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Supplementary Information for
SOD1 is an essential H_2S detoxifying enzyme

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Supplementary Information Text

MATERIALS AND EXTENDED METHODS

Experimental Models and Subject Details

Yeast strains
Parental wild type (BY4741) [MAT*a* *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0] and *sod1*Δ mutant yeast strains were obtained from Horizon Discovery. Yeast strains were cultured at 30°C on YPD agar or liquid media and *sod1*Δ yeast were maintained under selection pressure by adding 200 μg/mL G418 to YPD media.

Human cell lines
Human cell lines were obtained from ATCC. Human female embryonic kidney epithelial cell line (HEK293) was cultured in Dulbecco's Modified Eagles Medium (Gibco) supplemented with 10% v/v FBS. Amphotericin B (2.5 μg/mL) and 100U/mL penicillin and 100mg/mL streptomycin were added to media during cell expansion passages. Human female normal breast epithelial cell line (MCF10A) was cultured in Mammary Epithelial Growth Medium (Lonza) supplemented with 100 ng/mL cholera toxin as instructed by ATCC. Cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere and were detached from culture vessels by incubating cells with trypsin-EDTA diluted to 0.1% w/v with PBS at 37°C and quenched with Defined Trypsin Inhibitor. Cell viability was assessed by trypan blue exclusion assay and quantified with a Countessa II cell counting system (Invitrogen).

Plasmids
Wild type and H65R mutant human CBS was cloned into pcDNA3.1(+)-N-eGFP and wild type human SOD1 was cloned into pcDNA3.1+/C-(K)-DYK vectors, expressed in TOP10 E. coli and purified by GenScript Biotech. Plasmids are available via Addgene.

Purified protein
Purified human and bovine SOD1 were obtained from Sigma. Human SOD1 was loaded by incubating protein solutions with 100 μM $CuSO_4$ and $ZnCl_2$ in 50 mM acetate buffer, pH 4.5 overnight and dialyzing against 50 mM MOPS buffer, pH 7.5 at 4°C. SOD1 copper loading was confirmed by visible spectroscopy and protein concentration determined by BCA assay. Human apo-SOD1 was prepared by dialyzing SOD1 against 50 mM acetate pH 4.5 containing 100 μM EDTA and buffer exchanged into 50 mM MOPS pH 7.5 + 100 μM EDTA. SOD1 activity was confirmed by SOD1 activity assay (Abcam).

Method Details

Yeast toxicity
Frozen glycerol stocks of yeast were spread onto YPD agar plates and incubated for 24-48 hours at 30°C. Colonies were isolated and grown overnight in YPD at 30°C and with 500 rpm agitation. Yeast were diluted to OD_{600} = 0.1 and grown for 6-12 hours to achieve mid-log phase cultures. Yeast were supplemented with Mn by culturing strains as above in YPD containing 4 mM Mn $(acetate)_2$ (1). For OD_{600} growth experiments, yeast strains were then diluted into fresh YPD media, aliquoted into 96-well plates and NaSH added (from freshly prepared 100x stocks prepared in 50 mM Na_2CO_3, pH 10). Wells were sealed with an adhesive plate film and inserted into a CLARIOstar Plus set to 30°C and OD_{600} was monitored for 12 hours with shaking between readings. For spot assays, mid-log phase cultures were washed and serially diluted in PBS. Freshly poured YPD agar plates were supplemented with 100 μM (final conc.) methyl viologen, NaSH, cysteine trisulfide or potassium polysulfide and 3 μL of yeast suspension were spotted and incubated for 24 hours at 30°C. Plates were imaged on an iBright FL1500 Imaging System and densitometry performed with Gel-Pro Analyzer software (v3.1). To examine persulfide/polysulfide formation in yeast cells exposed to H_2S, mid-log phase yeast cultures in 5 mL YPD were stained with 3 drops of NucBlue Live (Hoechst 33342) for 30 minutes and cells washed in PBS. Yeast pellets were suspended in

500 μL PBS and SSP4 added to a final concentration of 20 μM. Yeast were incubated for 1 hour at 30°C and with 500 rpm agitation and yeast were washed again in PBS. SSP4/Hoechst 33342-loaded yeast were re-suspended in 5 mL YPD, diluted 1:20 and added to a white-walled clear bottom 96-well plate. NaSH was added as described above and plates were sealed with an adhesive plate film and inserted into a CLARIOstar Plus set to 30°C. Relative SSP4 (487±14 nm excitation, dichroic filter 507 nm, 535±30 nm emission; gain 900) and Hoechst 33342 (360±20 nm excitation, dichroic filter 407.5 nm, 460±30 nm emission; gain 900) fluorescence was measured every 15 minutes for 12 hours and compared to YPD alone without yeast (baseline). SSP4 fluorescence was normalized to Hoechst 33342 fluorescence at each time point and plotted versus time.

HEK293 cell SOD1-silencing

HEK293 cells were transfected with control or SOD1- or SOD2-specific siRNA (40 nM) using Lipofectamine 3000. After 48 hours, cells were isolated and used as described below and knockdown was confirmed by immunoblotting. For proliferation assays, 20,000 viable cells/100 μL DMEM + 10% v/v FBS were seeded into xCELLigence RCTA E-Plate 16 (Agilent, Cat# 5469813001) and GYY4137 added from 100x stock solutions freshly prepared in 1:1 PBS/DMSO mixture. Void space between wells in the E-Plates were filled with 37°C PBS before starting the experiment. E-Plates were then loaded into the xCELLigence RTCA DP analyzer installed in a 37°C incubator with a humidified, 5% CO_2 atmosphere and electrical impedance measured every 15 minutes for 96 hours. All data points were baseline (wells with media only) corrected using RTCA Software Pro. Alternatively, cells were seeded and allowed to grow for 24 hours before addition of GYY4137. To examine endogenous H_2S production, wild type or mutant (H65R) human CBS expression plasmids were co-transfected with siRNA as above. After 24 hours, NucBlue Live (Hoechst 33342) was added to media and cells were imaged using EVOS M7000 Cell Imaging System. Cells were then diluted and seeded into xCELLigence plates as above and analyzed for protein expression by immunoblotting. For mitochondrial respiration studies, the Seahorse XF96 Cell Mito Stress Test was performed as previously described (2). Briefly, 20,000 HEK293 cells were seeded into Seahorse XF Cell Culture Microplates 48 hours after siRNA transfection and allowed to adhere overnight. NaSH was then added to the wells and the plate assembled with oligomycin, FCCP, rotenone/antimycin A and analysed by Seahorse XF96 Cell Metabolic Analyzer. Oxygen consumption rates were normalized to total protein content determined by BCA assay directly after Seahorse analysis. For protein oxidation assays, cells were seeded into 12-well plates and cultured for 24 hours. To measure protein S-glutathionylation, cells were pre-treated with Glutathione Ethyl Ester, Biotin Amide (BioGEE) (Thermo Fisher Scientific) (150 μM final concentration) for 1 hour before addition of NaSH. Cells were incubated with NaSH for 2 hours and lysed in 2X Laemmli Sample Buffer (without 2-mercaptoethanol) supplemented with 50 mM maleimide. Samples were heated to 85°C with 1500 rpm agitation for 10 minutes and analyzed by immunoblotting.

HEK293 cell SOD1 overexpression

To examine the effect of SOD1 overexpression, HEK293 cells were transiently transfected with a FLAG-tagged wild type human SOD1 expression plasmid using Lipofectamine 3000 and cultured for 24 hours. Cells were then seeded into xCELLigence plates as above and grown for 48 hours before GYY4137 added and cellular proliferation measured for another 48 hours. The remaining cells were analyzed for protein expression by immunoblotting.

Immunoblotting

Cellular samples were measured for relative protein expression levels by immunoblotting. Cell lysates were denatured in 2X Laemmli Sample Buffer containing 5% v/v 2-mercaptoethanol with heating to 85°C with 1500 rpm agitation for 10 minutes. Proteins were then separated by SDS-PAGE using either 10% or 4-20% gradient Mini-PROTEAN TGX precast gels (Bio-Rad) with 25 mM Tris, 192 mM glycine, 0.1% SDS running buffer. Gels were then transferred to PVDF membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were then blocked with 5% w/v BSA in phosphate-buffered saline containing 0.1% Tween-20 (PBS-Tween) for 1 hour at room temperature and then incubated overnight with diluted primary antibody at 4°C with gentle rotation. Primary antibody dilutions: SOD1 (1:2000), α/β-tubulin (1:3000), PKG1alpha (1:1000), β-

actin (1:10,000), biotin (1:1000), GFP (1:1000). Blots were washed in PBS-Tween and incubated with secondary antibody diluted in 5% w/v BSA for 1-2 hours at room temperature with gentle rotation. Secondary antibody dilutions: anti-rabbit-HRP (1:5000), anti-mouse-HRP (1:5000), anti-mouse-Alexa 647 (1:10,000). Membranes were washed and proteins detected by ECL or fluorescent imaging using an iBright FL1500 Imaging System. Densitometry analyses were performed on images using Gel-Pro Analyzer software (v3.1).

Liquid chromatography-electrospray ionization–tandem mass spectrometry
Cells (1-2x10^6) were homogenized and sonicated in 100 µL 50 mM sodium acetate buffer (pH 6.5) containing 1 mM *N*-iodoacetyl l-tyrosine methyl ester (TME-IAM) and 70% methanol. Lysates were incubated at 37°C for 30 min followed by centrifugation at 20,000 g for 15 min at 4°C. The supernatants (100 µL) were collected and acidified with 10 µL of 10% formic acid (FA), mixed with 100 nM of stable isotope-labeled standards in 0.1% FA/water before liquid chromatography-electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) analysis. Insoluble pellets were dissolved in 150 µL of phosphate-buffered saline containing 1% sodium dodecyl sulfate and protein content was determined using the bicinchoninic acid assay.

LC-ESI-MS/MS analyses were carried out using a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, Milford, MA) coupled to a Shimadzu LC-20A system (Shimadzu, Kyoto, Shimadzu) equipped with an autosampler (SIL-20A), a communications bus module (CBM-20A), an on-line degasser (DGU-20A5), two liquid chromatographs (LC-20AD) and a column oven (CTO-20A) as described previously (3) with some modifications. Briefly, cell lysates were evaluated with the Shimadzu LC-20A system on a Mightysil-C18 column (50 mm × 2.0 mm inner diameter, Kanto Chemical, Tokyo, Japan) and then eluted using methanol as the mobile phase with a linear gradient (1%–99%, 5 min) in the presence of 0.1% FA at a flow rate of 0.3 mL/min at 40°C. The mass spectrometer was operated in the positive mode with the capillary voltage and de-solvation gas (nitrogen) set to 1000 V and 1000 L/h, respectively, at a temperature of 500°C. The respective adducts were detected in the multiple reaction monitoring (MRM) mode using the parameters shown in Table S1.

H_2S measurements
H_2S amperometric electrodes (ISO-H2S-2, World Precision Instruments) connected to a TBR4100 free radical analyzer (World Precision Instruments) set to 100nA range and coupled to a PowerLab 4SP data acquisition system controlled by LabChart 8 (ADInstruments) were used to measure sulfide in solution. Sulfide consumption experiments were performed in PBS containing 10 µM EDTA. A sulfide standard curve was established to correlate H_2S concertation with the pA output of the electrode. A sulfide stock solution was diluted to 10 µM in the reaction vessel containing the electrode with rapid stirring. The signal is allowed to equilibrate for approx. 3-5 minutes before the addition of human SOD1 or MOPS buffer (50 mM, pH 7.5). To measure steady-state H_2S levels in cell culture media, GYY4137 was diluted into DMEM + 10% v/v FBS and 100 µL are added to a 96-well plate and incubated at 37°C for 24 hours. A standard curve was established by adding 100x Na_2S stock solution to 100 µL media in a 96-well plate and the signal plateau was averaged to correlate H_2S concentration to electrode current. GYY4137 containing media was similarly measured to determine the steady-state H_2S levels in our *in vitro* assays. To measure H_2S formed from cysteine trisulfide, 100 mM stock solutions were diluted 1000-fold into 2 mL 500 mM MOPS buffer, pH 7.5 after a stable baseline was established. 2-ME was then added to the rapidly stirring solution at room temperature. Relative cellular H_2S measurements were measured by adding Difluorinated H_2S Fluorescent Probe 1 (7-azido-6,8-difluoro-4-methyl-2h-1-benzopyran-2-one; CAS# 2103919-91-9) (Cayman Chemical) to a final concentration of 1 µM in media (for HEK293) or PBS (yeast) and incubating for 30 minutes prior to measuring fluorescence (ex. 365/em. 450nm) on a CLARIOstar Plus. Alternatively, after probe loading, cysteine trisulfide was added and incubated 2 hours before fluorescence measurements.

Sulfate measurements
Sulfate production from the enzymatic oxidation of sulfide was measured by ion chromatography. All plastic and glassware including pipette tips used in ion chromatography experiments were

washed overnight in 30% v/v trace metal-grade nitric acid, thoroughly rinsed in distilled water and dried before use to remove contaminating sulfate. Human [Cu-Zn]- or apo-SOD1 and NaSH were dissolved in Water For Ion Chromatography (Fisher Scientific, Cat# 12903644) and mixed at room temperature. SOD1 protein was removed from reaction products by Amicon Ultra-0.5 Centrifugal Filter Unit (3 kDa NMWCO). Standards and samples were injected via autosampler into a Thermo Dionex IC 5000+ System with 2 x 50 mm guard and 2 x 250 mm Dionex IonPac anion-exchange analytical columns, a Dionex Anion Dynamically Regenerated Suppressor 600 and quantified with an electrochemical detector.

SOD1-catalyzed persulfide formation

Aerobic persulfide formation by was examined by mixing human SOD1 (10 or 25 μM) and SSP4 (100 μM) in 50 mM MOPS pH 7.5. The reaction was initiated by adding NaSH and the reaction was incubated at 37°C for 30 minutes with 300 rpm agitation and protected from light. Reactions were measured for SSP4 fluorescence (487±14 nm excitation, dichroic filter 507 nm, 535±30 nm emission; gain 800) on a CLARIOstar Plus at room temperature. SOD1-sulifde complex and persulfide formation were examined under anaerobic conditions. To study sulfide coordination, the visible spectrum of 100 μM bovine Cu-Zn SOD1 solutions in 50 mM MOPS buffer, pH 7.5 was recorded with a PicoDrop spectrophotometer inside the anaerobic glove box before and after the addition of 50 μM NaSH. The cuvette was removed from the glove box, pipetted up and down 2-3 times to exchange gases and returned to the anaerobic chamber for spectral recording. To examine the mechanism of SOD1-catalyzed persulfide formation, bovine SOD1 and NaSH were reacted in the presence of electron acceptors. All solutions were deoxygenated by multiple cycles of vacuum pumping and nitrogen filling in the small port of an anaerobic glove box maintained below 1 ppm O_2. To avoid denaturation via bubbling, bovine SOD1 solutions were prepared in the glove box with pre-weighed aliquots. SOD1 (10 or 25 μM) and either $K_3Fe(CN)_6$, VCl_3 or MV^{2+} (1 mM) were mixed in a microcuvette in 50 mM MOPS buffer, pH 7.5. The reaction was initiated by addition of NaSH and the change in absorbance 420 nm was recorded over 3 minutes at 23°C with a PicoDrop spectrophotometer inside the anaerobic glove box. $MV^{\bullet+}$ was formed by adding excess dithionite solid to MV^{2+} (1 mM in 50 mM MOPS buffer, pH 7.5).

Sodium persulfide reactivity

Sodium persulfide (Na_2S_2) (Dojindo) was used as supplied and stock solutions were freshly prepared in 10 mM NaOH and immediately further diluted into either MOPS or PBS + EDTA (10 μM) and added to cells or reactions. Glutathione (GSH) oxidation was measured by reacting GSH (5 μM) with Na_2S_2 in 50 mM MOPS buffer, pH 7.5 at 37°C for 30 minutes with 300 rpm agitation. Reaction products were then assayed with GSH-Glo (Promega) as instructed and compared to an authentic GSH standard curve. Adherent MCF10A cells were loaded with BioGEE (150 μM final concentration in culture media for 60 minutes, 37°C) briefly rinsed with PBS and treated with Na_2S_2 in PBS for 15 minutes. Similarly, adherent HEK293 cells were rinsed with PBS and treated with Na_2S_2 in PBS for 15 minutes. Cells were then lysed with 2X Laemmli Sample Buffer containing 50 mM maleimide. HEK293 cells (20,000 viable cells/100 μL DMEM + 10% v/v FBS) were seeded into xCELLigence RCTA E-Plate 16 and cell growth was monitored for approx. 24 hours before the addition of Na_2S_2 diluted into MOPS buffer. Cellular proliferation was monitored for an additional 48 hours.

In vitro hypoxia

To measure the cellular formation of persulfides under hypoxic conditions, adherent HEK293 cells in black-walled 96-well plates were loaded with SSP4 (20 μM final) and NucBlue (Hoechst 33342) stain for 1 hour. Media was exchanged with DMEM growth media with GYY4137 or vehicle and incubated in either a normal or X incubator with a 5% CO_2, 1% O_2 humidified atmosphere at 37°C for 24 hours. Relative SSP4 (487±14 nm excitation, dichroic filter 507 nm, 535±30 nm emission; gain 900) and Hoechst 33342 (360±20 nm excitation, dichroic filter 407.5 nm, 460±30 nm emission; gain 900) fluorescence was measured on a CLARIOstar Plus at 37°C. Overnight yeast cultures were diluted to OD_{600} = 0.1 and grown to mid-log phase for 3 hours. SSP4 (40 μM final) was added to the media for the last hour and yeast were washed 3x in PBS before fluorescent measurements.

GSSG/GSS^- equilibrium

Glutathione persulfide formation was measured by reacting GSSG with sulfide. Na_2S stock solutions prepared in 10 mM NaOH were diluted into 500 mM MOPS buffer, pH 7.5 and immediately added to oxidized glutathione (GSSG) (1 mM) in 50 mM MOPS buffer, pH 7.5. Reactions were agitated at 300 rpm for 15 minutes at 37°C and UV-visible spectra recorded in a quartz microcuvette on a Picodrop spectrophotometer. To measure the effect of SOD1 on HS^-/GSS^- equilibrium, GSSG (10mM) was mixed with NaSH (100 μM) in 50 mM MOPS buffer, pH 7.5 and added to a quartz microcuvette. Bovine SOD1 (100 μM final) or MOPS buffer were added to the glutathione/sulfide mixture and Abs 340 nm measured every 2 seconds. GSS^- concentration converted from absorbance values with Beer's law using ε = 19,800 M^{-1} cm^{-1} (4).

Potassium superoxide reactions

KO_2 (100 mM in 10 mM NaOH) stock solutions were freshly prepared and immediately used. KO2 was reacted with Na_2S solutions (also freshly prepared in 10 mM NaOH) in 500 mM MOPS buffer, pH 7.5 for 30 minutes at room temperature. mBBr was added to a final concentration of 1 mM and incubated at room temperature for 15 minutes protected from light. Relative fluorescence recorded on a CLARIOstar Plus at room temperature. Alternatively, CBA (1 mM final) or SSP4 (200 μM final) was added to reaction buffer and fluorescence measured on a CLARIOstar Plus. Polysulfide formation was monitored at room temperature over time at 300 nm. Na_2S (50 mM, final) was added to a quartz microcuvette, and the reaction was initiated by adding 5 mM (final) KO_2.

Cellular ROS production

HEK293 cells (20,000 in 100 μL media) were seeded into black-walled, clear bottomed 96-well plates and cultured overnight. Cells were then loaded with MitoSOX Red (50 μM for 30 minutes), washed gently with media and rotenone (100 nM final) or DMSO vehicle was added to the media and then NaSH added (500 μM final) and cells were incubated at 37°C for 2 hours. Before fluorescent measurements, NucBlue (Hoechst 33342) was carefully added to the media and incubated a further 10 minutes at 37°C. Fluorescence (MitoSOX:510/580nm and Hoechst 33342: 360/460nm) was measured on a CLARIOstar Plus at room temperature and RFU ratios calculated and plotted. In parallel experiments, cells were treated with rotenone and NaSH as above and incubated 24 hours at 37°C. Cell viability was then measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) as instructed.

Statistical Analysis

Data are shown as mean ± SEM unless otherwise indicated in figure legends. The number of biological replicates for cellular experiments is expressed as n in the figure legends and each replicate is the mean value of technical replicates as indicated. The number of replicates for chemical and biochemical experiments is expressed as n in the figure legends. Statistical analyses were conducted using Graph Pad Prism9 software. Yeast growth curve data (Figure 1C) was smoothed by averaging 8 data points on either side using a second-order polynomial and raw data were fit to log growth curve using MARS Data Analysis Software (BMG LABTECH) and calculated sloped were calculated by four-parameter logistic curve. SOD1-Cu^{2+} reduction was fit to a first order decay curve and calculated goodness of fit. Significance was assessed using an unpaired, two-tailed t test or by one- or two-way ANOVA with Dunnett or Sîdak multiple test correction. Statistical significance was determined by $p < 0.05$.

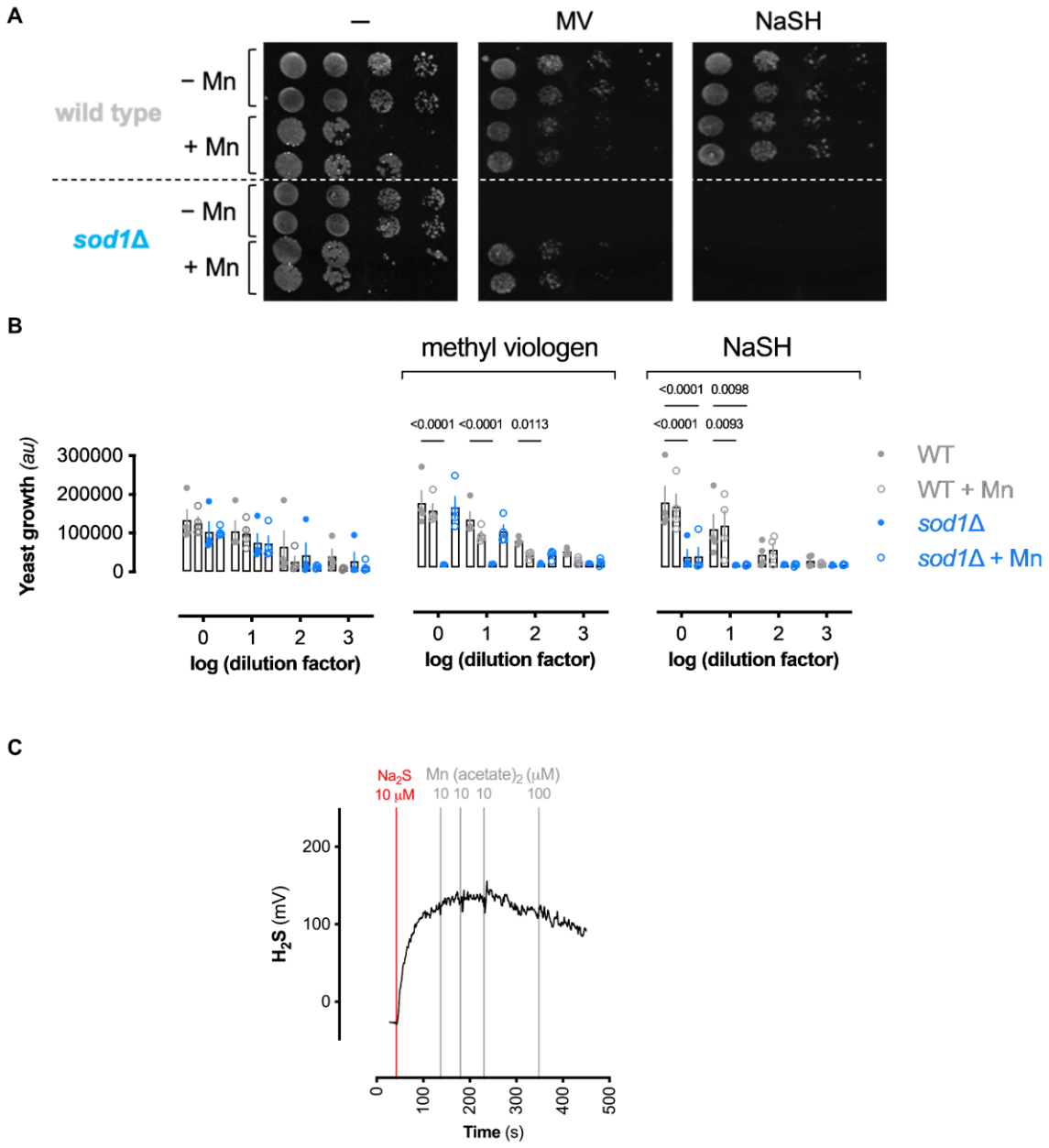


Fig. S1. Oxidative stress does not mediate sulfide-induced cytotoxicity in *sod1*Δ yeast.

(**A**) Representative image of yeast spot assay of wild type or *sod1*Δ yeast cultured with or without manganese diacetate (4 mM) and grown on YPD agar media or YPD containing either 100 μM MV^{2+} or NaSH. (**B**) Bar graph showing mean values from densitometric analysis of yeast spot assay images (±SEM; n=4 experiments, n=2 spots per experiment). Significance calculated by two-way ANOVA with Sîdak's test. (**C**) Amperometric measurement of H_2S (10 μM) in the presence of Mn $(acetate)_2$, indicating that Mn^{2+} does not accelerate sulfide decay.

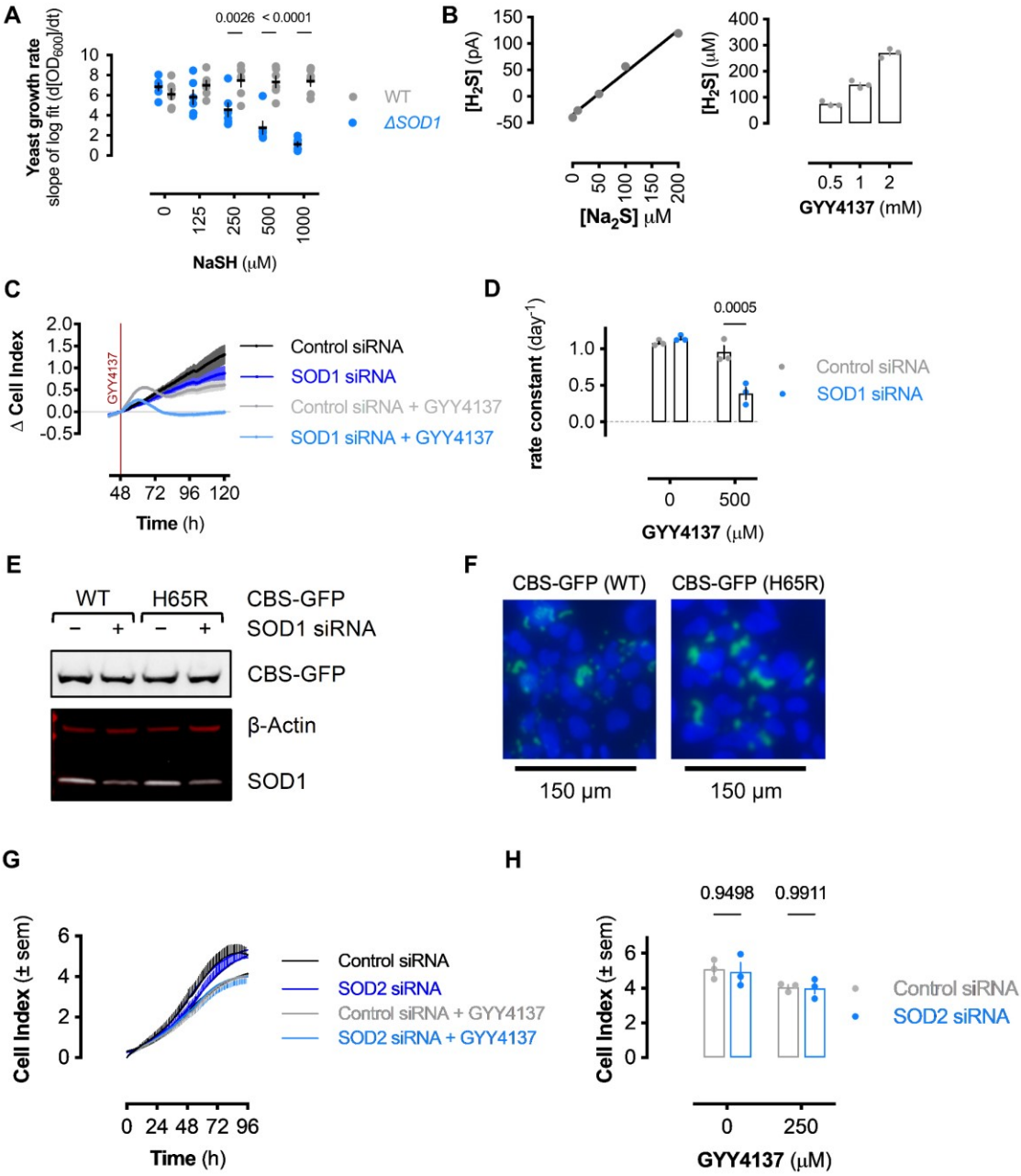


Fig. S2. SOD1 expression protects cells from H_2S toxicity.

(**A**) Yeast growth rates calculated from Figure 1B plotted against NaSH concentration. Horizontal lines represent mean rates (±SEM; n=6 experiments, n=3-6 wells per experiment). (**B**) (*left*) H_2S amperometric electrode standard curve generated from Na_2S stock solutions diluted into PBS at 25°C. (*right*) Steady-state H_2S concentrations in DMEM media released from GYY4137 after 24 hours at 37°C. Bars represent mean concentrations (±SEM; n=3 wells). (**C**) Representative cellular proliferation of adherent control or SOD1-silenced HEK293 treated with GYY4137 (500 μM) at the indicated time point. (**D**) Growth rate constants calculated from (C). Bars represent mean rate constants (±SEM; n=3 experiments, n=3-4 wells per replicate). (**E**) Immunoblot showing relative CBS and SOD1 expression in HEK293 cells. (**F**) Fluorescent images of wild type or mutant CBS-GFP expressing HEK293 cells. Images recorded at 20x magnification and scale bar represents 150 μm. (G) Representative cellular proliferation of control or SOD2-silenced HEK293 treated with

GYY4137 (500 μM). Data shown are mean values (±sd; n=3-4 wells). (**H**) Growth rate constants calculated from (G). Bars represent mean rate constants (±SEM; n=3 experiments, n=3-4 wells per replicate).

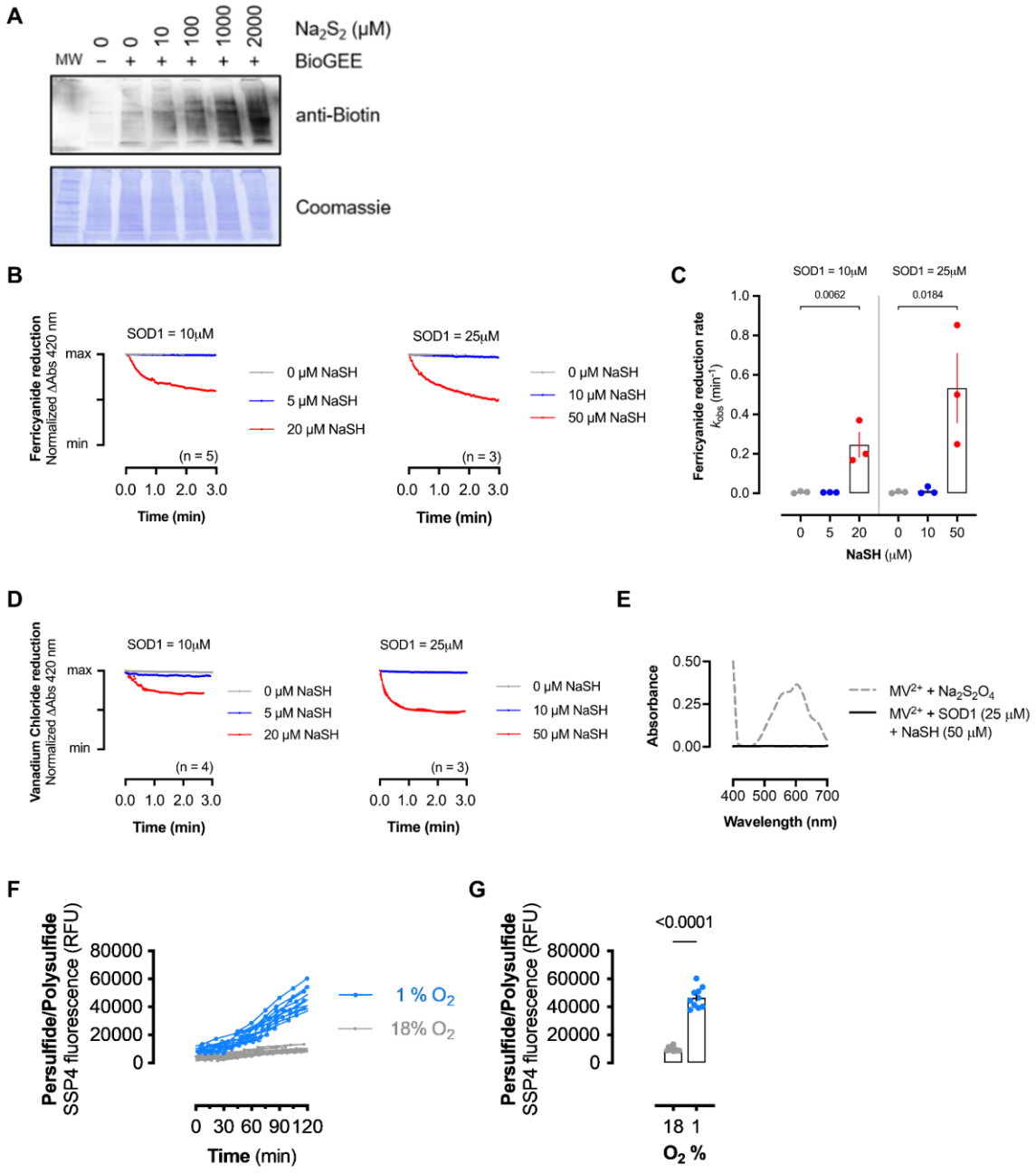


Fig. S3. SOD1-persulfide complex is a potent reducing agent.

(**A**) Representative immunoblot and Coomassie-stained membrane showing relative protein S-glutathionylation from MCF10A human breast epithelial cell line treated with Na_2S_2. (**B**) Kinetics of $K_3Fe(CN)_6$ reduction by SOD1 reacted with limiting or excess H_2S under anaerobic conditions. Data shown are from representative reactions (n=3-5 reactions). (**C**) Observed reaction rate constants calculated from A by nonlinear regression analysis. Rate constants are shown as mean values (±SEM; n=3-5 reactions). Significance was calculated with one-way ANOVA with Dunnett's multiple comparisons test. (**D**) Kinetics of VCl_3 reduction by SOD1 reacted with limiting or excess H_2S under anaerobic conditions. Data shown are from representative reactions (n=3-4 reactions). (**E**) Visible spectra of methyl viologen radical cation ($MV^{\bullet+}$) formed from the reduction of methyl viologen (MV^{2+}) by excess $Na_2S_2O_4$ (dashed line) compared to the spectrum of MV^{2+} reacted the complexed formed from SOD1 (25 μM) and NaSH (50 μM) (solid line). Spectra recorded at 23°C

in an anaerobic atmosphere. (**F**) Relative intracellular RSS formation measured by SSP4 fluorescence from wild type yeast cultured in either 18% or 1% O_2 atmosphere at 30ºC for 120 minutes. Each line represents an individual replicate (n=10). (**G**). Bar graph showing mean SSP4 fluorescence after 120 minutes of 1% or 18% O_2 (±SEM; n=10 wells). Significance calculated by unpaired, two-tailed t test.

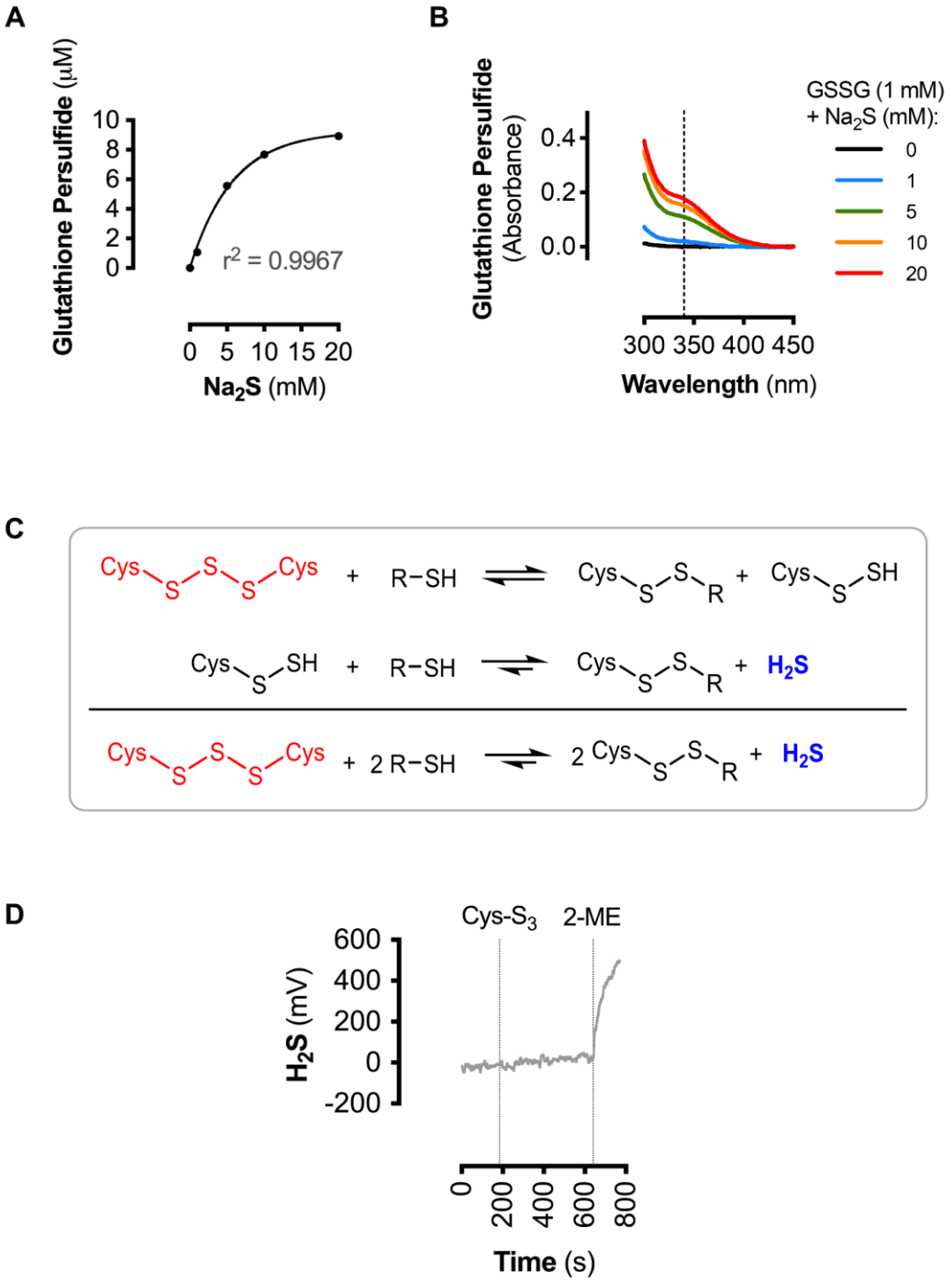


Fig. S4. SOD1 limits RSS formation.

(**A**) Glutathione persulfide formation from the reaction of Na_2S with glutathione disulfide (1 mM) at 23°C, pH 7.5 (black circles). Data was fit to a one-phase exponential association curve (black line) with r^2 = 0.9967. (**B**) UV-visible spectra showing glutathione persulfide formation (340 nm) from Na_2S reacting with GSSG at pH 7.5. (**C**) Equilibria showing H_2S formation from the reaction of

cysteine trisulfide with thiols, via persulfides intermediates. (**D**) Amperometric electrode measurements of H_2S formed from the reaction of 100 μM cysteine trisulfide (Cys-S_3) and 100 μM 2-mercaptoethanol (2-ME).

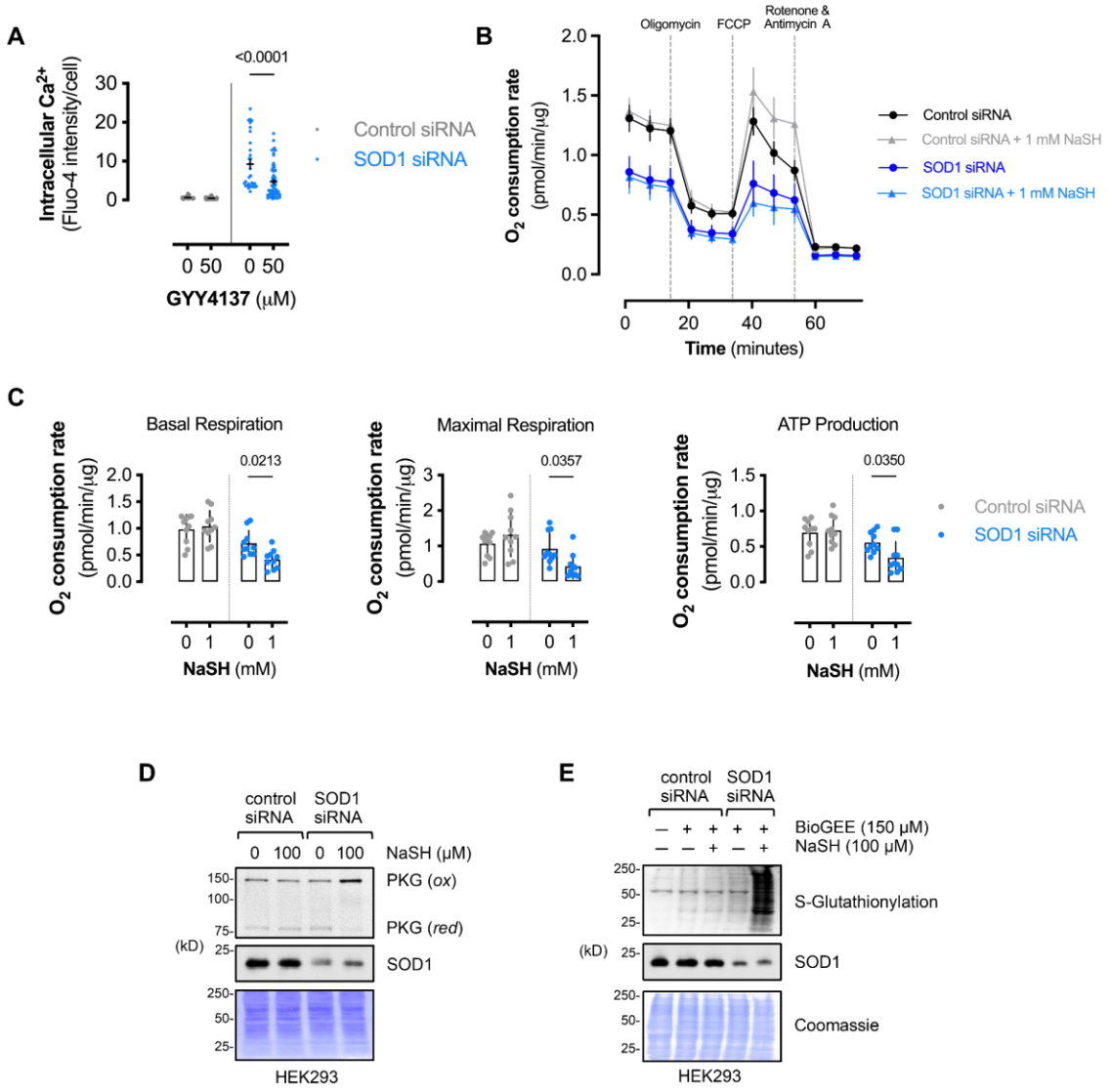


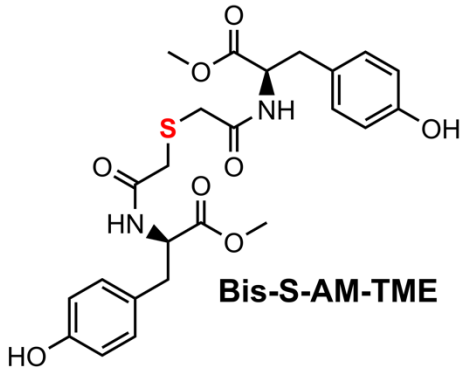
Fig. S5. SOD1 catalyzed RSS induces cellular thiol oxidation.

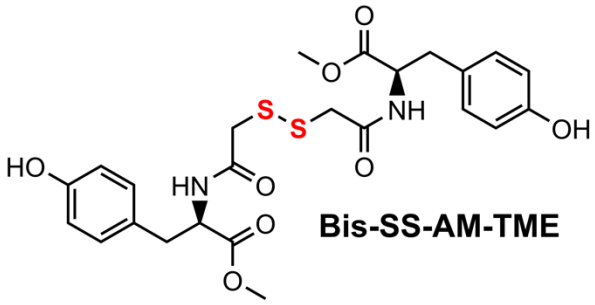
(**A**) Relative free intracellular calcium levels in control or SOD1-silenced HEK293 cells cultured with GYY4137 (50 μM) or vehicle. Data shown are fluo-4 fluorescence intensity per cell and black lines represent the mean values (±SEM; n= cells from 3 wells). Significance calculated by two-way ANOVA with Sîdak test. (**B**) Seahorse mitochondrial stress test measuring changes in oxygen consumption rate over time and normalized to protein content of control or SOD1-silenced HEK293 cells treated with vehicle or NaSH and challenged with oligomycin, FCCP, and rotenone/antimycin A. Data represents mean values (±sd; n=6 wells) of a representative experiment. (**C**) Respiration (basal and maximal) and ATP production calculated from Seahorse Mitochondrial Stress Test. Bars represent mean values (±SEM; n=10 wells) and significance was calculated by two-way ANOVA with Sîdak test. (**D**) Reduced and oxidized PKG and SOD1 expression from control or SOD1-silenced HEK293 cells treated with 100 μM NaSH. (**E**) Protein S-glutathionylation and SOD1 expression from control or SOD1-silenced HEK293 cells loaded with BioGEE and treated with 100 μM NaSH.

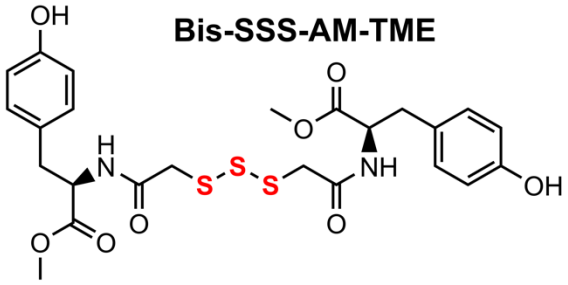
Table S1. Multiple reaction monitoring parameters of various persulfide/polysulfide derivatives, used for LC-ESI-MS/MS analyses.

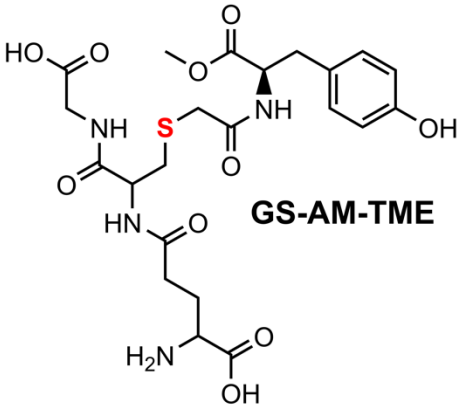
Species trapped	Analyte measured‡	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)
HS^-	Bis-S-AM-TME	+	505.5	136.0	40
	*Bis-S-AM-TME-d4	+	509.5	137.0	40
HSS^-	Bis-SS-AM-TME	+	537.5	136.0	45
	*Bis-SS-AM-TME-d4	+	541.5	137.0	45
$HSSS^-$	Bis-SSS-AM-TME	+	569.5	136.0	45
	*Bis-SSS-AM-TME-d4	+	573.5	137.0	45
GSH	GS-AM-TME	+	543.2	414.0	20
	*GS-AM-TME-d2	+	545.2	416.0	20
GSSH	GSS-AM-TME	+	575.5	446.0	20
	GSS-AM-TME-d2	+	577.5	448.0	20

*Stable isotope-labeled standards.
‡Chemical structures of analytes:









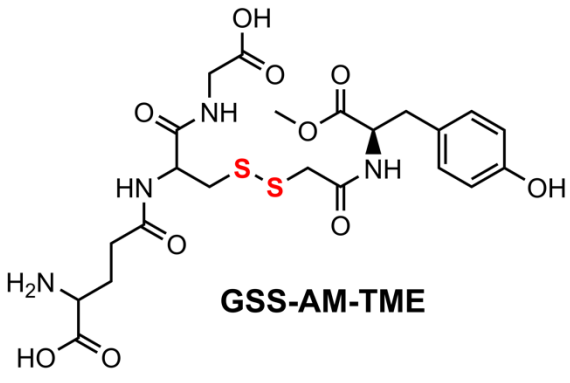


Table S2. Reagents and resources list.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α/β-Tubulin	Cell Signaling Technology	RRID:AB_2288042
Anti-SOD1	Cell Signaling Technology	RRID:AB_2302392
Anti-GFP	Cell Signaling Technology	RRID:AB_1196614
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	RRID:AB_2099233
Anti-mouse IgG, HRP-linked	Cell Signaling Technology	RRID:AB_330924
Anti-β-Actin	Sigma-Aldrich	RRID:AB_476743
Anti-biotin (D5A7)	Cell Signaling Technology	Cat#5597
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 647	Invitrogen	Cat#A28181, RRID:AB_2536165
PKG Polyclonal Antibody	Enzo Life Sciences	Cat# KAP-PK005D, RRID:AB_2170659
Chemicals, Peptides, and Recombinant Proteins		
Superoxide Dismutase from bovine erythrocytes	Sigma-Aldrich	Cat# S7571
Superoxide Dismutase from human erythrocytes	Sigma-Aldrich	Cat# S9636
Sodium hydrosulfide	Sigma-Aldrich	Cat# 161527
Sodium sulfide	Sigma-Aldrich	Cat# 407410
Sodium disulfide	Dojindo	Cat# SB02
Difluorinated H_2S Fluorescent Probe 1	Cayman Chemical	Cat# 29835
L-Glutathione reduced	Sigma-Aldrich	Cat# G4251
GYY4137 Dichloromethane complex	Sigma-Aldrich	Cat# SML0100
Geneticin™ Selective Antibiotic (G418 Sulfate), Powder	ThermoFisher Scientific	Cat# 11811023
Manganese diacetate	Sigma-Aldrich	Cat# 330825
Methyl viologen dichloride hydrate	Sigma-Aldrich	Cat# 856177
SSP4	Dojindo	Cat# SB10
Glutathione Ethyl Ester, Biotin Amide (BioGEE)	Invitrogen	Cat# G36000
Iron(III) chloride	Sigma-Aldrich	Cat# 157740
Potassium superoxide	Sigma-Aldrich	
Cysteine trisulfide, > 90% purity	Prof Jon Fukuto	SI Ref. (5)
2-mercaptoethanol	Sigma-Aldrich	Cat# M6250
Potassium polysulfide	Sigma-Aldrich	Cat# 12665
rotenone	Sigma-Aldrich	Cat# R8875

Vanadium(III) chloride	Thermo Scientific Acros	Cat# 197000250
Sodium dithionite	Sigma-Aldrich	Cat# 157953
Bovine Serum Albumin	Tocris	Cat# 5217
UltraPure™ 0.5M EDTA, pH 8.0	Invitrogen	Cat# 15575020
Yeast Extract	Gibco	Cat# 211929
Peptone	Gibco	Cat# BP1420-500
DPBS, no calcium, no magnesium	Gibco	Cat# 14190144
Trypsin-EDTA (0.25%), phenol red	Gibco	Cat# 25200056
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat# 15140
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	Gibco	Cat# 31966021
Fetal Bovine serum	PAN BIOTECH	Cat# P40-39500
Lipofectamine™ 3000 Transfection Reagent	Invitrogen	Cat# L3000008
L-Glutathione oxidized	Sigma-Aldrich	Cat# G4376
NucBlue™ Live ReadyProbes™ Reagent	Invitrogen	Cat# R37605
B-(2-oxo-2H-1-benzopyran-7-yl)-boronic acid (CBA)	Cayman Chemical	Cat# 14051
Copper(II) sulfate pentahydrate	Thermo Scientific	Cat#197720010
Zinc nitrate hexahydrate	Sigma-Aldrich	Cat#228737
Water for Ion Chromatography	Fisher Chemical	Cat#12993634
Nitric Acid (TraceMetal™ Grade)	Fisher Chemical	Cat#A509-P212
Defined Trypsin Inhibitor	Gibco	Cat#R007100
MEGM™ Mammary Epithelial Cell Growth Medium BulletKit™	Lonza	Cat#CC-3150
Cholera Toxin from *Vibrio cholerae*	Sigma-Aldrich	Cat#C8052
Critical Commercial Assays		
GSH-Glo™ Glutathione Assay	Promega	Cat# V6911
Complex IV Human Enzyme Activity Microplate Assay Kit	Abcam	Cat# ab109909
Superoxide Dismutase Activity Assay Kit (Colorimetric)	Abcam	Cat#ab65354
MitoSOX Red	Invitrogen	Cat# M36008
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)	Promega	Cat# G3582
Amersham ECL Prime Western Blotting Detection Reagent	Cytiva	Cat# RPN2232768
Experimental Models: Cell Lines		
HEK293 cell line	ATCC	RRID:CVCL_0045
MCF10A cell line	ATCC	RRID:CVCL_0598
Experimental Models: Organisms/Strains		

yeast: *sod1*Δ: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sod1Δ	Horizon	Cat#YSC6273-201937842
yeast: wild type: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Horizon	Cat#YSC1048
Oligonucleotides		
siRNA targeting sequence: SOD1 Fw: GGUGGUCCAUGAAAAAGCATT	ThermoFisher Scientific	Cat#4390824; siRNA ID s451
siRNA targeting sequence: SOD1 Rev: UGCUUUUUCAUGGACCACCAG	ThermoFisher Scientific	Cat#4390824; siRNA ID s451
siRNA targeting sequence: SOD2 Fw: UGUCAGGCCUGAUUAUCUATT	ThermoFisher Scientific	Cat#4390824; siRNA ID s13268
siRNA targeting sequence: SOD2 Rev: UAGAUAAUCAGGCCUGACATT	ThermoFisher Scientific	Cat#4390824; siRNA ID s13268
Silencer™ Select Negative Control No. 1 siRNA	ThermoFisher Scientific	Cat#4390843
Recombinant DNA		
CBS-GFP (WT)	this manuscript	Addgene; Plasmid ID 182923
CBS-GFP (H65R)	this manuscript	Addgene; Plasmid ID 182923
pcDNA3.1-hSOD1-FLAG	this manuscript	Addgene; Plasmid ID 182922
Software and Algorithms		
GelPro Analyzer 3.1	Media Cybernetics	https://www.mediacy.com/
Prism9	Graphpad	https://www.graphpad.com/
LabChart8	ADInstruments	https://www.adinstruments.com/
RTCA Software Pro	Agilent	https://www.agilent.com/
MARS Data Analysis Software 3.31	BMG LABTECH	https://www.bmglabtech.com/microplate-reader-software/

SI References

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