Supplemental Methods

Statistical analysis

We used multiple linear regression analysis to test the effect of SOD genotype (GG, AG vs. AA) on clinical and laboratory outcome. In each, model the beta represents the additive effect minor allele (G) on clinical outcome.

A multiple linear regression model with SOD genotype, LDH (natural log) and their interaction was used to predict the TRV. In this model the effect of interaction was statistically significant. Then we used marginal (post hoc) estimation to plot TRV by LDH in each genotype group. LDH was transformed to a normal distribution using natural log to be compatible with assumption of statistical analysis. Also, we used a subgroup analysis to test the correlation between TRV and LDH in each genotype group. Three P values from this subgroup analysis are presented in **Figure 1E**.

Cell Culture and Reagents

Human pulmonary arterial endothelial cells (HPAECs) were obtained from Lonza. Cells were cultured in Endothelial Basal Medium^{TM}-2 supplemented with Endothelial Growth Medium^{TM}-2 SingleQuots^{TM} (Lonza) at 37°C and 5% CO_2

Primers Used for Plasmid Construction

$SOD2^{WT}$ primers: ATCCGCTAGCGCCAGCATGTTGAG (forward) and TTGAGCTCGAGCTTAATGGTGATGGTGATGATGAGCGGCCGCCTTTTTGCAAGCCA (reverse).

$SOD2^{V16A}$ primers: CAGCTGGCTCCGGCTTTGGGGTATC (forward) and GATACCCCAAAGCCGGAGCCAGCTG (reverse).

Hydrogen Peroxide Measurement

HPAECs were transduced with 20 μL lentivirus in a 6 well plate then replated onto a 96 well plate (~10,000 cells/well). The next day cells were placed in assay buffer (25nM HEPES, 10μM EDTA, 100μM L-NAME (N(G)-Nitro-L-arginine methyl ester), 1mM Taurine and 0.01% BSA in HBSS without phenol red). Catalase (500μg/mL) was added to select wells as a negative control. Cells were pretreated with 2mM antimycin A with or without 10nm aminotriazole and then 20μM coumarin boronate (Cayman) was added to all wells and fluorescence (350/450 nm) was measured kinetically for four hours. Automatic gain adjustment was set to scale to low wells using the wells treated with catalase. The slope of the linear portion of the curve was used to determine the rate of hydrogen peroxide production. The slope was calculated after removing production detected from catalase negative controls. Following the assay, cells were fixed in 2% PFA for 10 minutes and stained with 0.5% Crystal Violet for 15 minutes. The wells were then washed with water to remove excess dye. 1% SDS was then added to the wells for 20 minutes. Absorbance was read at 590nm using a BioTek Synergy HT Microplate reader. Slopes calculated from the hydrogen peroxide assay were normalized to the total cell number measured by crystal violet absorbance.

Respiratory Complex and Oxygen Consumption Assays

Complex I activity was determined by monitoring the rate of oxidation of 5mM NADH in the presence of 5mM coenzyme Q2 at 340 nm in the presence and absence of 1mM rotenone. Complex III activity was determined by monitoring the rate of reduction of 15μM cytochrome c at 550nm in the presence of 8mM ubiquinol. Complex IV activity was determined by measuring oxidation of cytochrome c at 550nm. Complex V activity was determined by measuring oxidation of 300μM NADH at 340nm in the presence and absence of 250μM oligomycin. Complex I, II, and IV activities were normalized to Complex V activity. Oxygen consumption was performed using a Seahorse as previously described[1].

SOD Activity

SOD activity was measured using SOD Activity Assay Kit (Abcam, ab65354). To inhibit SOD1 activity 3mM potassium cyanide was added to the cell lysate for 15 minutes[2] before adding reaction solutions.

Mitochondrial DNA (mtDNA) Measurements

Total DNA was extracted as previously described with minor modifications[3]. Snap-frozen cells were lysed in proteinase K buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl), 0.6 mg/mL proteinase K (Fisher Scientific) and 0.01% 2-mercaptoethanol, overnight at 55° C. NaCl (1. 27M) was added to precipitate proteins and cell membranes by centrifugation at 28,000 RCF for 15 minutes at 4℃. The supernatant was collected and mixed to 100% ethanol. The nucleic acids were precipitated by centrifugation at 28,000 RCF for 15 minutes at 4 °C. The pellet was washed with 70% ethanol, air-dried overnight in the dark, and resuspended in DNases-free water. DNA was treated with 0.4 mg/mL RNase A (Sigma-Aldrich) for 2 hours at 37 °C to degrade RNAs, then stored at -20 °C, and successively quantified by AccuBlue Broad Range dsDNA Quantitation kit (Biotium).

Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) were measured by duplex qPCR using the TaqMan chemistry in a 96-well StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The reaction contained four ng of DNA template, Luna Universal qPCR Master Mix (New England Biolabs), two probes and primer set (ND1PL for mtDNA and B2M for nDNA, respectively). Thermocycling condition and probes/primer set were previously validated[4].

Mitochondrial DNA was normalized to the nDNA by ΔCt method for each sample. The mtDNA/nDNA in $SOD2^{V16A}$ was calculated as variation of $SOD2^{WT}$ (arbitrary unit=1), by $2^{-\Delta\Delta Ct}$ method for each biological replicate.

Mitochondria Superoxide Measurements

Transduced HPAECs were trypsinized and resuspend in Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS). 150,000 cells were added to each well of a 96 well plate along with 5 μM MitoSOX Red™ (Invitrogen). After addition of MitoSOX, fluorescent signal (510nm/580nm) was recorded at 37°C for 2 hours. Fluorescent intensities were averaged every 6 minutes to reduce variation before the log phase slope was calculated to express the reaction rate. The results were normalized to protein concentration.

Electron Microscope (EM) Imaging and Affinity Pulldown of APEX2-SOD2 Constructs

Transfected HPAECs were rinsed and fixed in 1 volume 2% glutaraldehyde, 1 volume deionized water, and two volumes 0.2M cacodylate on ice for one hour. Following fixation cells were washed with 100 mM cacodylate and then incubated with 20 mM glycine 2 mM $CaCl_2$ in 100mM cacodylate on ice for 5 minutes. After washing cells were incubated in 0.5 mg/mL 3,3'-diaminobenzidine (DAB) for 30 minutes. After incubation with DAB, H_2O_2 was added to a final concentration of 10mM until brown precipitate was observed. Cells were then rinsed with 100mM cacodylate, post-fixed in 1% osmium tetroxide with 1% potassium ferricyanide, rinsed in PBS, dehydrated through a graded series of ethanol and embedded in Poly/Bed® 812 (Luft formulations). Semi-thin (300 nm) sections were cut on a Leica Reichart Ultracut, stained with 0.5% Toluidine Blue in 1% sodium borate and examined under the light microscope. Ultrathin sections (65 nm) were examined on JEOL 1400 Plus transmission electron microscope (grant #1S10RR016236-01 NIH for Simon Watkins) with a side mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA) at the Center for Biologic Imaging at the University of Pittsburgh.

For affinity pulldown, transfected cells were incubated with 500μM biotin-phenol (Cayman) for 30 minutes. APEX2 was activated by adding H_2O_2 at a final concentration of 100μM for 60 seconds. The reaction was quenched by repeated washing using 10mM sodium ascorbate, 10mM sodium azide, 5 mM Trolox (Cayman) in phosphate buffered saline, pH 8. Biotinylated proteins were enriched utilizing Dynabeads™ M-280 Streptavidin (Invitrogen) and eluted in 2X Laemmli Buffer by heating the beads before immunoblotting.

Quantification of APEX Imaging

To quantify the localization of the SOD2 variants, regions of interest were drawn within the mitochondrial matrix and the cytoplasm adjacent to the measured mitochondria matrix. The inverse of the raw integrated intensities was taken and the inverse of the intensity within the mitochondria matrix was normalized to the inverse of the intensity measured in the cytoplasm.

Measurement of Metabolic Pathways

Oxygen consumption rate (OCR) was measured in HPAECs transduced with either SOD2 variant using the Seahorse Extracellular Flux (XF96) Analyzer (Seahorse Bioscience, Inc., North Billerica, MA) as previously described[1,5]. HPAECs were transduced in 6 well dishes and grown in Endothelial Basal Medium. 72 hours after transduction 2.2 $x10^4$ cells were loaded into each well of a standard XF24 plate. 24 hours after replating, the Endothelial Basal Medium was replaced with Dulbecco's Modified Eagle's Media (DMEM containing 25mM glucose, 2mM glutamine and 1mM pyruvate). Once the medium was changed, cells were consecutively treated with oligomycin A (2.0 μM), FCCP (carbonyl cyanide-ρ-trifluoromethoxyphenylhydrazone) (0.5 μM), Antimycin-A (Ant A) (2 μM), and Rotenone (2 μM).After addition of each agent the wells were mixed and three measurements of OCR were made. After the end of the experiment, measurements were normalized to cell number using crystal violet staining.

ATP-linked OCR is represented as oligomycin A values subtracted from basal respiration. Proton leak is the difference of oligomycin A and Rotenone/Antimycin values. Non-mitochondrial respiration is the Rotenone/Antimycin values subtracted from basal respiration. Values are expressed in terms of percentage basal respiration for each run.

Fatty acid oxidation (FAO) was measured by treating cells with 40 mM etomoxir (ETO) and subtracting ETO values from basal respiration. Pyruvate metabolism was measured by treating cells with 10 mM UK5099 and subtracting UK5099 values from basal respiration. Glutamine oxidation was measured by treating cells with 5 mM BPTES and subtracting BPTES values from basal respiration. Basal extracellular acidification rate (ECAR) was measured to determine glycolytic rate.

Western Blot Analysis

Cell lysates were prepared as previously described[6]. Goat anti-GFP polyclonal antibody (Abcam, ab6673), mouse 6X-his tag monoclonal ab (Invitrogen, 37-2900), mouse anti-tubulin monoclonal (Sigma, T5168), mouse anti-ATP citrate synthase polyclonal ab (Santa Cruz, 5F8D11), mouse total OXPHOS human ab cocktail (Abcam, ab110411, Complex I-subunit NDUFB8, Complex II-subunit 30kDa, Complex III-subunit Core 2, Complex IV-subunit II, Complex V-subunit alpha), rabbit anti-catalase monoclonal ab (Cell Signaling, D4P7B), mouse anti-TOM20 monoclonal ab (Santa Cruz, sc17764), rabbit anti-HIF-2α monoclonal ab (Cell Signaling, 7096), mouse anti-eNOS monoclonal ab (BD Transduction Labs, 610308), rabbit anti-peNOS S1177 polyclonal ab (Cell Signaling, 9571), and mouse anti-actin monoclonal ab (Santa Cruz, sc47778) were used in western analyses.

ATP Quantification

ATP was quantified using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G9241) according to manufacturer's instructions.

Measurement of $\Delta\psi$

Transfected HPAECs were incubated with 10 μg/mL JC-1 probe (ThermoFisher Scientific, T3168) for 20 minutes at 37°C then subjected to 2 color flow cytometry (514/529nm and 585/590nm) to quantify the proportion of cells containing JC-1 green monomer (indicative of low $\Delta\psi$) and JC-1 red aggregates (indicative of high $\Delta\psi$) . The ratio of cells containing red aggregates to green monomer was calculated and expressed relative to $SOD2^{WT}$ transfected HPAECs.

In vitro Wound Healing Assay

HPAECs were plated onto 12 well cell culture plates, transduced and grown to confluence. Scratches were made to the bottom of the well using a pipet tip and then imaged. Cells were then incubated in growth factor free medium for approximately 16 hours and reimaged. Percent wound closure was then calculated. Four measurements were made from three wells on three separate days. Results from each day were averaged and SOD2 V16A closure was normalized to SOD2 WT closure from the respective day.

Cell Survival

22,000 SOD2 WT or SOD2 V16A cells were seeded onto a cell culture plate. The next day nonadherent cells were washed off and the remaining living cells were fixed and stained with crystal violet. Cells were then dissolved with 1% SDS and protein concentration was measured by absorbance at 590 nm. SOD2 V16A cell survival was normalized to SOD2 WT value from the respective day.

Nitrite Measurement

HPAECs were plated and transduced in 6 well cell culture plates. Cells were scraped and collected in a nitrite preservation buffer (potassium ferricyanide 801.1 mM, N-ethylmaleimide 17.6 mM, NP-40 substitute 6%). Samples were then injected in a glass sparger where nitrite was reduced to nitric oxide using the tri-iodide method [potassium iodide 1.11% (w/v), iodine 0.72% (w/v), acetic acid 77.8% (v/v)][7]. The resultant nitric oxide is carried by helium into Sievers Nitrite Oxide Analyzer 280i (General Electric). The chemiluminescent signal from the reaction between nitric oxide and oxygen was recorded. Nitrite concentrations were calculated using the area under the curve and sodium nitrite standards. SOD2 V16A and Ant A treatment nitrite measurements were normalized to SOD2 WT values from the respective day.

SOD2 Immunoprecipitation

HPAECs were plated onto a 10 cm cell culture dish and then transduced with either SOD2 WT or SOD2 V16A. 72 hours after transduction, cells were split and half the cells were treated with 2 μM Ant A for 30 minutes at 37 ºC. Cells were then lysed with immunoprecipitation (IP) buffer (1% NP-40, 137 mM NaCl, 2 mM EDTA, 20 mM Tris) supplemented with proteinase, phosphatase, and deacetylase inhibitors and rotated at 4 ºC for 20-30 minutes. An aliquot was placed aside for whole cell lysate sample and 50 μL Dynabeads™ His-Tag Isolation and Pulldown (ThermoFisher, 10103D) was added to the rest of the lysate and rotated at 4 ºC overnight. The next day, beads were washed 4 times with 0.1% Tween-20. Protein was eluted in 50 μL 2x Laemmli buffer at 95 ºC for 15 minutes, vortexing every 2-3 minutes. IP samples were run on a gel and transferred. Primary antibodies rabbit anti-SOD2 (Abcam, ab13533) and rabbit anti-Acetylated Lysine (Cell Signaling, 9441) were used in Western analysis.

Supplemental Figure Legends

Supplemental Table 1. Baseline clinical parameters of SCD cohort. Distribution of clinical and ECHO marker in SS patients by SOD2 genotype. Results for LDH values are given as mean (SD). All other results are in mean (SE) unless otherwise specified. Hemolytic index is measured by a principal component analysis of four factors: aspartate transaminase (AST), LDH, absolute reticulocytes, and total bilirubin.

Supplemental Figure 1. SOD2 V16A has increased catalase activity without changes in catalase and SOD1 protein in transduced HPAECs. (A) Hydrogen peroxide assay utilizing the catalase inhibitor amino triazole demonstrates an increase in hydrogen peroxide production inhibitable by catalase in SOD2 V16A transduced cells. (B-C) Western blot and quantification of catalase protein in SOD2 variants. (D-E) Western blot and quantification of SOD1 protein in SOD2 variants.

Supplemental Figure 2. No change in SOD2 enzymatic activity, localization between SOD2 variants, or other metabolic pathways. (A) Schematic of experimental design to specifically measure SOD2 activity. (B) SOD2 specific activity in SOD2 variants. (C) Quantification of SOD2 APEX EM Imaging. (D, E) Increase in fatty acid oxidation (FAO) and glycolysis.

Supplemental Figure 3. No changes in ATP production or mitochondria potential in SOD2 V16A transduced HPAECs. (A) ATP production, (B) mitochondria potential, and (C) mitochondria DNA in transduced HPAECs. (D) Mitochondria area normalized to perimeter, EM image taken at 100,000x magnification with JEM 1400Plus TEM.

Supplemental Figure 4. Increased NO metabolism in SOD2 V16A transduced HPAECs. (A, B) Representative images and quantification of *in vitro* scratch assay to measure cell proliferation and migration. No difference was observed between SOD2 variants. (C) Cell survival was measured by plating 22,000 transduced cells and assaying for survivability with crystal violet the next day. (D, E) Western blot and quantification of HIF-2α protein in SOD2 variants. (F) Nitrite measurements at baseline in transduced cells. (G-I) Western blot and quantification of eNOS and eNOS pSer1177 protein in SOD2 variants.

Supplemental Figure 5. Increased mitochondrial superoxide production in SOD2 V16A HPAECs is not attributable to changes in acetylation. (A, B) His-tag beads were used to immunoprecipitate SOD2 WT or SOD2 V16A from transduced HPAECs and degree of acetylation was measured by Western blot. No changes in acetylation were detected at baseline or after treatment with antimycin A.

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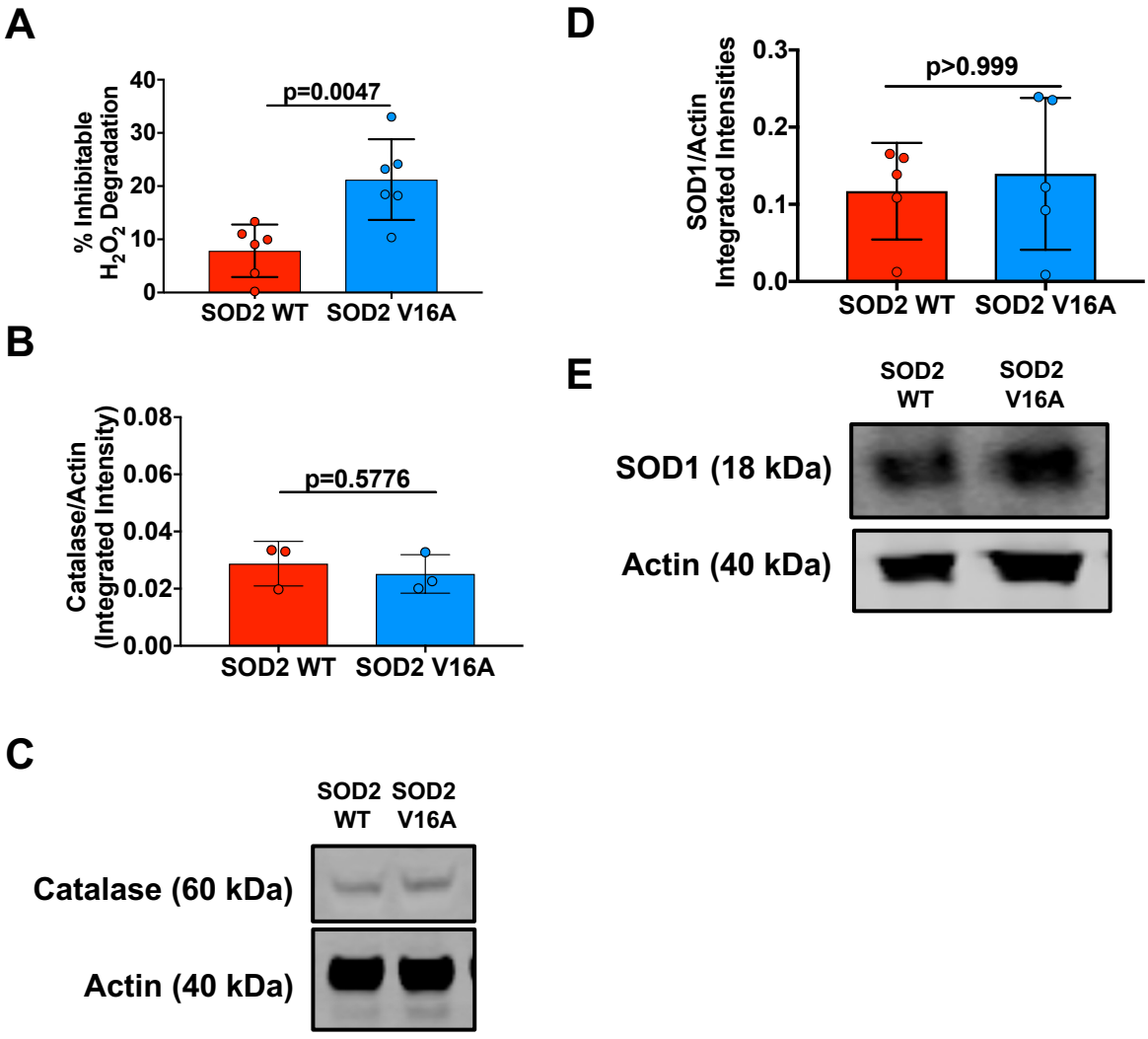
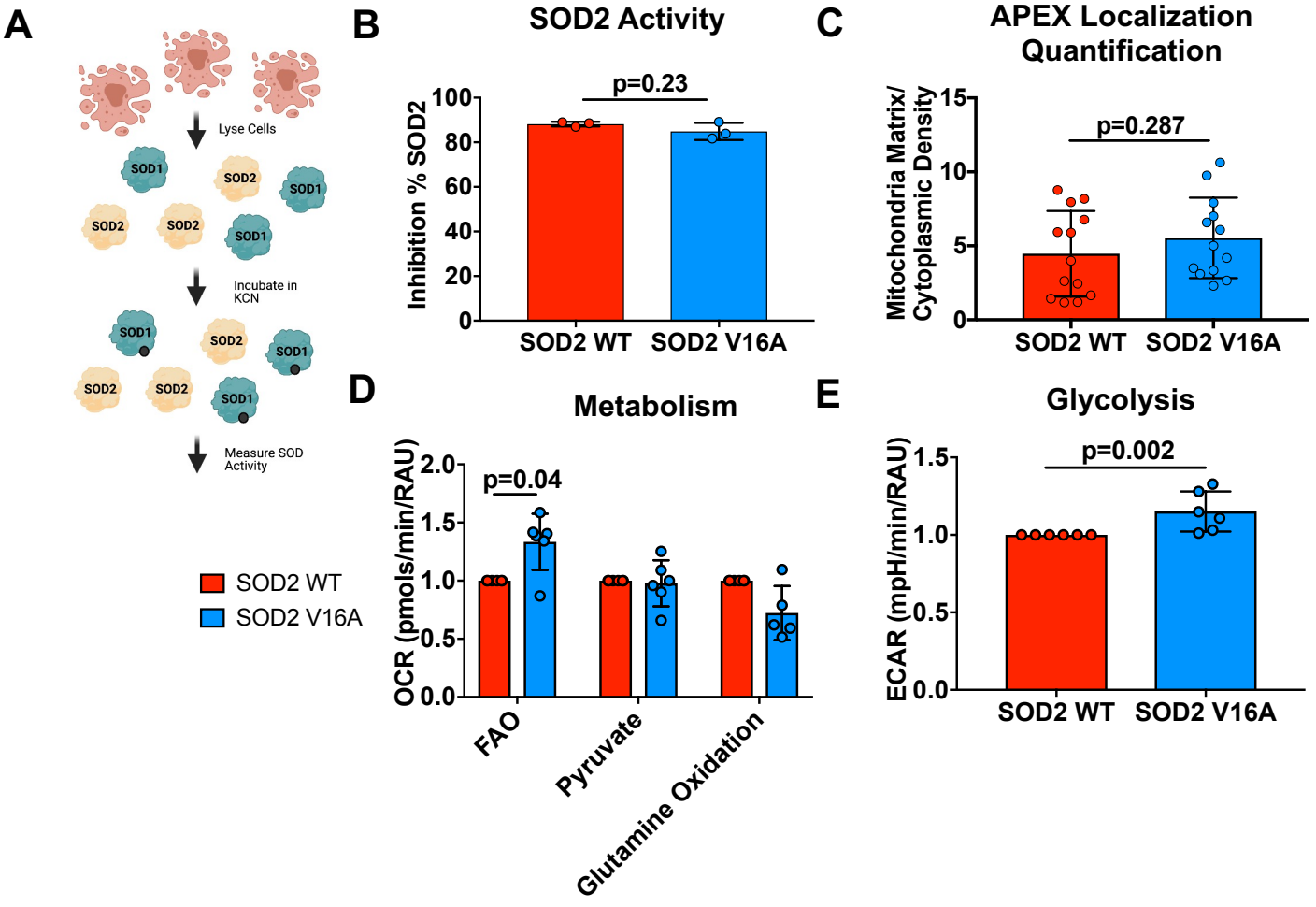
Supplemental Table 1

Distribution of clinical and echo marker in SS patients by SOD2 genotype. Results are in mean (SE) unless otherwise specified.				
	AA	AG	GG	P value for linear trend
	N = 129	N = 217	N = 64	
Age	35 (1.1)	36 (0.8)	38 (1.6)	0.09
History of pulmonary embolism %	5%	3%	6%	0.82
Hemoglobin	8.9 (1.4)	8.6 (1.1)	8.5 (2.0)	0.06
MCV	94 (1.0)	93 (0.8)	93 (1.4)	0.62
Reticulocyte count	26 (1.2)	26 (0.9)	28 (1.7)	0.27
WBC count	9.7 (0.33)	10.4 (0.25)	10.4 (0.46)	0.21
Platelet count	375 (11.6)	380 (9.0)	357 (16.4)	0.37
Left ventricular e/ea'	6.8 (0.26)	7.2 (0.20)	6.8 (0.37)	0.95
Left mass index	113 (3.0)	117 (2.3)	116 (4.3)	0.52
Right atrial area	19 (0.4)	20 (0.3)	20 (0.6)	0.76
Creatinine	0.9 (0.09)	0.9 (0.07)	0.9 (0.13)	0.82
Hemolytic Index	0.4 (0.18)	0.5 (0.12)	0.9 (0.22)	0.037
LDH	493 (368)	473 (294)	506 (258)	0.39

Supplemental Figure 1

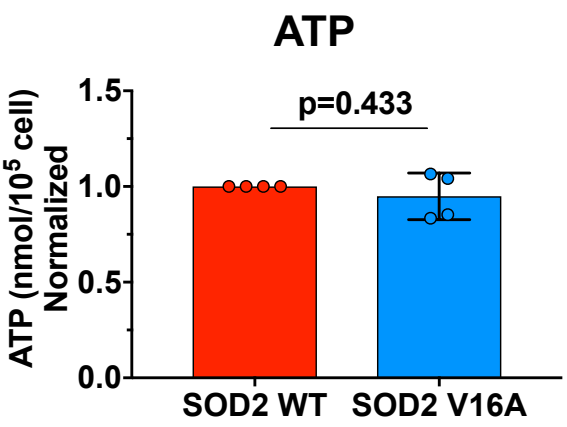


Supplemental Figure 2



Supplemental Figure 3

A



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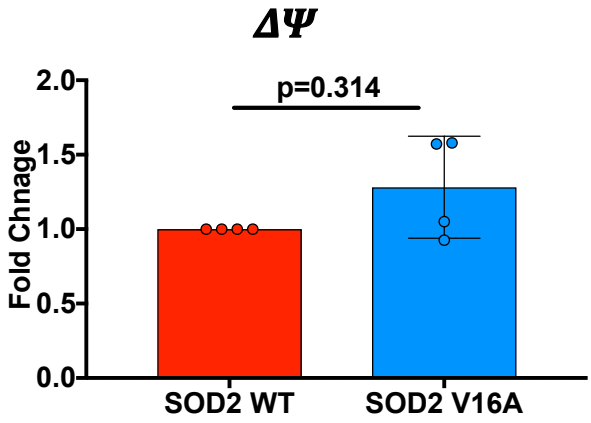

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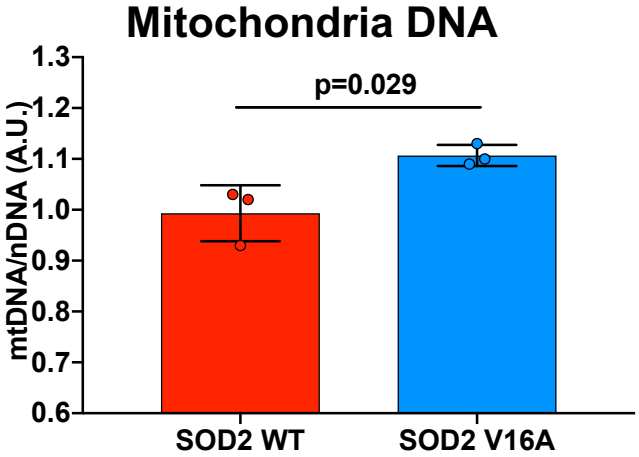

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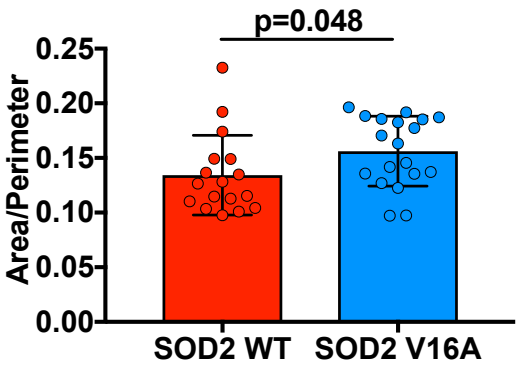
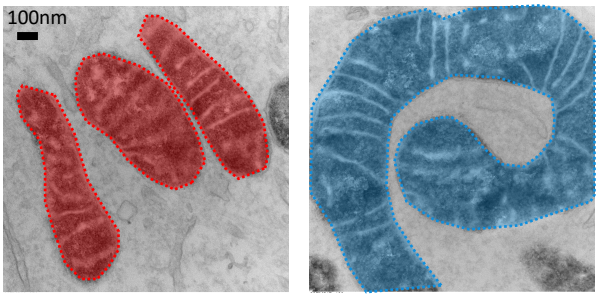
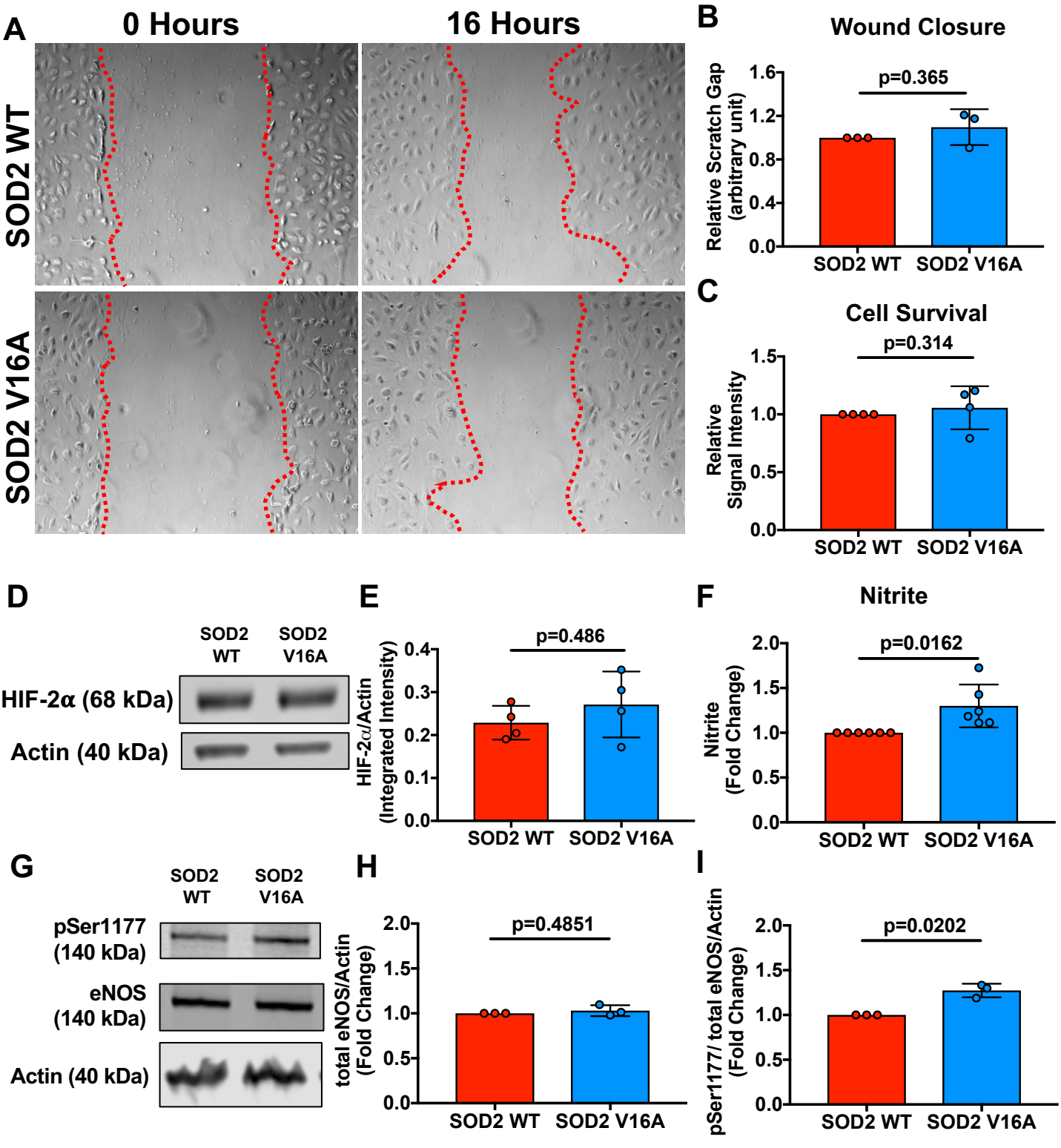




Supplemental Figure 4



Supplemental Figure 5