Supporting Information

Cytochrome c Reduction Potentiates H_2S Signaling

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Running Title: Cytochrome c reduction by H_2S

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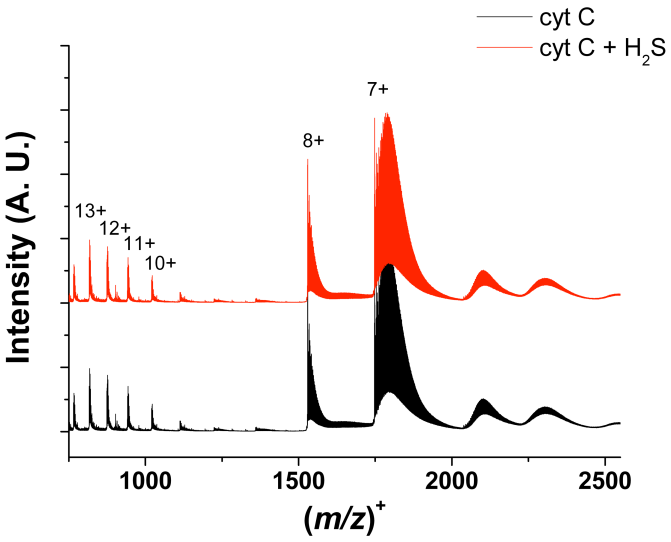
Supporting Figures 1–5

Supporting Material and Methods

Supporting References

A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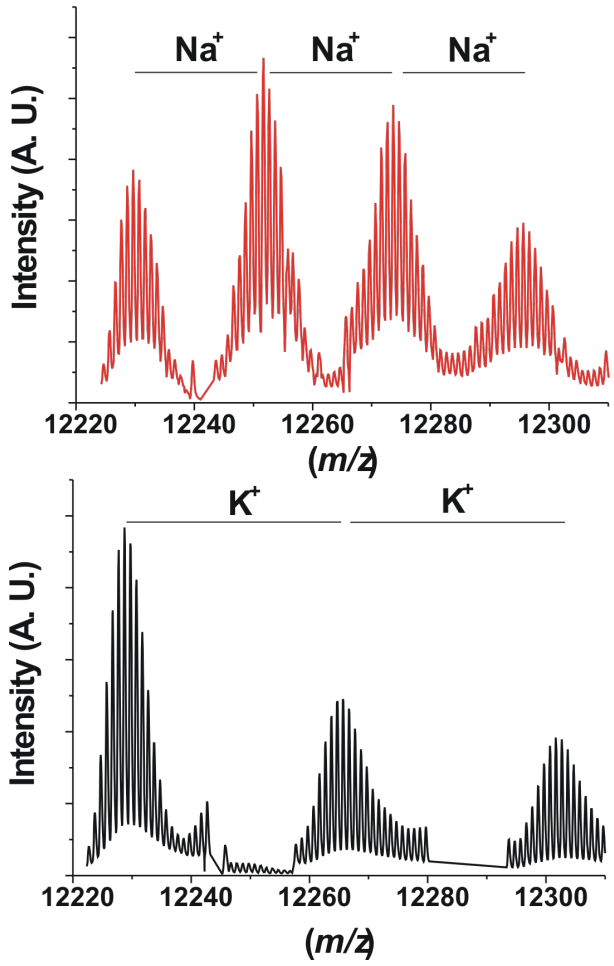
(m/z)+

B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(m/z)
K+
K+
Intensity (A. U.)
12220
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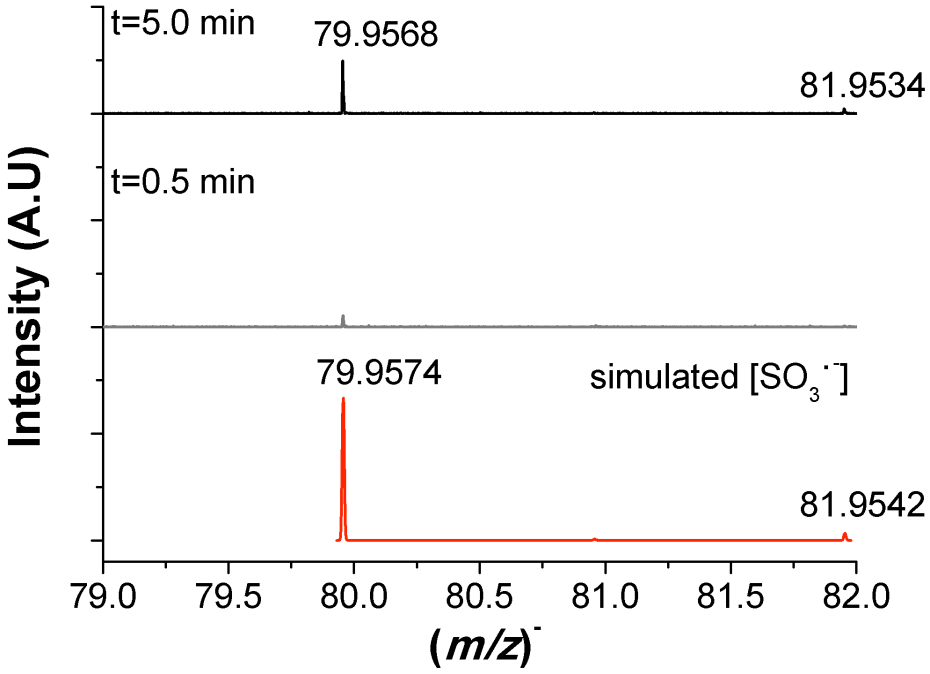
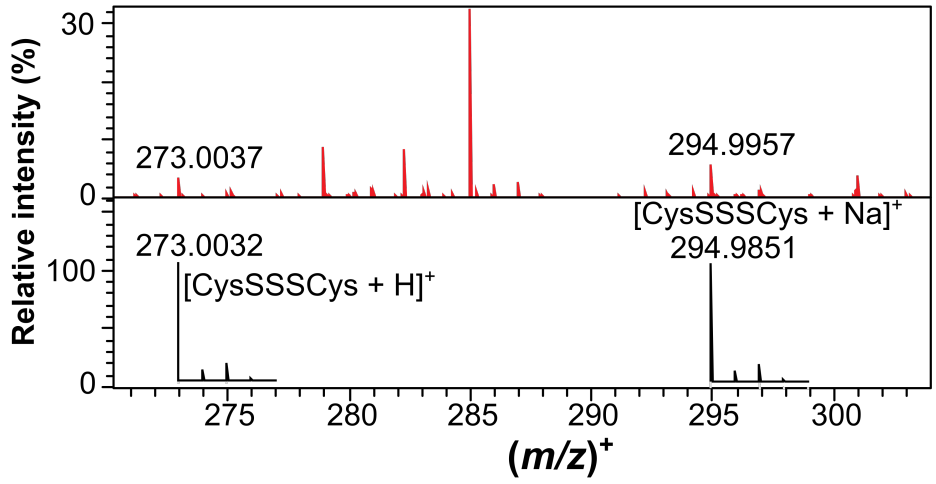
12260
12280
12300
(m/z)

Supporting Figure 1. Mass spectra of cyt c (10 μM) treated (red) or not (black) with 100 μM Na_2S in ammonium carbonate buffer pH 7.7. A) Original spectra. B) Deconvoluted spectra. The aerobic reaction was monitored continuously during 15 min, at room temperature. In the control spectrum (black), speciation matching the addition of potassium ions ($\Delta m/z$ = 38) could be observed, while in the sample treated with Na_2S (red) species with different sodium content predominate ($\Delta m/z$ = 22). No additional species could be detected.



Supporting Figure 2. Time-dependent increase in $SO_3^{\bullet-}$ formed in the reaction of cyt c (10 μM) with 100 μM H_2S in ammonium carbonate buffer pH 7.7 under aerobic condition at room temperature. The simulated mass spectrum is shown in red. The spectra were recorded in negative ion mode.

A



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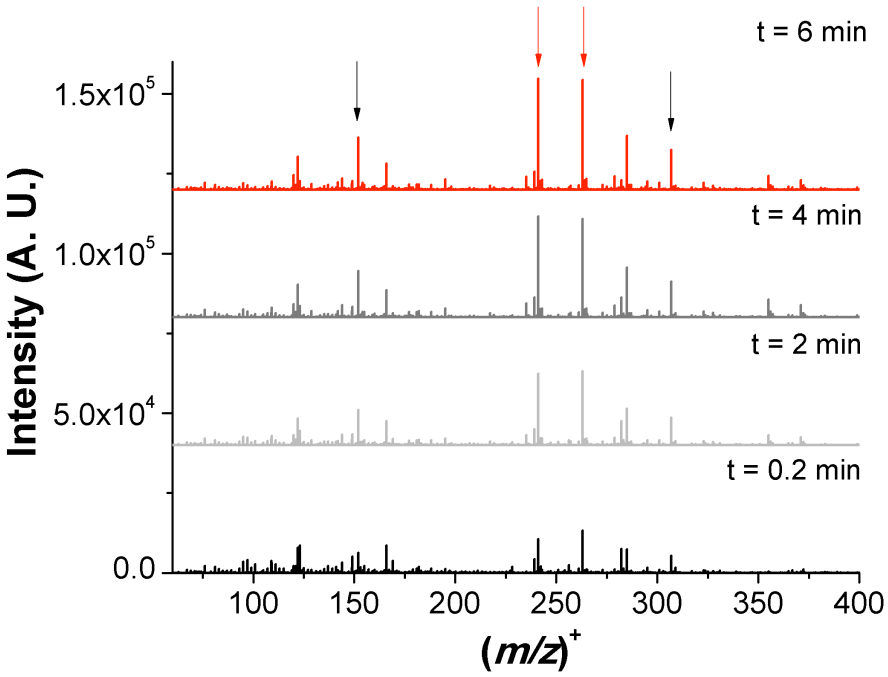
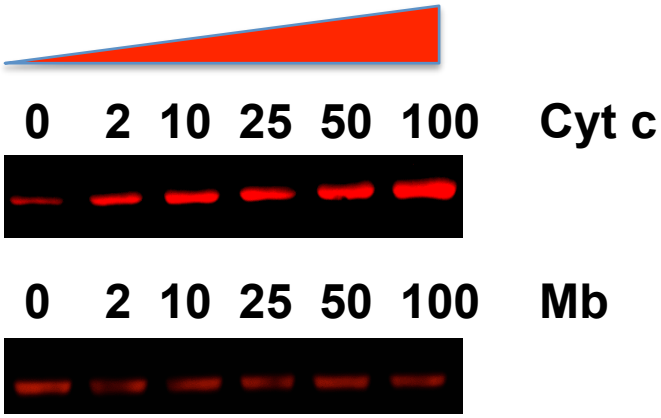
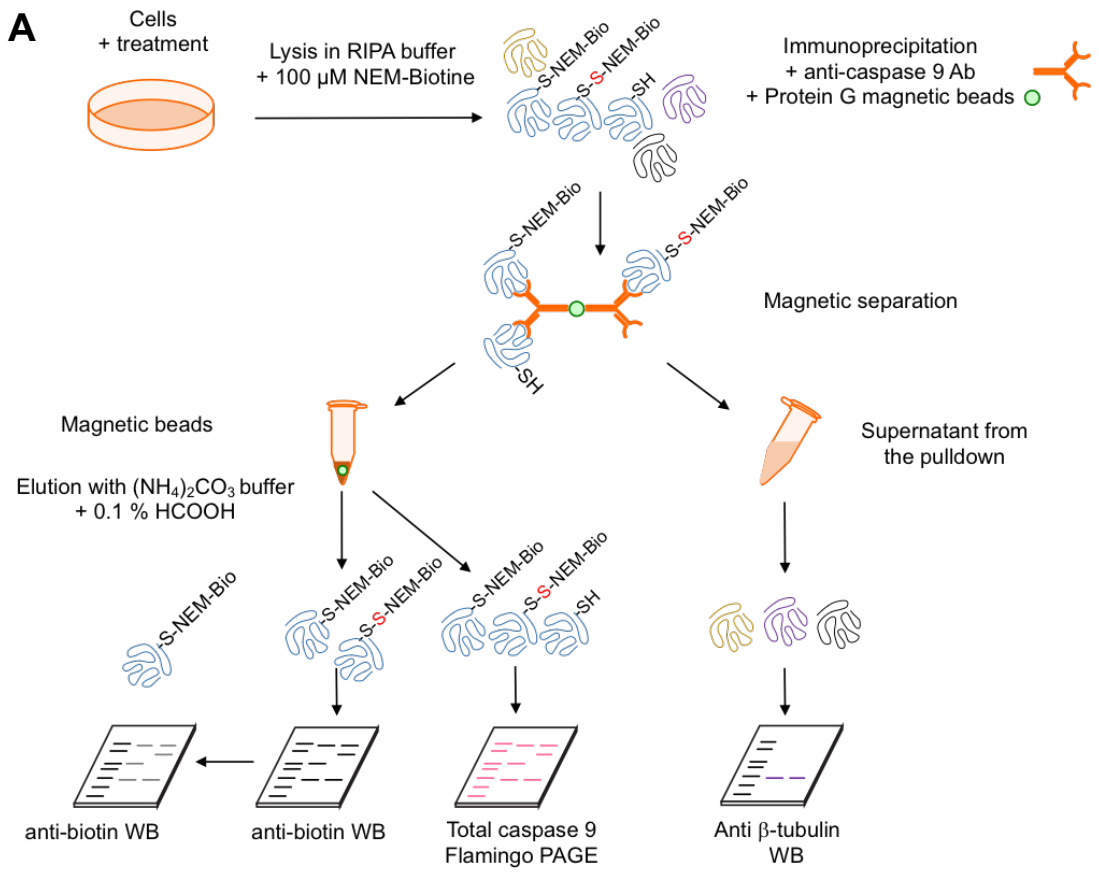


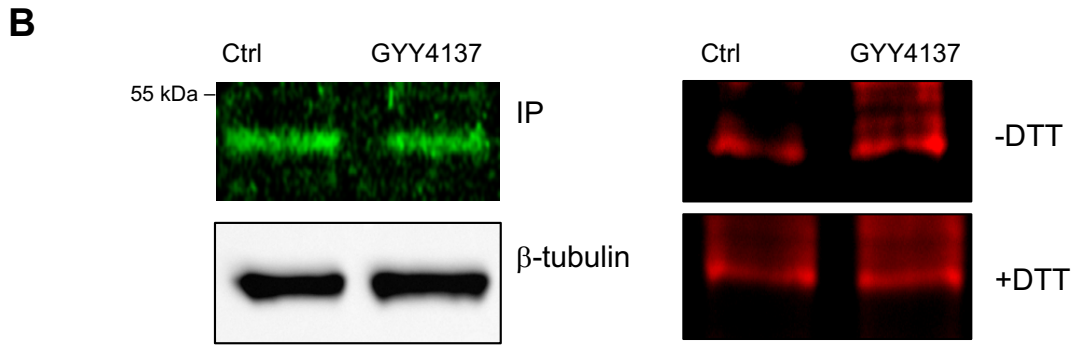
Supporting Figure 3. Cysteine persulfidation by a cyt c/H_2S mixture. A) Selected *m/z* range for the mass spectrum of the reaction mixture containing 20 μM cyt c, 100 μM H_2S and 200 μM cysteine in ammonium carbonate buffer, pH 7.7 (red) and simulated isotopic distributions (black) for cysteine trisulfide ions, $[CysSSSCys + H]^+$ and $[CysSSSCys + Na]^+$. B) Time-resolved MS spectra of the reaction mixture containing 20 μM cyt c, 100 μM H_2S and 200 μM cysteine in ammonium carbonate buffer, pH 7.7. Black arrows show species

corresponding to CysSSH while the red arrows indicate the CysSSCys species. The complete speciation is shown in Figure 4B. The reaction is performed at room temperature.



Supporting Figure 4. HSA persulfidation by cyt c/H_2S or Mb/H_2S mixtures. HSA (10 μM) was incubated with 100 μM Na_2S and cyt c or myoglobin (Mb) in 50 mM phosphate buffer, pH 7.4 for 30 min at 37 ˚C using protein concentrations (in μM) indicated in the figure. HSA persulfidation was detected using the tag switch method.





Supporting Figure 5. Detection of procaspase 9 persulfidation. A) Depiction of the protocol used to assess total vs. persulfidated caspase 9 from HeLa cell lysates. B) Total procaspase 9 (left) and biotinylated procaspase 9 before and after DTT treatment (right).

Supporting Material and Methods

Reduction of Cyt C by H_2S or H_2S_2. All experiments were performed in 100 mM HEPES buffer, pH 7.4 at 25 °C unless specified otherwise. Stock solutions of Na_2S (1 mM in water) and Na_2S_2 (1 mM in 100 mM Tris buffer, pH 8.0) were prepared fresh daily. Anaerobic experiments were performed inside an anaerobic chamber (VAC, Hawthorne, CA) filled with N_2 (with ~0.2–0.5 ppm O_2).

The pH dependence of the rate of Cyt C reduction by sulfide was studied under anaerobic conditions using a stopped-flow spectrophotometer (SX.MV18) placed inside an anaerobic chamber. Cyt C (5 μM) was prepared in 100 mM buffer with pH values ranging from 5.5-9.5, and rapidly mixed with 1 mM Na_2S prepared in the same buffer. The final concentrations of Cyt C and Na_2S were 2.5 and 500 μM, respectively in the following buffers: MES (pH 5.5 to 6.5), HEPES (pH 7.0 to 8.0), TES (pH 7.4 and 8.0), Tris (pH 8.0 and 8.5) and CHES (pH 8.6-9.5). The change in absorbance at 415 nm was monitored and the k_{obs} value at each pH was determined from 3-5 replicates and represents the mean ± SD. The data were fitted to the equation described below as reported previously.[1]

$$V = \frac{V_{max}}{1+10^{(pK_{a1}-pH)}+10^{(pH-pK_{a2})}}$$

Here, V_{max} is the maximal reaction rate, pK_{a1} and pK_{a2} are the lower and higher pKa values.

Analysis of H_2S oxidation products. An aerobic solution of ferric Cyt C (100 μM in 100 mM HEPES buffer, pH 7.4) was incubated with a 10–20-fold excess of Na_2S at 25 °C in sealed sample tubes with a liquid:air volume ratio of 2:1. Buffer alone with the same concentration of Na_2S was used as a control. Samples were collected at the desired times, derivatized with monobromobimane and analyzed by HPLC as described previously.[2]

Alkylation of HSA. HSA was precleaned and prepared as described previously.[3] Thiols in HSA (1 mM solution in 100 mM HEPES buffer pH 7.4) were alkylated by incubation for 1 h

with iodoacetamide (20 mM) in the dark at 25 °C. The iodoacetamide stock solution (200 mM) was prepared in 0.1 M Tris buffer pH 8.0. Excess iodoacetamide was removed using a 2 ml Zeba Spin Desalting Column (Thermo Scientific, Rockford, IL) and the protein was concentrated using an Ultracel-10 K Amicon Ultra-0.5 ml Centrifugal Filters (Merck Millipore). The concentration of HSA was determined spectrophotometrically at 280 nm using ε = 38.55 mM^{-1} cm^{-1} and alkylation was confirmed using the DTNB assay.[4]

Mass Spectrometry. Ultra-high resolution cryo-electron spray ionization MS was performed using HR-qToF Bruker Daltonics (Bremen, Germany) maXis plus, an ESI-quadrupole time-of-flight (qToF) mass spectrometer capable with a resolution of at least 60.000 (full width at half-maximum). Detection was in either the positive or negative ion mode; the source voltage was 3.8 kV (positive ion mode) or 2.0 kV (negative ion mode). The flow rates were 180 $\mu L\ h^{-1}$ and the spraying temperature was 4 °C. The spectrometer was calibrated prior to every experiment via direct infusion of the Agilent ESI-TOF low concentration tuning mixture (for recording Cyt C spectra) or with lithium formate (for recording compounds with low *m/z*). Spectra were processed and analysed by Data Analysis software (Bruker Daltonics, Bremen, Germany).

Oxygen consumption analysis. O_2 consumption was measured using a 1.5 mL Gilson type chamber equipped with a Clark O_2 electrode and a magnetic stirrer. The reaction mixture initially contained ferric cyt c (50–150 μM) ± HSA in 100 mM HEPES buffer, pH 7.4. After stabilization of the background signal, a solution of Na_2S (100 mM stock solution in water) was injected into the chamber and the decrease in O_2 was recorded at 25 °C.

To measure O_2 consumption in cell suspensions, confluent cells from 10 cm plates were trypsinized, resuspended in cell culture medium and centrifuged for 5 min at 2,000 x *g* at 4 °C. The medium was aspirated and the cell pellet was resuspended in 2 mL Dulbecco's phosphate-buffered saline containing calcium and magnesium chloride but without glucose

and pyruvate (Gibco, Fisher Scientific), supplemented with 20 mM HEPES buffer, pH 7.4 and kept on ice. Just prior to the start of the experiment, the cell suspension was diluted 3-fold with the same solution, placed in the chamber and incubated with constant stirring at 37 ^{o}C. After stabilization of the O_2 consumption rate, antimycin A (0.5 mg/mL stock solution in ethanol) was added to a final concentration of 2 μg mL^{-1} and the O_2 consumption rate was allowed to stabilize. Then, Na_2S (2 mM stock solution in water) was added to a final concentration of 20 μM and the rate of O_2 consumption was measured. KCN was added to a final concentration of 5 mM to block ETC-dependent O_2 consumption. The rate of O_2 consumption was normalized to protein concentration in the cell suspension. To measure protein concentration, aliquots of stock cell suspension were collected and stored at –20 ^{o}C. For protein analysis, the aliquots were thawed, mixed with an equal volume of lysis buffer (20 mM HEPES, pH 7.4, 25 mM KCl, NP 40, 0.5%, protease inhibitor cocktail for mammalian tissue (Sigma), 1% (v/v)), the mixture was frozen/thawed, centrifuged, and the protein was measured in the supernatant using the Bradford reagent (Bio-Rad) and BSA as a standard.

Detection of endogenous H_2S in HeLa cells under hypoxic conditions

HeLa cells were seeded at 4 x 10^4 cells per sample in µ-Dish 35 mm, high Glass Bottom dishes (ibidi, Germany) in complete DMEM medium and grown overnight in an atmosphere of 5 % CO_2 at 37 ˚C. Cells were treated with 5 µM MeRho-Az either in DMEM supplemented with 3 % FCS or in argon-purged DMEM supplemented with 3 % FCS and 1 mM sodium dithionite in air-sealed dishes at 37 ˚C for 1 h. After this treatment, cells were washed three times with PBS, fixed with 4 % paraformaldehyde/PBS solution at 4 ˚C for 20 min and washed thoroughly with PBS. Images were obtained by analyzing the samples on an Olympus IX81 fluorescent microscope using 488/525 nm filter set and 63x oil objectives. All images were post processed in ImageJ software (NIH, USA).

Detection of protein persulfidation by Cy3-based tag switch method

HSA, murine recombinant procaspase 9, or purified recombinant human ETHE-1 and ATR were treated with H_2S, cyt c or both, for 30 min in 50 mM phosphate buffer, pH 7.4 at 37 ˚C. The details of each experiment are given in corresponding figure legends. Proteins were then purified using biospin columns (BioRad), incubated with 10 mM 2-(methylsulfonyl)-1,3-benzothiazole (Santa Cruz, USA) for 1 h and precipitated using water/methanol/chloroform (4:4:1, v/v/v). After resuspension of the pellet in PBS containing 2 % SDS, proteins were incubated with 10 µM Cy3-CN[5] and separated by SDS PAGE. The gels were fixed with methanol/acetic acid/water (12.5%/4%/83.5%) for 20 min and scanned using a Typhoon 9500 fluorescent gel scanner (GE Healthcare, USA).

Detection of protein persulfidation in cyt C depleted cells under normoxic and hypoxic conditions

HeLa cells were seeded at 4 x 10^4 cells per µ-Dish 35 mm, high Glass Bottom dishes (ibidi, Martinsried. Germany) in complete DMEM medium and grown overnight in an atmosphere of 5% CO_2 at 37 ˚C. The following day, cyt C was depleted using the Santa Cruz cyt C siRNA kit (Santa Cruz, USA, sc-29292) according to the vendor's protocol and control treated with scrambled siRNA. After gene silencing, both control and cyt C-depleted cells were treated with complete DMEM or with argon-purged complete DMEM medium supplemented with 1 mM sodium dithionite for 1 h in air sealed dishes at 37 ˚C, in order to induce hypoxic conditions (concentration of O_2< 2%). Then, the cells were washed 5x with PBS, fixed with ice cold methanol for 20 min at –20 ˚C and permeabilized with ice cold acetone for 5 min at –20 ˚C. Following permeabilization, cells were washed 3x with PBS and thiols and protein persulfides were blocked with 10 mM 2-(methylsulfonyl)-1,3-benzothiazole (Santa Cruz, USA) in 50 mM HEPES buffer (pH 8) overnight at room temperature. Next day, cells were washed extensively with 30% methanol in PBS and stained with 25 µM Cy3-CN in 50 mM HEPES buffer (pH 8) in the dark at room temperature for 1 h. After staining, cells were washed extensively with PBS, the nuclei stained with 1 µg mL^{-1} DAPI for 10 min and washed 3x with PBS prior to analysis. Detection of protein persulfidation was performed

using an Olympus IX81 fluorescent microscope equipped with a Cy3 excitation and emission filter set and 63x oil objectives. All images were post processed using the ImageJ software (NIH, USA).[5]

The efficiency of silencing was quantified by Western blot. After SDS-PAGE (15%) proteins were transferred to nitrocellulose membrane, blocked with 5 % nonfat dried milk/TPBS (PBS with 0.01% Tween 20) at room temperature for 1 h and exposed to mouse monoclonal anti cytochrome C antibody (sc-13156, Santa Cruz, USA) at 1: 1000 dilution in 1% nonfat dried milk/TPBS at 4 ˚C overnight. Then, the membrane was washed three times with TPBS, exposed to HRP-conjugated mouse IgG kappa binding protein (Santa Cruz, USA, sc-516102) at a dilution of 1:4000 in 1% nonfat dried milk/TPBS at room temperature for 2 h. The membrane was subsequently washed three times with TPBS and the signal was obtained using the Clarity™ Western ECL Substrate (Bio-Rad) and G:BOX chemiluminescence imager (Syngene, UK).

Detection of protein persulfidation in functional mitochondria

Functional mitochondria were isolated from *Saccharomyces cerevisiae* as described previously[6] and stored at –80 ^{0}C. An aliquot of frozen mitochondria (9 mg total protein) was resuspended in respiration buffer (100 mM Tris, 5 mM $MgCl_2$, 200 mM KH_2PO_4, 20 µM EDTA, 250 mM sucrose, pH 7.4) supplemented with 5 mM glutamic acid and 2.5 mM malic acid and divided into nine experimental groups each containing 1 mg mL^{-1} of mitochondrial proteins. Selected samples were preincubated with 2.5 µg mL^{-1} of antimycin A or 10 mM of KCN for 10 min at 37 ^{0}C and further treated with 1 µM H_2S for 20 min at 37 ˚C. After the treatment, samples were centrifuged for 10 min at 12,000 x *g*, washed two times with 10 mM Tris, pH 7.4 and processed for protein persulfidation using the tag switch method.[1] Protein samples (30 µg) were resolved on 10% reducing polyacrylamide gels in the dark. Following electrophoresis, the gels were fixed with methanol/acetic acid/water (12.5%/4%/83.5%) for 20 min and scanned using the Typhoon 9500 fluorescent gel scanner (GE Healthcare).

Determination of Caspase 9 activity in cells

Immortalized human T-lymphocyte cells (Jurkat) were grown in complete RPMI medium in an atmosphere of 5% CO_2 at 37 ^{0}C. 1 x 10^6 cells were used per experimental group. The cells were treated with either DMSO (vehicle), 100 μM GYY4137 or 200 μM ammonium tetrathiomolybdate and/or 2.5 μM staurosporine in complete RPMI medium for 6 h at 37 ˚C. Aliquots (2.5 x 10^5 cells) were taken at indicated time points and cells were stained for 45 min with the Caspase 9 (active) FITC Staining Kit (Abcam, UK; cat no: ab65615) according to the manufacturer's protocol. Prior to the measurement, cells were additionally stained with 1 μg mL^{-1} propidium iodide. Caspase 9 activity and cell viability were recorded using FL1 and FL2 channels on an Accuri C6 PLUS Flow cytometer (BD, USA) where each recording contained 3 x 10^4 events.

Determination of caspase 9 activity in cell lysates

Activity of caspase 9 was determined using the modified Caspase-9 Fluorometric Assay Kit (Enzo, cat no: ALX-850-224-KI01) following the manufacturer's protocol. Briefly, HeLa cells (1 x 10^6 cells) were lysed in 500 μL of Cell Lysis Buffer on ice for 10 min. After lysis, 50 μL of cell lysate was treated with buffer (control), or with 500 nM cyt c, or with 500 nM cyt c and 10 μM H_2S for 30 min at 37 ˚C. Samples were subsequently diluted with 50 μL of Reaction Buffer (omitting DTT) and stained with 50 μM Ac-LEHD-AFC substrate for 1 h in the dark at 37 ˚C. After staining, samples were transferred to the black 96-well fluorescent plate and fluorescence was measured using a CLARIOstar fluorescent plate reader (BMG LABTECH, Germany).

Determination of caspase 3 activity in antimycin-treated cells

Hela cells (1 x 10^6 cells/sample) were grown in complete DMEM medium and treated with 300 μM antimycin A, 100 μM GYY4137 or co-incubated with 300 μM antimycin A and 100 μM GYY4137 overnight in an atmosphere of 5% CO_2 at 37 ˚C. After 16 h, cells were washed

with sterile PBS and caspase 3 activity was determined using the modified Caspase-3 Fluorometric Assay Kit (Enzo, cat no: ALX-850-216-KI01). Briefly, Hela cells were lysed in 500 μL of Cell Lysis Buffer on ice for 10 min. Cell lysate (50 μL) was mixed with an equal volume of Reaction Buffer (omitting DTT) and stained with 50 μM Ac-DEVD-AFC substrate for 1 h in the dark at 37 ˚C. After staining, the samples were transferred to black 96-well fluorescent plate and fluorescence was measured.

Detection of total and persulfidated procaspase 9 from Hela cells

Hela cells were grown and treated with vehicle (DMSO), 100 μM GYY4137 and 2.5 μM staurosporine. In addition, staurosporine-treated cells were pretreated for 2 h or treated for the last 2 h with 100 μM GYY4137. Following these treatments, cells were lysed in 300 μL ice-cold RIPA buffer supplemented with 100 μM biotin-maleimide (Sigma Aldrich) and 1% proteinase inhibitor cocktail for 10 min on ice. Prior to immunoprecipitation, samples were diluted to 1 mL with 25 mM Tris buffer (pH 7.5) and mixed for 45 min at room temperature. SureBead Protein G Magnetic Beads (Bio Rad) were washed twice and suspended in PBS, mixed with murine monoclonal anti caspase 9 antibody (1.37 μg/sample, Santa Cruz Biotechnology, sc-56076) and incubated at room temperature for 2 h. Premixed antibody-bead slurry (100 μL) was mixed with 1 mL of samples and incubated overnight at 4 ˚C. Following immunocapture, samples were magnetized for 1 min using a magnetic rack, the supernatant was collected and the frozen and beads were washed three times with PBS. Samples were eluted by incubating beads with 50 μL of 20 mM ammonium bicarbonate buffer, pH 7.4 containing 0.1 % formic acid at 37 ˚C for 2 h. After elution, samples were neutralized with 1 μL of 1 M Tris buffer (pH 8) and resolved on a non-reducing 12 % polyacrylamide gel. Following electrophoresis, the gel was rinsed with distilled H_2O, fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for 2 h and stained overnight with Flamingo protein fluorescent dye (Bio Rad). Total caspase 9 was visualized by scanning the Flamingo stained gel on a Typhoon 9500 fluorescent imager (GE Healthcare).

Supernatants obtained from the initial immunoprecipitation of (pro)caspase 9 were resolved on a 10 % polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, blocked with 5 % nonfat dried milk/TPBS (PBS with 0.01% Tween 20) at room temperature for 1 h and exposed to murine monoclonal anti caspase 9 antibody (sc-56076, Santa Cruz Biotechnology) at 1:4000 dilution in 1% nonfat dried milk/TPBS at 4 ˚C overnight. Then, the membrane was washed three times with TPBS, exposed to HRP-conjugated mouse IgG kappa binding protein (sc-516102, Santa Cruz Biotechnology) at a dilution of 1:4000 in 1% nonfat dried milk/TPBS at room temperature for 2 h. The membrane was subsequently washed three times with TPBS and the signal was obtained using the Clarity™ Western ECL Substrate (Bio-Rad) and G:BOX chemiluminescence imager (Syngene).

To detect persulfidated procaspase 9, 15 μL of immunoprecipitated samples were resolved on a 12 % polyacrylamide gels under non-reducing conditions and transferred to nitrocellulose membranes as described above. The membrane was then exposed to HRP-conjugated mouse monoclonal anti-biotin antibody (Sigma Aldrich, cat no: A0185) at 1:10000 dilution in 1% nonfat dried milk/TPBS at 4 ˚C overnight. Then, the membrane was washed three times with TPBS and bands were visualized using the Clarity™ Western ECL Substrate (Bio-Rad). To quantify the persulfidation levels, membranes from non-reducing blots were then incubated with 3 mM DTT in PBS for 15 min, then two times with 0.2 M NaOH for 5 min each and finally with 1 % β-mercaptoethanol in PBS for 15 min. Then, the membranes were washed three times with PBS, blocked and probed again using same antibody and imaging technique. All images were post processed and quantified using ImageJ software (NIH, USA).

Supporting References

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