Supporting Information

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SI Methods

Liquid Chromatography/Mass Spectrometry (LCMS). Proteins in lysates from cells expressing GFP-tagged WT or M80A cytochrome *c* were immunoprecipitated using an anti-GFP antibody. Proteins present in immunoprecipitates were eluted in formic acid and separated by microscale reverse phase chromatography-electrospray mass spectrometry and the resulting spectra deconvoluted to obtain the molecular weights of the WT and M80A cytochrome *c*-GFP proteins. Chromatography was performed using a 0.5 mm ID X 150 mm Zorbax SB octadecyl (300 Å pore size, 3.5 μm particle size) column (Aglient) with a 60 min solvent gradient from 18% to 81% acetonitrile in 0.1% formic acid, 0.01% trifluoroacetic acid delivered at 10 μl/min. Positive ion electrospray spectra were acquired on-line using a ThermoFisher LTQ linear ion trap mass spectrometry system with the capillary at 225 °C and 36 V, the source at 4.0 kV, the skimmer at 0 V, and the tube lens at 90 V. The cytochrome *c*-GFP proteins eluted at 34 min and the spectrum averaged over the elution profile was deconvoluted using PROMASS software (ThermoFisher) to obtain the molecular weights for both cytochrome *c*-GFP proteins.

Virus Production. HEK293GP cells were grown to 20–40% confluence in a 75 cm^2 flask and transfected using GeneJuice transfection reagent (Novagen) with vector expressing the VSV-G gene and the previously described vector pBABE mouse cytochrome *c*-GFP (1) or the same vector in which M80 of cytochrome *c* was mutated to an alanine as confirmed by sequence analysis using a QuikChange site-directed mutagenesis kit (Stratagene). At 24, 48 and 72 h after transfection, the virus-containing culture supernatants were collected and polybrene (5 mg/ml) (Sigma-Aldrich) was added to the viral supernates. The supernates were then either frozen at −80 °C or used immediately.

Derivation of Cell Lines Expressing Cytochrome *c*-GFP. The cell line to be transduced was grown to 60–80% confluence in 6-well plates. The cells were then infected with 3 rounds of pBABE mouse cytochrome *c*-GFP virus. After infection, the medium was replaced with puromycin selection medium (5 mg/ml for HeLa and Bax/Bak DKO MEFs, up to 20 mg/ml for MCF-7 cells) and grown until colonies formed. The cells were then detached, replated and grown in selection media, and analyzed by confocal microscopy to determine efficiency of the infection. They were then sorted by flow cytometry if necessary and maintained in complete medium.

Immunoprecipitation of Cytochrome *c*. WT and M80A cytochrome *c*-GFP-expressing HeLa cells were lysed in RIPA buffer (apocytochrome c IPs) [50 mM Tris·HCl pH 7.4, 1% Triton X-100, 1% sodium deoxicholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM ethylenediamine tetraacetic acid (EDTA), 5 μg/ml aprotinin, 5 μg/ml leupeptin] or Nonidet P-40 buffer (holocytochrome c and GFP IPs) (50 mM Tris base pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors) for 30 min while rocking at 4 °C. The Nonidet P-40 lysates were then centrifuged 2 times at 2,000 rpm, the supernates were collected, and then the NaCl concentration of the supernatants was increased to 500 mM. The Nonidet P-40 supernates and the RIPA lysates were then centrifuged for 10 min at 14,000 rpm and the supernates were collected. The supernates were precleared for 2 h while rocking at 4 °C with Protein G-Sepharose beads (Invitrogen) preincubated with mouse IgG1. Precleared lysates were then split into 3 equal volumes and incubated overnight with 4 μg of an anti holocytochrome *c* mouse monoclonal antibody 1G1.E9 (2), an antibody that recognizes apocytochrome *c* (BD Biosciences, clone 7H8.2C12), or equal concentrations of isotype-matched IgG1 control antibody. Antigen/antibody complexes were isolated with Protein G-Sepharose beads. The beads were then washed 5 times in high-salt buffer (50 mM Tris base pH 8.0, 500 mM NaCl, 1% Nonidet P-40, and protease inhibitors) followed by a single wash with PBS. Finally, the beads were resuspended in 2X Laemmli buffer and boiled for 5 min, and samples were separated on 12% polyacrylamide gels. Proteins were then either visualized using a ProteoSilver Silver Stain kit (Sigma), or transferred to nitrocellulose and analyzed by Western blot using an anti-cytochrome *c* antibody (BD Biosciences) as described previously (3).

Preparation of Cell-Free Extracts and Caspase 3 Assay. Cell-free extracts were generated from Jurkat T lymphocyte cells (ATCC, TIB-152) as previously described (4). Cells (5×10^6/ml) were pelleted and washed twice with ice-cold PBS. The cell pellet was suspended in 5 volumes of ice-cold buffer [20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), and 1 mM DTT (DTT)] supplemented with protease inhibitors (protease inhibitor mixture P8340 from Sigma). After sitting on ice for 15 min, the cells were disrupted with 10 strokes at 500 rpm with a Teflon pestle. Nuclei and undisrupted cells were removed by centrifugation at 1000 g for 10 min at 4 °C. The supernatant was then centrifuged at 100,000 g for 1 h at 4 °C. The resulting supernatant was carefully removed and stored at −80 °C until use.

Caspase 3 activity was evaluated following the cleavage of the chromogenic substrate AcDEVDpNA (Upstate) at 405 nm, using 200 microliters final volume of cell-free extract (2–4 mg/ml), 2 mM ATP, 2 mM dATP, 0.2 mM AcDEVDpNA and different concentrations of WT or M80A cytochrome *c*.

Detection of Nitrated GFP-Tagged Cytochrome *c*. HeLa cells expressing GFP-tagged WT cytochrome *c* (1×10^8 cells) were washed, resuspended in 20 ml PBS, and were left untreated or were treated with 25 μM peroxynitrite (R & D Systems) in PBS as previously described (5). The peroxynitrite was added to the side of the tube and then rapidly mixed with the cells by vortexing for 30 sec. All further steps were performed at 4 °C. Cells were then pelletted by centrifugation, resuspended in hypotonic buffer (10 mM Tris-Mops pH 7.5, 15 mM $MgCl_2$, 10 mM KCl and protease inhibitors), and allowed to swell on ice for 15 min. After addition of an equal volume of 10 mM TES pH 7.2, 400 mM sucrose, 0.1 EGTA, and 2 μM DTT, the suspension was homogenized using a tight pestle glass Dounce homogenizer until 60% of more of the cells stained with trypan blue. The homogenized cells were then centrifuged at $900 \times g$ for 10 min. The nuclear-enriched pellet was resuspended in 2 ml of lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors) and passed 10 times through a 28-gauge needle to disrupt nuclei. The lysate was rocked for 10 min, 140 μl of 5 M NaCl was added to the samples, and the samples were rocked for an additional 10 min. The lysate was then centrifuged at $14,000 \times g$ for 10 min and protein concentrations in the supernatants were measured using the BCA assay (Pierce). Twenty mg of protein was subsequently immunoprecipitated using a rabbit anti-GFP antibody (Clon-

tech) or using equal concentrations of normal rabbit serum. Immunoprecipitates were then analyzed on immunoblots using an anti-cytochrome *c* (BD PharMingen) and an anti-nitrotyrosine (mouse monoclonal 1A2.9, a gift from Dr. A. Estevez, Burke Medical Research Institute, Weill Medical College of Cornell University, Ithaca, NY) antibody.

Confocal Microscopy. For confocal microscopic analysis, cells were grown to 70% confluence in 35 mm glass bottom dishes (MatTek Cultureware). For experiments involving DEANO treatment, cells were treated with 2 mM DEANO (Acros Organics) for 8 h followed by confocal microscopic analysis. For experiments involving peroxynitrite treatment, cells were washed 3 times with PBS, and then resuspended in 1 ml of PBS. Peroxynitrite (1 ml in 0.9% NaCl) (R&D Systems) was then added to the cells resulting in a final concentration of 25 μM peroxynitrite. In control wells, 1 ml of 0.9% NaCl alone was added to the cells. After a 30 sec incubation at room temperature with gentle swirling of the dishes, the solution was replaced by complete medium and the cells were incubated for 15 min at 37 °C. The cells were then analyzed by confocal microscopy. Confocal imaging of live cells was performed with a Leica DMIRE2 inverted microscope and Leica True Confocal Scanner TCS SP2. Mitochondrial labeling was carried out with either Mito Tracker Red CM-H_2 XRos or MitoFluor 589 (Invitrogen - Molecular Probes) according to the manufacturer's instructions.

Production of Recombinant WT and M80A Mutant Cytochrome *c*. The pJRhrsN2 plasmid (a gift from Dr. Jon Rumbley, University of Minnesota, Duluth, MN) was used to produce recombinant WT and M80A mutant cytochrome *c*. pJRhrsN2 codes for a horse heart cytochrome *c* described in ref. 6. The M80A mutant was obtained by PCR using QuikChange II site-directed mutagenesis kit (Stratagene). Recombinant cytochrome *c* wt or mutant M80A were expressed in *E. coli* BL21 star strain and purified as described previously (6) with minor modifications. Twenty-five ml of starter culture grown in LB medium supplemented with 100 mg/ml ampicillin was inoculated in 1 L of Terrific broth supplemented with 100 mg/ml ampicillin. The culture was grown with vigorous shaking (220 rpm) until $A_{550\ nm} = 0.8$. Cytochrome *c* M80A expression was induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 20 h. The protein was purified using a CM-Sepharose fast flow column (Amersham Pharmacia Biotechnology). Fractions with a 410/280 absorbance ratio >4.0 were considered pure, as confirmed by SDS/PAGE analysis.

WST-1 Assay. The WST-1 reagent (Roche) was used to analyze cell survival after the treatment with tBuOOH or peroxynitrite. The assay is based on the same principle of the MTT assay (cleavage of tetrazolium salts to formazan by mitochondrial dehydrogenases), with the advantage that WST-1 is more sensitive and requires no solubilization in alcohol for its detection (WST-1 yields water-soluble cleavage products).

For experiments involving tBuOOH treatment, cells were seeded in 96-well culture plates at 2.5×10^3 cells in 100 microliters per well in complete medium and incubated overnight. tBuOOH (12.5 μM) was then added to the cells. For experiments involving peroxynitrite treatment, cells were washed 3 times with PBS and resuspended in 500 μl of PBS. Peroxynitrite (R&D Systems) was diluted in saline solution (0.9% NaCl) at the indicated concentrations and 500 μl was mixed with the cell suspensions following gentle vortexing for 30 seconds. The cells were pelleted by centrifugation at 2,000 $\times$ *g* for 1 min and resuspended in 1 ml of supplemented medium, followed by distribution in 96-well culture plates ($5–10 \times 10^3$ cells per 100 μl per well). Cells were then incubated for 18 h at 37 °C after tBuOOH or peroxynitrite treatment. Survival was then assessed by adding 10 μl per well of WST-1 reagent (Roche) and reading the absorbance at 450 nm after incubation for 30 min at 37 °C.

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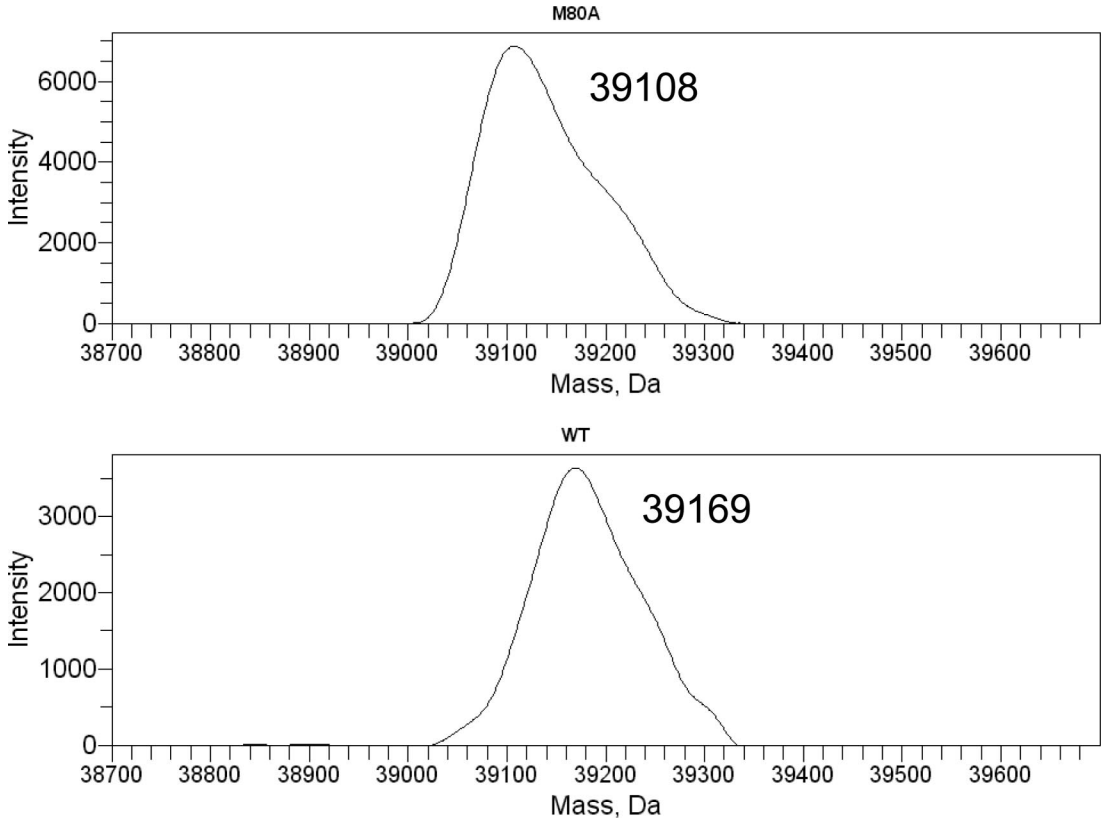

Fig. S1. Deconvoluted positive ion electrospray mass spectra of WT and M80A mutant cytochrome *c*-GFP proteins obtained during LCMS analysis. The measured molecular weights of 39,169 and 39,108, respectively, are in agreement with the calculated molecular weights for the holo forms of the proteins (theoretical values of 39,159 and 39,099, respectively). The theoretical calculated molecular weight for the apo forms of WT and M80A mutant cytochrome *c*-GFP are 38,475 and 38,415, respectively.

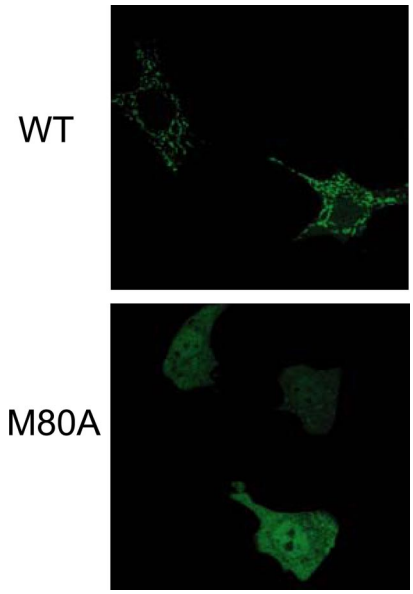

Fig. S2. M80A cyt *c* release from mitochondria is Bax/Bak-independent. To determine if the spontaneous release of M80A cyt *c* from mitochondria in the absence of apoptosis was Bax or Bak-dependent, GFP-tagged M80A or WT cyt *c* was expressed in mouse embryonic fibroblasts (MEFs) obtained from Bax and Bak DKO mice. M80A cyt *c* has a cytoplasmic and nuclear distribution in these cells.

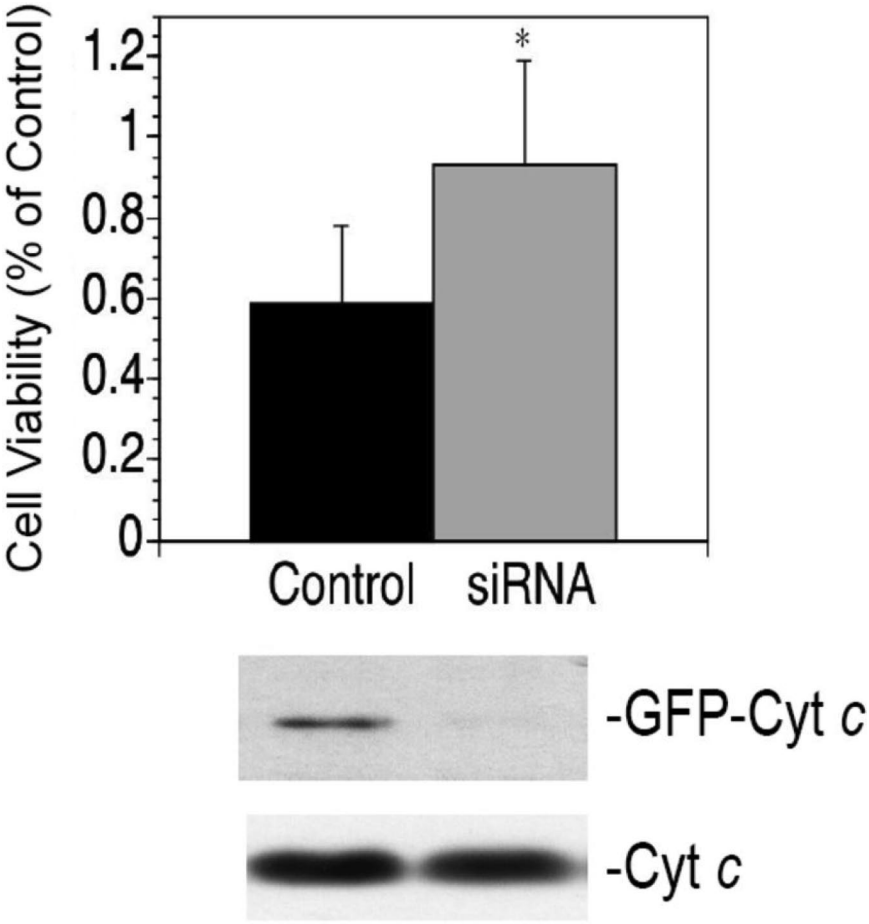

Fig. S3. Knock-down of M80A expression protects cells from oxidative stress-induced death. Cells expressing M80A cyt *c*-GFP were mock transfected (Control) or were transfected with anti-GFP siRNA (siRNA). After 96 h, the cells were treated with tBuOOH (12.5 μM) for 18 h and cell viability was assessed using a WST-1 assay. The data indicates the mean absorbance (percent of untreated controls) plus SD in 3 separate experiments. * indicates $P < 0.05$, paired *t* test, $n = 3$. Cyt *c*-GFP and cyt *c* WBs of equal concentrations of the control and siRNA-treated cells at the time of tBuOOH treatment are shown.