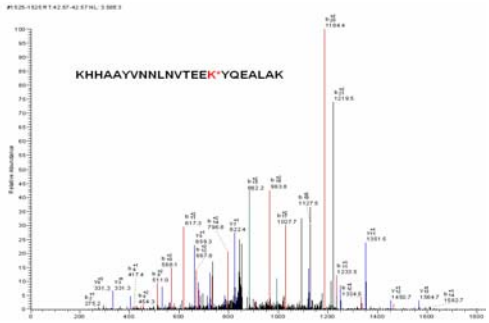
Supplementary Fig. 1. Identification of acetylation of K68 of SOD2

A



B

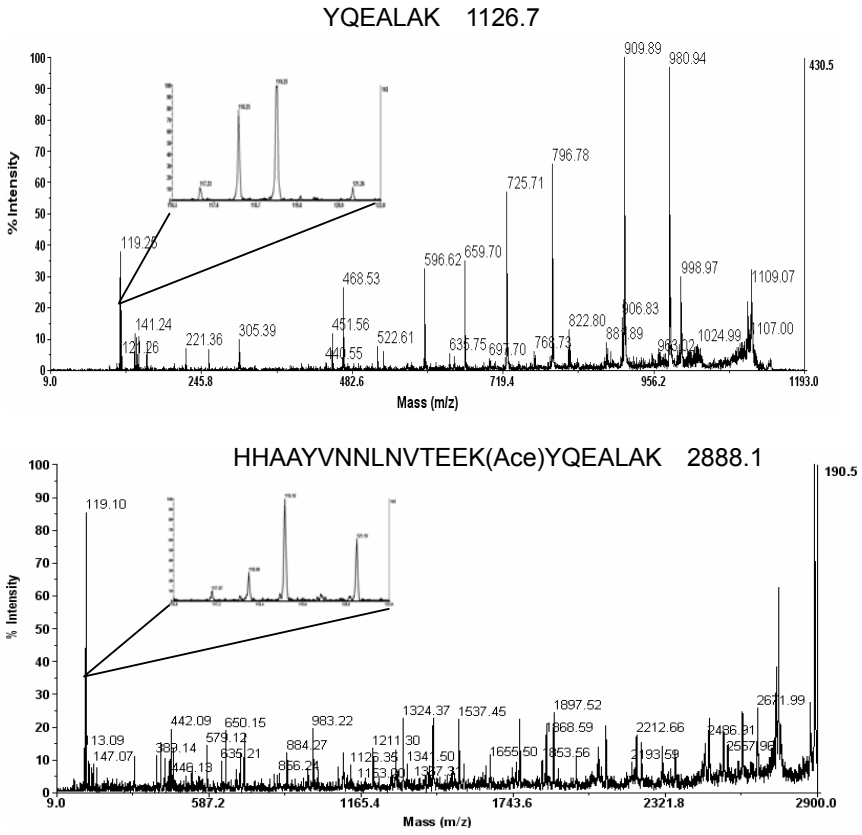
H. sapiens	54	KHHAAYVNNLNVTEE**K**YQEALAK	75
M. musculus	54	KHHAAYVNNLNATEE**K**YHEALAK	75
X. laevis	55	KHHATYVNNLNITEE**K**YAEALAK	77
D. rerio	55	KHHATYVNNLNVTEE**K**YQEALAK	77
D. melanogaster	47	KHHQTYVNNLNAAEEQLEEAKSK	68
A. thaliana	70	KHHKTYVENLNKQILGTDLDALS	92
C. elegans	54	KHHATYVNNLNQIEE**K**LHEAVSK	75
S. pombe	54	KHHQTYVNNLNAAQE**K**LADPNLD	74
S. cerevisiae	55	KHHQTYVNGFNTAVDQFQELSDL	77

Figure S1. Lys68 is the primary acetylation site of SOD2.

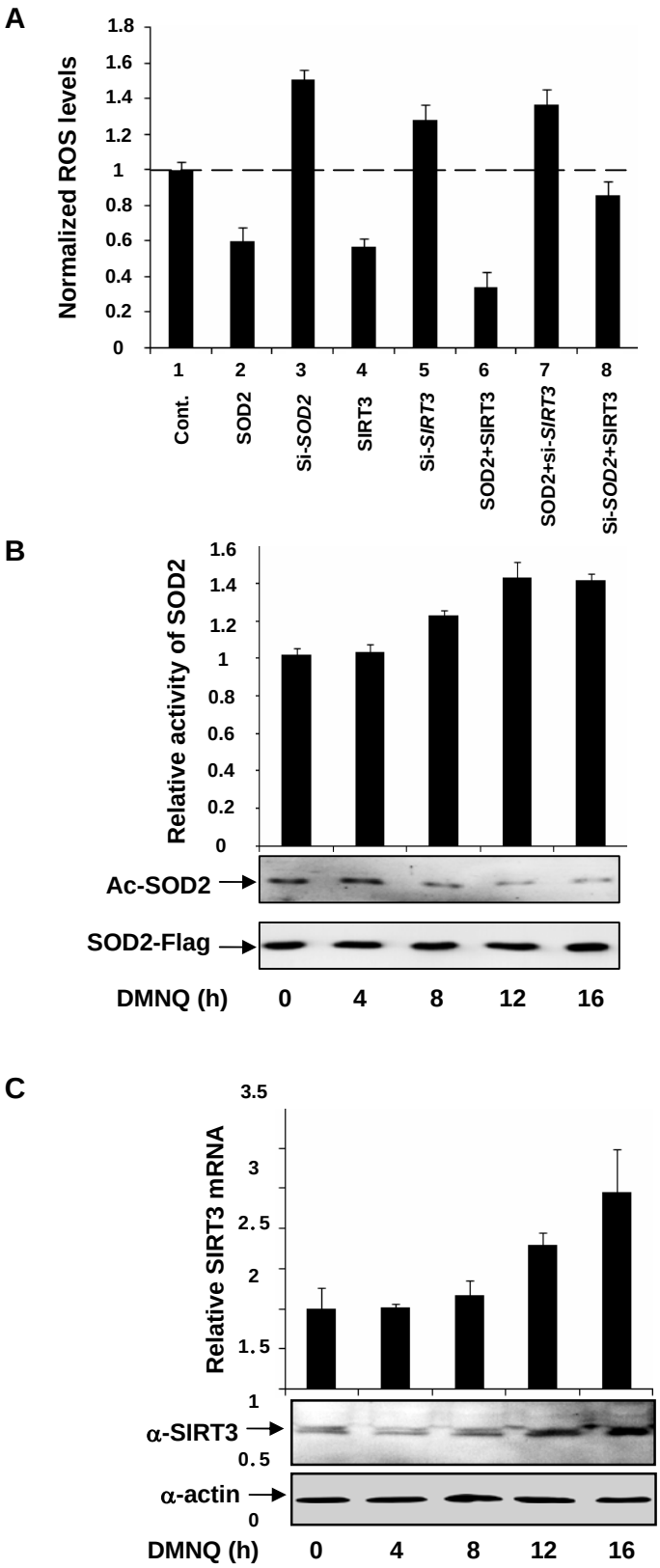
A. MS/MS spectrum of Lys68 acetylated peptide of SOD2.

B. Sequence alignment of SOD2 proteins from different species. SOD2 from different species were aligned, sequences flanking Lys68 are shown.

Supplementary Fig. 2. MS spectra of iTRAQ quantification of acetylation of K68 of SOD2



Supplementary Fig. 3. K68 of SOD2 acetylation regulates ROS level



(**A**) Expression of SOD2 and SIRT3 reduce ROS levels. ROS levels in HEK293T cells with either increased or decreased levels of SOD2 and SIRT3 were determined.

(**B**). DMNQ treatment decreases SOD2 acetylation level and activates SOD2. Acetylation levels and specific activities of SOD2 after treatment with DMNQ for different lengths of time were determined by western blotting and enzyme assay, respectively.

(**C**). DMNQ treatment increases SIRT3 mRNA and protein levels. mRNA and protein levels of Sirt3 in HEK293T cells under different DMNQ treatment times were determined by qRT-PCR and western blotting, respectively.

Methods

Cell culture, transfection and chemical treatments. Cells were cultured under stand conditions. Transfection of cells were carried out by using Trans-EZ of Sunbio. Inc. Treatments for different chemicals including TSA (0.5 μM), NAM (10mM), H_2O_2 (1.5 mM), DMNQ (20 μM).

RNA interference. The target sequence used were as follow: SIRT3, 5' GCTTGATGGACCAGACAAA 3'; SOD2，5' CCCTGGAACCTCACATCAA 3'

Western blotting. Antibodies recognizing SIRT3 (Cell Signaling), SOD2 (Abcam), β-actin, Flag, HA (Santa Cruz) were purchased commercially. Pan-acetyllysine polyclonal antibodies were previously described (Zhao et al., 2010).

SOD2 assay. The SOD2 activity assay was carried out with the SOD Assay Kit-WST (Dojindo Molecular Technology Inc. Rockville). Briefly, 10ul Flag peptides eluent was added to the 96 wells plate, followed by the addition of 200ul WST working and then 20ul Enzyme working solution. The plates were incubated at 37°C for 20 min and 450 nm absorbance was read using a microplate reader.

In vitro deacetylation. His-CobB was purified with nickel beads and stored at -80°C in 10% glycerol. For in vitro deacetylation assay, 5 ug SOD2-Flag proteins were incubated with 15 μg His-cobB proteins in a HEPES buffer (HEPES 40 mM; $MgCl_2$, 1mM; DTT 1mM; NAD+ 5MM) at 30°C for 6 h.

Real-time PCR. Total cellular RNA was isolated using the TRIzol Reagent (Invitrogen). SIRT3 and MnSOD mRNA levels were quantified by SYBR Premix Ex Taq Perfect Real Time (TAKARA) according to the protocol described on the operating manual. The nucleotide sequences of the PCR primers used were as follows:

SIRT3, forward 5' CAGTCTGCCAAAGACCCTTC 3' and
reverse 5' AACACAATGTCGGGCTTCAC 3';

MnSOD2, forward 5' TAGGGCTGAGGTTTGTCCAG 3' and
reverse 5' CACCGAGGAGAAGTACCAGG 3';

β-actin, forward 5’ TCCCTGGAGAAGAGCTACG 3’ and
reverse 5’ GTAGTTTCGTGGATGCCACA 3’

Measurement of intracellular ROS level. Reactive oxygen species (ROS) production was determined by incubating the HEK392T cells in PBS containing 20 μM fluorescent dye 2’,7’-dichlorofluorenscin diacetate (H(2)DCF) (SIGMA) at 37°C for 30 min, followed by flow cytometry analysis.

Measurement of Mitochondrial Superoxide Levels: Superoxide production was determined by measuring MitoSOX (5 μM) (Invitrogen) oxidation in cells following the manufacturer’s instructions. Cells were cultured as described above and incubated for 10 min. at 37℃ before being trypsinized, resuspended, and measured by flow cytometry.

Peptide preparation for iTRAQ quantification: SOD2-Flag protein was ectopically expressed in and immunopurified from HEK293T cellsalone or in combination with SIRT3 knocking down. Purified proteins were resolved on 12% SDS PAGE and stained by Coomassie blue. The amount of protein was estimated by band intensity. Protein bands were sliced and dye was removed by soaking in 50 mM NH_4HCO_3 with 50% acetonitrile. The resulted gel slice was soaked in100 μl water to remove salts, followed by soaking in acetonitrile. The gel slice was then dried in 37°C incubator and then digested in 100 μl 50 mM NH_4HCO_3 with trypsin (trypsin:protein at 1:30) and incubated at 37°C overnight. The resulted peptides were extracted by a solution containing 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) followed by vacuum dry.

Synthesize of internal control peptides: two pairs of peptides, shown in Fig.3G corresponding to the acetylated and unacetylated SOD2 peptide pairs, were synthesized and purified to >99% purity.

Labeling of samples: samples and internal control peptides were labeled by

iTRAQ labeling reagents (ABI, Foster City, CA 94404 USA) as indicated in the table:

Sample	Labeling reagent
SOD2	117
SOD2+SIRT3	118
Internal	119
SOD2+shSIRT3	121

Mass spectrometric analysis and data process: equal amount (5 nmole, equal molar ratio) of 117,118,119 and 121 labeled samples were mixed and subjected to LTQ-OrbiTrap (Thermal) analysis under PQD mode. The resulted MS spectra were used to determine the peptide identity and abundance of each peptide in the same spectrum. Relative abundance of a peptide was calculated by comparing the intensity of the corresponding tag. The absolute quantification was calculated by comparing to the internal control peptides. The sum of the acetylated and unacetylated peptide for each pair was set as 100%. Relative abundance of acetylated peptide and unacetylated peptide in SIRT3 over-expression and knocking-down are shown in Fig. 3G.

DATA analysis

Each experiment has been repeated at least three times. Results are presented as mean and standard deviation (±SD).