaataaagcgttcaagagacgctttattgctccatgaatta-3′ and antisense 5′-ctagtaattcatggagcaataaagcgtctcttgaacgctttattgctccatgaac-3′; for shSirT1, sense 5′-tcgagcatgaagtatgacaaagatttcaagagaatctttgtcatacttcatggca-3′ and antisense 5′-ctagtgccatgaagtatgacaaagattctcttgaaatctttgtcatacttcatgc-3′.

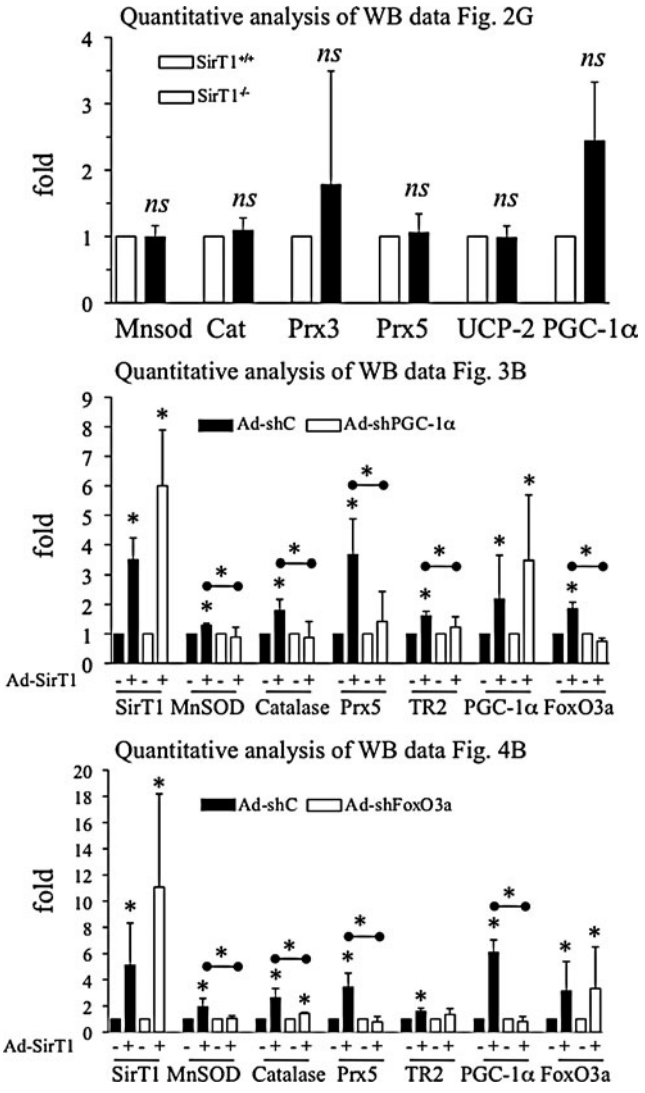
Quantitative analysis of confocal images

Every experimental point was done in triplicates (for each point of each experiment the cells were seeded on three cover slips). Additional seeded cover slips where used for technical controls. Images were taken with a 63× objective and analyzed using the Image J software. For each one of the cells in each one of the image fields, the cell surface and the nuclear surface were determined. After correcting for the background,



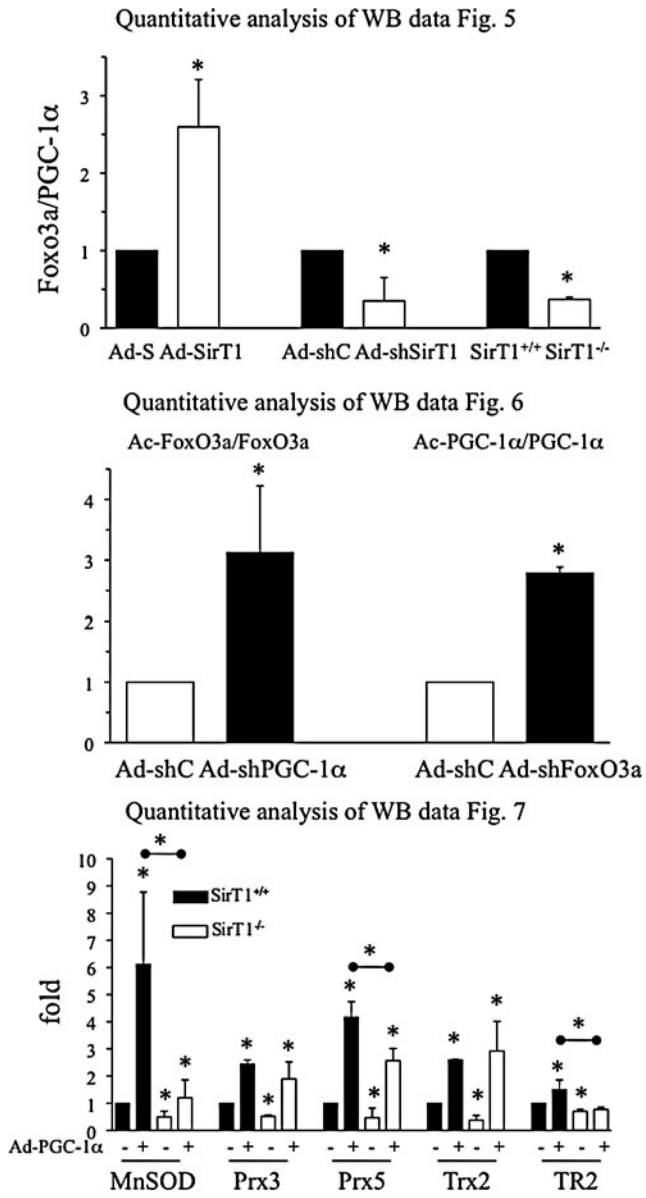
SUPPLEMENTARY FIG. S1. Original full-sized Western blots (WBs) with molecular weight markers (Fig. 1–7).



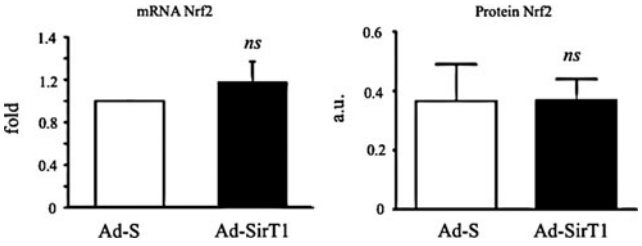


SUPPLEMENTARY FIG. S2. The graphs show the statistical analysis of western blot data corresponding to Fig. 1–7. Data were analyzed using β-actin as a loading control. Data are means + SD (*) $p \leq 0.05$ versus control.

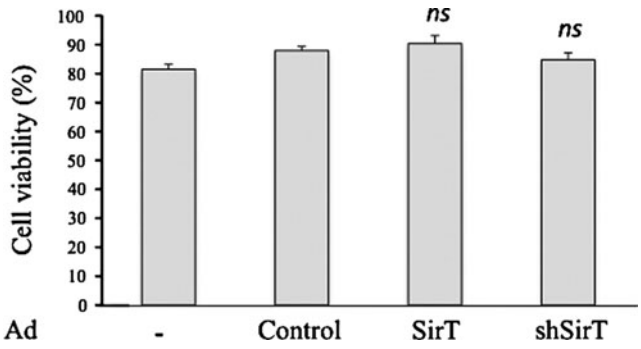
the total intensity of the fluorescence within the cell and within the nucleus was calculated. Finally, the intensity/area ratio was calculated for each cell. Approximately 10 cells per cover slip were analyzed, at least of 30 cells per point per experiment. The data presented is the average intensity per cell/nucleus for three independent experiments.



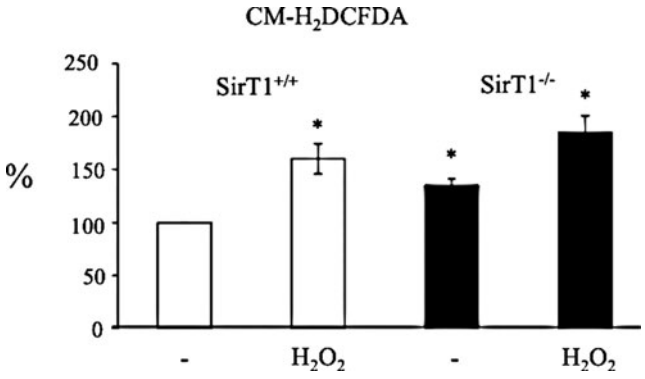
SUPPLEMENTARY FIG. S2. (Continued).



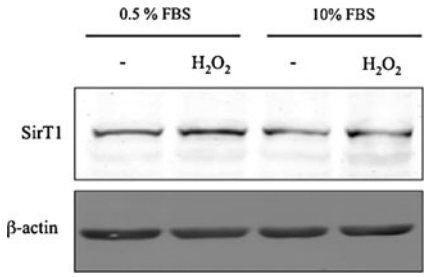
SUPPLEMENTARY FIG. S3. SirT1 does not regulate the expression of Nrf2. qRT-PCR and western blot analysis of Nrf2 levels in bovine aortic endothelial cells (BAEC) that were infected o/n with Ad-SirT1recombinant adenovirus or the control adenovirus, Ad-S and harvested 24 h postinfection. Data are means + SD.



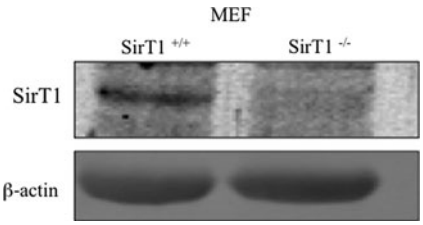
SUPPLEMENTARY FIG. S4. Viability test. BAEC cells were infected with the indicated Adenovirus and cell viability was tested 48 h post infection by IP staining and analyzed by flow cytometry. Data are from ≥3 independent experiments. Data are means + SD.



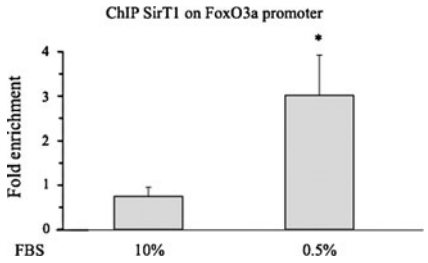
SUPPLEMENTARY FIG. S5. SirT1$^{-/-}$ MEFs have higher ROS levels than SirT1$^{+/+}$ MEFs. Confluent SirT1$^{+/+}$ and SirT1$^{-/-}$ MEFs were serum starved o/n and were indicated, treated for 30 min with 250 μM H_2O_2, labeled with CM-H_2DCFDA and analyzed by flow cytometry. Data are means + SD. (*) $p \leq 0.05$ versus control.



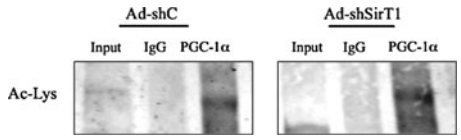
SUPPLEMENTARY FIG. S6. H_2O_2 increases SirT1 levels in serum starved BAEC. Confluent BAEC in 10% FBS or following o/n serum starvation were treated with 50 μM H_2O_2 for 4 h before harvesting. SirT1 levels were analyzed by WB in WCE.



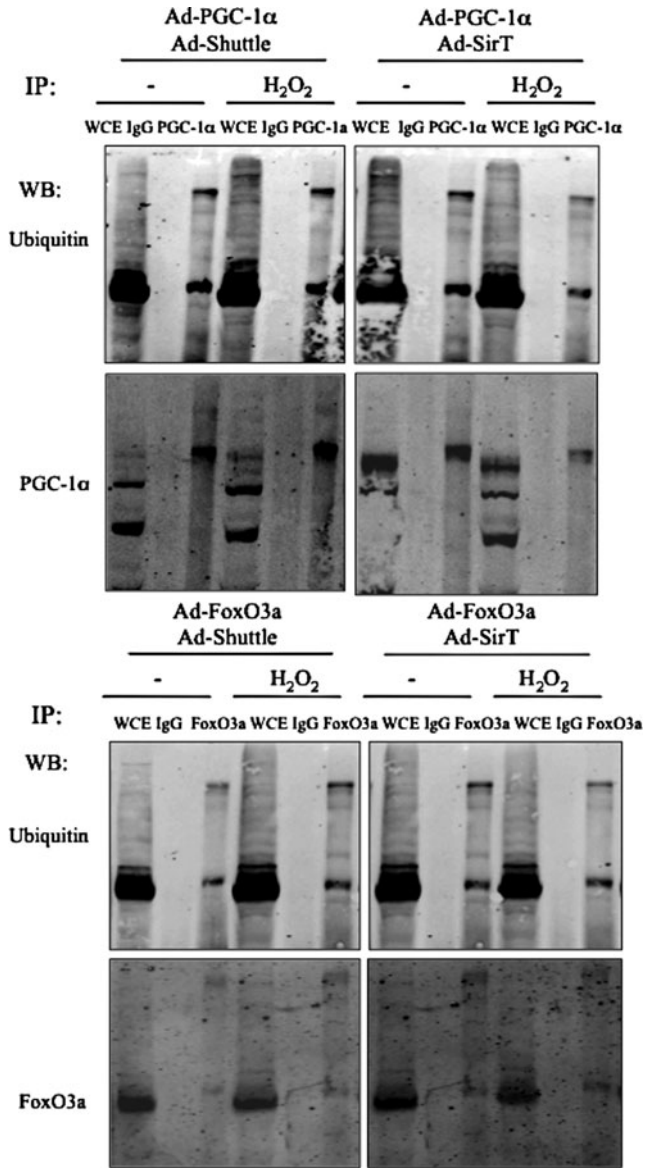
SUPPLEMENTARY FIG. S7. The specificity of the SirT1 antibody was tested in WCE of MEF SirT1$^{+/+}$ and SirT1$^{-/-}$ by WB.



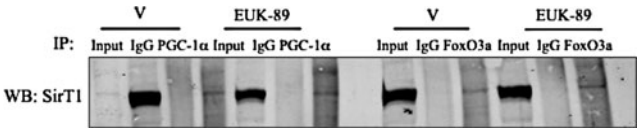
SUPPLEMENTARY FIG. S8. SirT1 is recruited the FoxO3a promoter region ChIP assay of FoxO3a promoter region. Crosslinked chromatin from confluent BAEC cells in 10% FBS or serum deprived o/n was precipitated with SirT1 antibodies or normal IgG, to control for nonspecific binding. Promoter occupancy was analyzed by qPCR using specific primers. Analysis of β-actin CDS was used to control for non specific enrichment. Data are means + SD. (*) $p \leq 0.05$ versus control.



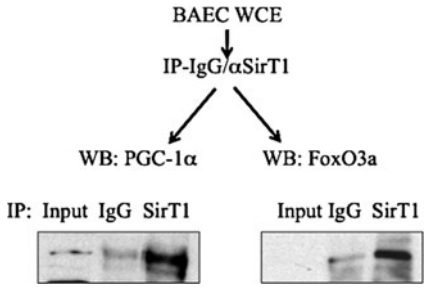
SUPPLEMENTARY FIG. S9. Knockdown of SirT1 increases PGC-1α acetylation in BAEC. BAEC cells were infected with Ad-PGC-1α and Ad-shSirT1 or control adenovirus as indicated. 12 h postinfection, cells were deprived of serum o/n and harvested. BAEC whole cell extracts were subjected to immunoprecipitation (IP) with the monoclonal antibody 12CA5 or normal IgG, as a control, and analyzed by western blot as indicated.



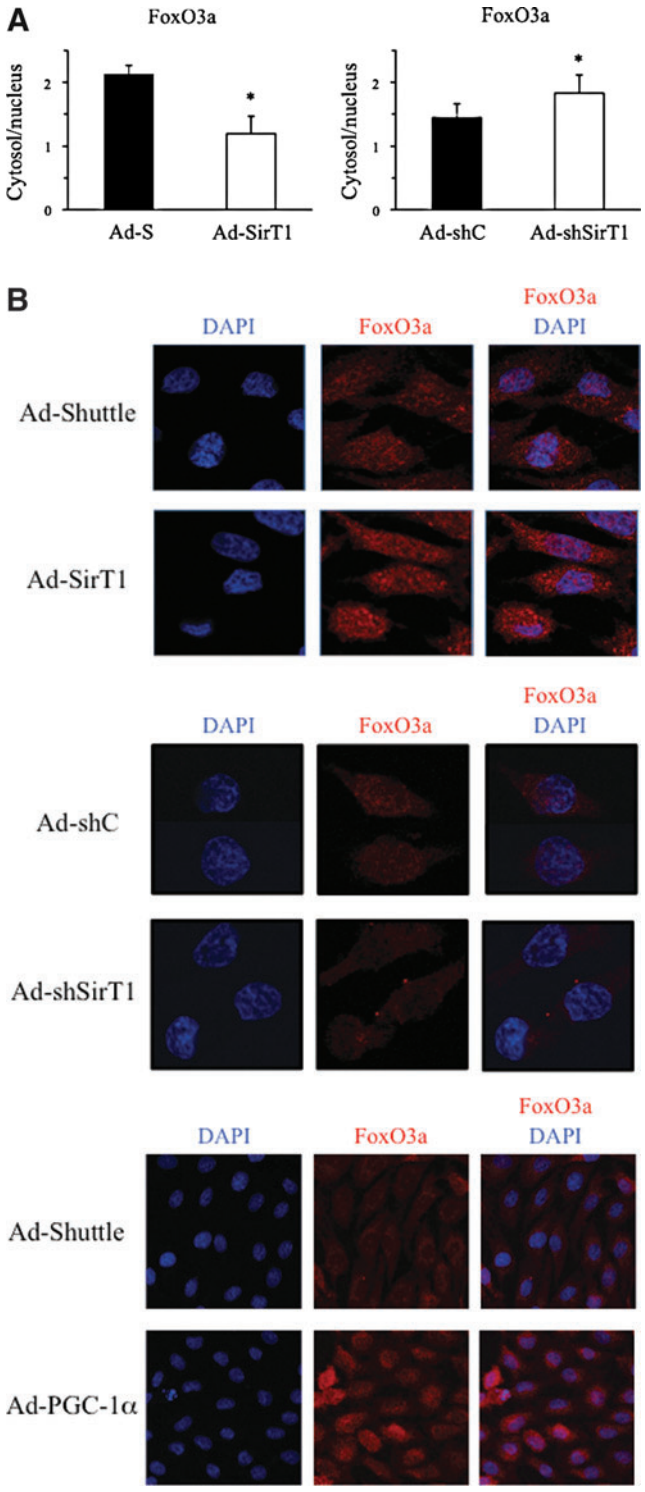
SUPPLEMENTARY FIG. S10. H_2O_2 increased ubiquitination of FoxO3a and PGC-1α is prevented by SirT1 overexpression. BAEC were coinfected with Ad-PGC-1α, Ad-FoxO3a, Ad-SirT1, or control adenovirus as indicated. 12 h postinfection, cells were deprived of serum o/n, then pretreated with the proteasome inhibitor MG132 (20 μM) for 30 min before H_2O_2 treatment for 4 h and harvested. BAEC whole cell extracts were subjected to IP with the monoclonal antibody 12CA5 or normal IgG, as a control, and analyzed by western blot as indicated.



SUPPLEMENTARY FIG. S11. Antioxidant treatment does reduce the interaction of SirT1 with PGC-1α. BAEC cells were infected with Ad-PGC-1α, 12 h post infection they were serum deprived o/n and treated for 2 h with EUK-189 50 μM before harvesting. Whole cell extracts were subjected to IP with a specific PGC-1α antibody or normal IgG, as a control, and analyzed by western blot as indicated.



SUPPLEMENTARY FIG. S12. SirT1/PGC-1α/FoxO3a co-immunoprecipitation. Confluent BAEC cells were cultured in galactose media and in the absence of serum for 48 h. SirT1 was immunoprecipitated from WEC and the presence of PGC-1α or Foxo3a in the co-immunoprecipitated material was analyzed by WB.



SUPPLEMENTARY FIG. S13. FoxO3a cytoplasm/nuclear ratio (A) Quantitation of images corresponding to Fig. 5D. Data are from ≥3 independent experiments. Data are means + SD (*) $p \leq 0.05$ versus control. **(B)** Confocal microscopy images coresponding to Figs. 5D and 6D that include nuclei labeled with DAPI (left panels) and FoxO3a/DAPI staining overlay (right panels).