Supplementary Information for

Respiration triggers heme transfer from cytochrome c peroxidase to catalase in yeast mitochondria

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SI Materials and Methods

Materials: The fluorescent dyes (MitoTracker® Red CMXRos, Hoescht and FM4-64) were purchased from Molecular Probes, Invitrogen. Proteins were obtained from the following suppliers: bovine catalase, bovine serum albumin (BSA), horse heart ferrocytochrome c type III, myoglobin (Mb) (Sigma), sequencing grade modified trypsin (Promega), thrombin (Calbiochem) and Zymolase 20T (Amsbio). Other suppliers were as follows: SuperSignal West Pico enhanced chemiluminscence kit, Tween 20 (Thermo Fisher); Ficoll 400, Coomassie (MP Biomedicals); hemin chloride, phenylmethylsulfonyl fluoride (PMSF), Percoll (Sigma); 5 mL HiTrap Q anion-exchange column, Benzamidine Sepharose 4 Fast Flow and DEAE Sepharose resins (GE Healthcare); Ni-NTA resin (Qiagen); pET15b vector (Novagen), and C18 Zip tips (Millipore).

Antibodies: The following antibodies were used for Western blotting: Rabbit anti-Ccp1 serum was kindly provided by Professor David Goodin (University of California, Davis); rabbit anti-GFP antibody (sc-8334 Santa Cruz); mouse anti-porin (ab110326 Abcam); mouse anti-Cyc raised against bovine heart cytochrome c (ab110325 Abcam); mouse anti-homocitrate synthase (MCA-31F5 EnCor Biotechnology); goat anti-rabbit horseradish peroxidase conjugated secondary antibody and goat anti-mouse horseradish peroxidase conjugated secondary antibody (Biorad). Porin, Cyc and homocitrate synthase are used in this study as mitochondrial outer membrane, mitochondrial intermembrane space (IMS) and nuclear markers, respectively.

Imaging of Live Yeast Cells by Wide-field Fluorescence Microscopy

Cells expressing Ccp1-GFP were cultured in YPD medium, washed with PBS (10 mM sodium phosphate and 150 mM NaCl pH 7.4), diluted to $1x10^4$ cells/mL in PBS, and co-stained for 30 min at room temperature with probes selective for mitochondria (MitoTracker® at 0.25 μM) and nuclei (Hoescht at 2.0 μM). Vacuoles were stained in 7-day cells by incubation with 40 μM FM4-64 for 60 min on ice and cells were transferred to fresh YPD media for 16 h at 14 °C (1, 2). Following staining, all cells were mounted on a glass slide with a coverslip (Thermo Fisher), and illuminated by a Heliphor LED or halogen light source (for differential interference contrast, DIC) of an inverted epifluorescence microscope (Nikon Eclipse Ti) and examined through an oil-immersed 100x objective using filters selective for GFP (480, 535/25 nm), MitoTracker (555, 630/75 nm), Hoechst (405, 488/75 nm) or FM4-64 (480 nm, 550 long-pass filter). Digital images were collected with an EMCCD camera (Photometrics Evolve 512) using

an exposure time of 100 ms, which was increased to 500 ms for GFP imaging. Image analysis was performed using ImageJ (v. 1.47; NIH), including background subtraction, brightness and contrast adjustment.

Subcellular Fractionation of Yeast Lysates and Isolation of Crude Nuclei

Denucleated (S2), mitochondria-enriched (P10) and mitochondria-depleted (S10) subcellular fractions were isolated from yeast as described previously (3, 4). Western blotting with anti-porin and anti-Cyc confirmed that P10 and S10 were highly enriched and depleted in mitochondria, respectively. Nuclei were isolated following the published protocol (5) with minor modifications. Briefly, yeast grown in YPD medium were harvested at the times indicated, washed twice with 0.85% NaCl and 7-day and older cells were incubated for 15 min at 35 °C in 100 mM EDTA (pH 8.0)/0.5% β-mercaptoethanol to prepare for spheroplasting. This washing step was bypassed for 1- to 4-day cells, which are more susceptible to spheroplasting (5). Cells were pelleted by centrifugation at 2000xg for 5 min at room temperature, resuspended in S buffer (1.2 M sorbitol, 0.5 mM $CaCl_2$ in 20 mM KPi, pH 6.5) containing 0.5% β-mercaptoethanol, and treated with Zymolase 20T (3 mg/g cell pellet) at 30 °C. Spheroplast formation, monitored by light microscopy, was complete after 1-2 h incubation at 30 °C with gentle agitation. Spheroplasts were pelleted by centrifugation at 2000xg and 4 °C for 10 min, resuspended in FB buffer (18% Ficoll 400, 0.5 mM $CaCl_2$, 0.5 mM PMSF in 20 mM KPi, pH 6.5), disrupted with 15 strokes of a Teflon Dounce homogenizer, centrifuged at 1500xg and 4 °C for 10 min to remove cell debris and the supernatant was spun at 24000xg for 15 min at 4 °C in a Beckman SW 41 Ti rotor. The crude nuclear pellet was resuspended at 4 mL/g in FB buffer, homogenized with 10 strokes of the homogenizer, layered onto a 32.5% Percoll gradient and spun at 38700xg for 45 min at 4 °C. Nuclei were recovered as a whitish band in the bottom third of the tube, collected and washed 3x in FB buffer to remove cytoplasmic contaminants. Immunoblotting with anti-homocitrate synthase confirmed the presence of nuclei.

Western Blotting of Ccp1 and Ccp1-GFP

The method of equal volumes (6) was used to quantify the relative amounts of Ccp1 in the subcellular fractions by Western blotting. Briefly, the S2 lysate was diluted to 1 mg/mL in homogenization buffer (0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail in 10 mM Tris HCl, pH 7.4), 1 mL was centrifuged at 12000xg at 4 °C for 20 min to obtain ~1 mL of mitochondria-free supernatant (S10) and the mitochondria-enriched pellet (P10) was resuspended in 1 mL of homogenization buffer. Equal volumes (10–20 µL) of the S2, P10 and S10 fractions were loaded onto a 12% resolving (10x6x0.1 cm)

SDS-PAGE gel and wet transfer of the electrophoresed proteins to a methanol-activated polyvinylidene fluoride (PVDF, BioRad) membrane was carried out at 100 mA for 2 h. After blocking for 1 h with 5% (w/v) skim milk in TBST (150 mM NaCl, 0.05% v/v Tween 20 in 50 mM Tris, pH 7.6), membranes were incubated with anti-Ccp1 (1:5000 dilution) or anti-GFP (1:1000) for 1 h, washed 3x with TBST, and incubated with the secondary antibody (1:20000) for 1 h. Signals were visualized by ECL using 30 s (Ccp1) and 1 min (Ccp1-GFP) exposure times and digitized in an AlphaImager (Alpha Innotech). After Ccp1 immunodetection, membranes were probed with anti-porin or stained with Coomassie as loading controls. Note that Ccp1 signals are normalized to the porin signal in Fig. 4, which compares Ccp1 levels in the P10 fractions from different yeast strains. Figs. 2 and S1 compare Ccp1 levels in S2, S10 and P10 fractions from wild-type and Ccp1-GFP cells vs age so these Ccp1 signals are normalized to the sum of the Coomassie signals in the same fraction (7) since porin is not present in all subcellular fractions.

Linear Range for Ccp1 Detection and the Response of Anti-Ccp1 Toward Different Chemical Forms of Recombinant Ccp1

A standard curve was generated by loading solutions of recombinant holoCcp1 in the range of 4 fmol–2 nmol onto a 12% resolving SDS-PAGE gel and performing Western blotting with anti-Ccp1 as described in the previous section. The response is found to be linear between 4–120 fmol Ccp1 (Fig. S7C). Solutions of 1 μM recombinant apoCcp1, recombinant holoCcp1 and recombinant holoCcp1 treated with 1 and 10 μM H_2O_2 in 20 mM KPi/100 μM DTPA (pH 7.5) for 60 min at room temperature were diluted to 30 nM and 2 μL of each (60 fmol of Ccp1) was loaded onto a reducing 12% SDS PAGE gel for Western blotting as described above. Anti-Ccp1 detects all chemical forms of the peroxidase equally (Fig. S7A,B).

Heme Blotting of Ccp1 Isolated from P10 and S10 Fractions

Mitochondria-enriched P10 and mitochondria-free S10 fractions from 500 mL cultures of 2- and 7-day cells were dialyzed against 20 mM KPi buffer (pH 6.0) overnight and centrifuged at 14000xg for 10 min. The supernatants (1 mL) were applied to the HiTrap Q anion-exchange column equilibrated with the same buffer and attached to a Biotech AKTA Purifier 10 (GE Healthcare). Elution was performed using a linear 0–1 M NaCl gradient over 25 min at a flow rate of 2.0 mL/min, 1 mL fractions were collected and 15 μL of each was dot blotted onto two PVDF membranes. One membrane was probed with anti-Ccp1 as described above for Western blotting and the second membrane was probed with the ECL reagent (luminol/H_2O_2) for 5 min to detect heme-containing proteins based on their pseudoperoxidase activity (8). BSA and Mb were used as negative (heme-free) and positive (heme-containing) protein controls,

respectively. The Ccp1 and heme signals were digitized using the AlphaImager with an exposure time of 2 min.

CCP and Catalase Activity Assays

CCP activity, the catalysis of ferrocytochrome c oxidation by H_2O_2 (9), was monitored in the subcellular fractions. The assay solution contained 90 µM H_2O_2, 27 µM (~95% dithionite-reduced) horse heart ferrocytochrome c and 10 mM EDTA in 50 mM KPi (pH 7.0). Following addition of the fractionated lysate (0.1–0.5 mg/mL protein) to 1 mL of assay solution, CCP activity was determined from the initial rates of ferrocytochrome c oxidation (ε_{550} = 27.6 mM^{-1} cm^{-1}), and 1 unit of specific CCP activity catalyzes the peroxidation of 1 µmol of ferrocytochrome c/min/mg protein (9). Before assaying for CCP activity, P10 fractions were incubated with 0.02% digitonin in homogenization buffer solution for 10 min at 4 °C to permeabilize the mitochondrial outer membrane (10). Also, the S10 fractions (1 mg) were pre-incubated with 0.1 µM hemin in homogenization buffer for 10 min at 4 °C in attempts to reconstitute extramitochondrial apoCcp1 with heme.

To determine catalase activity, 5.0–20 µL aliquots of soluble protein lysates, prepared as previously described (11), were added to 1.0 mL of 20 mM H_2O_2 in 50 mM KPi (pH 7.0). H_2O_2 decomposition was monitored at 240 nm (ε_{240}=43.6 M^{-1} cm^{-1}) (12), and 1 unit of catalase activity catalyzed the degradation of 1 µmol of H_2O_2 per min. Cta1 and Ctt1 activities were assayed separately following extract fractionation by native PAGE as reported (11).

Expression and Purification of Recombinant Ccp1

The cDNA for yeast Ccp1 encoding an extra methionine and isoleucine at positions -2 and -1 of the mature protein (9) was subcloned into the NdeI and EcoRI sites of the pET15b vector. *E. coli* BL21(DE3) cells were transformed with pET15b-Ccp1, and grown to an OD_{600} of 0.6 in LB medium with 100 µg mL^{-1} ampicillin at 37 °C and 250 rpm. IPTG (0.5 mM) was added to induce Ccp1 overexpression and the culture was further incubated at 37 °C and 250 rpm for 16 h. Cells expressing Ccp1 with an N-terminal His_6-tag were lysed by 3 freeze/thaw cycles in liquid nitrogen/37 °C water bath, and the lysate was added to loading buffer (500 mM NaCl, 10 mM imidiazole in 100 mM Hepes, pH 7.5). Following 10 x 10 s sonication cycles, the cell debris was removed by centrifugation at 12000xg for 20 min, 1 mL of Ni-NTA resin was added to 10 mL of supernatant, incubated at 4 °C for 1 h, and the resin was washed with loading buffer containing 10 and then 25 mM imidazole. His_6-Ccp1 was eluted from the resin with 500 mM imidiazole in the same buffer, dialyzed against 20 mM KPi (pH 7.5) overnight and the His_6-tag was cleaved by incubation with 5 U of thrombin per mg His_6-Ccp1 for 16 h at 4 °C. Ni-NTA resin was added to

remove any uncleaved protein and the free His tag, and the supernatant containing tag-free Ccp1 and thrombin was transferred to an Eppendorf tube with 100 µL of Benzamidine Sepharose 4 Fast Flow resin (pre-equilibrated with 20 mM KPi, pH 7.5) to bind the thrombin. The resultant Ccp1 solution (60 mg/L culture) had a 410/280 nm absorbance ratio of 0.16, indicating that Ccp1 was isolated mainly as the apoform, which was stored in 20 mM KPi (pH 7.5) at -80 °C until use.

***In Vitro* Heme Transfer**

Since removal of the buried heme from catalase results in poorly defined products, apoMb, the prototypical heme acceptor (13, 14), was used as a surrogate acceptor of Ccp1 heme *in vitro*. ApoMb was prepared from the horse heart holoprotein by the acid/methyl ethyl ketone method (15). Heme removal was confirmed by the loss of Soret absorbance at 408 nm, and apoMb in 20 mM KPi (pH 7.5) was stored at -20 °C until use. A stock solution of 0.70 mM hemin was prepared in 0.1 M NaOH and its concentration determined by forming the pyridine hemochrome (16). ApoMb or apoCcp1 was incubated with 1.1 molar equivalents of hemin in 20 mM KPi (pH 7.5) for 1 h at 4 °C to reconstitute the holoproteins, unbound hemin was removed on a 0.8 x 4 cm DEAE Sepharose column equilibrated with the same buffer and the holoproteins were eluted by adding 500 mM NaCl to the buffer. Protein concentrations were determined spectrophotometrically using the following ε values (mM^{-1} cm^{-1}): apoMb (ε_{280}=59.4), holoMb (ε_{408}=188) (17); apoCcp1 (ε_{280}=15.8), holoCcp1 (ε_{410}=98) (18).

Heme donation from 20 µM holoCcp1 to 60 µM apoMb was examined in 20 mM KPi (pH 7.5) at 30 °C. Following incubation for 60 min with gentle stirring, the proteins were separated on the DEAE column as described above and the percent heme transfer was estimated from the Soret absorbance of the two proteins (Fig. S2A,C). To examine if Ccp1 hyperoxidation by H_2O_2 accelerates heme transfer, 20 µM Ccp1 was pretreated with 200 µM H_2O_2 at 4 °C for 60 min, 0.1 nM catalase was added to remove any unreacted H_2O_2, and 20 µM hyperoxidized Ccp1 was incubated with 60 µM apoMb. The Soret maximum of the Fe^{III} heme of Ccp1 red shifts from 410 to 419 nm indicative of Fe^{IV} heme formation on H_2O_2 addition (Fig. S2A). However, the Fe^{IV} heme decayed to Fe^{III} heme during anion-exchange chromatography so heme loading of Ccp1 was monitored at 410 nm (Fig. S2B).

Mass Spectrometric Analysis of Ccp1 Hyperoxidation In Vitro and In Vivo

After standing in 20 mM KPi pH (7.5)/100 µM DTPA at 4° C for 60 min, solutions of 5 µM recombinant Ccp1 or apoCcp1 $\pm$ 50 µM H_2O_2 were diluted 5-fold into 2% acetonitrile/0.1% formic acid. Aliquots (2 µL) were loaded onto a reversed-phase Zorbax 300SB-C3 (2.1 x 150 mm, 5 mm) column equilibrated with

5% acetonitrile/0.1% formic and acid attached to an Agilent 1100 HPLC system. The protein was eluted at 0.2 mL/min into the electrospray (ESI) source of a QToF3 Ultima API Mass Spectrometer (Waters) using a 5-95% acetonitrile gradient over 5 min, 95% acetonitrile for 3 min, and 95–5 % acetonitrile over 3 min, all in 0.1% formic acid. The column was re-equilibrated with 5% acetonitrile/0.1% formic acid for 8 min prior to the next injection. The mass measurements were performed using the following QToF3 parameters: capillary voltage 3.5 kV, cone voltage 35 V, RF lens 50 V, source temperature 80°C and desolvation temperature 300 °C. Protein envelopes were deconvoluted by the MaxEnt1 algorithm to obtain the protein masses given in Fig. S4.

The exact mass of protein-derived heme and authentic hemin was recorded on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Hemin or hemoprotein in 5% acteontrile/0.1% formic acid was injected onto a reversed-phase C4 column (100 µm x 4.0 cm) prepared in-house and attached to an Easy-nLC 1000 (Thermo Fisher). The column was equilibrated with the same solution and samples were eluted at 200 nL/min into the ESI source using a 5–95% acetonitrile gradient and analyzed in full-scan mode (m/z 100–2000) in the Orbitrap high resolution mass analyzer (R = 60,000 at m/z 400). Other instrumental parameters were: electrospray voltage 3 kV, CID collision energy 30 V and heated capillary temperature 200 °C.

To identify sites of polypeptide oxidation, 1 µM oxidized Ccp1 was digested overnight with 12.5 ng/µL (1:20) of trypsin in 50 mM Tris (pH 7.4)/100 µM DTPA at 37 °C. The digest was desalted on C18 Zip tips and the tryptic peptides (5 µL/injection) were separated on a reversed-phase C18 column (100 µm x 6.5 cm) prepared in-house and attached to the nanoLC. The column was equilibrated with 2% acetonitrile/0.1% formic acid and peptides were eluted at 200 nL/min into the ESI source using a 2–94% acetonitrile gradient and analyzed in full-scan mode (m/z 350–2000) with the instrumental parameters given above for hemin analysis. Precursor ions of the Ccp1 peptides were selected using a mass exclusion threshold of 10 ppm and subjected to MS/MS in the Velos linear ion trap mass analyzer using a mass tolerance of 0.8 u for the fragment ions. MS/MS fragments with an intensity count of 20 or greater were analyzed using Proteome Discoverer 1.3.0 (Thermo Scientific) and the Sequest search engine with mass filters for oxidation (+16, +32, +48 u) of Met, Cys, Trp, Tyr and His, and for Cys alkylation by iodoacetamide (+57 u). Sequest correlated the MS/MS spectra with peptide sequences in the Ccp1 Fasta file downloaded from the NCBI website (ftp://ftp.ncbi.nlm.nih.gov/). For confident peptide identification, the following Sequest filters were implemented: XCorr ≥ 2 and False Discovery Rate < 0.01. XCorr is the cross-correlation between the theoretical and experimental MS/MS spectra of the

sequenced peptides. The percent oxidation of His175 or Met172 is based on the relative integrated peak areas in the extracted ion chromatograms (XICs) from the primary mass spectra (MS1) (Table S4).

Ccp1 was isolated from 2- and 7-day yeast cells as described under *SI Heme Blotting of Ccp1 Isolated from P10 and S10 Fractions.* The anti-Ccp1 reactive fractions were further subjected to SDS-PAGE on a 12% resolving gel and Coomassie stained. The gel was cut into 1-cm bands and the proteins in each band were reduced with dithiothreitol, alkylated with iodoacetamide, tryptic digested and the peptides were analyzed by LC-MS/MS as described above.

Table S1. *S. cerevisiae* strains

Strain	Genotype	Reference
BY4741 Wild-type	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al. 1998 (19)
YDR256C	BY4741; cta1Δ::kanMX4	Winzeler et al. 1999 (20)
YGR088W	BY4741; ctt1Δ::kanMX4	Winzeler et al. 1999 (20)
YKR066C	BY4741; CCP1:GFP-HIS3MX6	Huh et al, 2003 (21)

Table S2. Normalized CCP activity of subcellular fractions from wild-type and Ccp1-GFP-expressing yeast [a-c]

Strain/Cell age	S2 fraction	P10 fraction	S10 fraction[d]
WT 1-day	0.61 ± 0.05	0.84 ± 0.10	ND
Ccp1-GFP 1-day	0.51 ± 0.05	0.80 ± 0.09	ND
WT 2-day	1.00 ± 0.12	1.00 ± 0.03	ND
Ccp1-GFP 2-day	1.00 ± 0.08	1.00 ± 0.09	ND
WT 3-day	1.09 ± 0.07	0.61 ± 0.11	ND
Ccp1-GFP 3-day	1.03 ± 0.22	0.62 ± 0.03	ND
WT 7 day	0.98 ± 0.05	0.34 ± 0.04	ND
Ccp1-GFP 7 day	0.96 ± 0.20	0.38 ± 0.03	ND

[a] CCP activity of denucleated (S2), mitochondria-enriched (P10), mitochondria-free (S10) fractions (see SI *Materials and Methods*).

[b] One unit of CCP specific activity catalyzes the peroxidation of 1 µmol of horse heart ferrocytochrome c per min per mg total protein. The specific activity of each fraction was ratioed by the relative amount of Ccp1 protein in that fraction (Fig 2*B*, Fig S1*B*), and normalized to the specific activity (µmol/min/mg total protein) of 2-day WT S2 (5.71 ± 0.5) and P10 (13.8 ± 1.2) fractions, and Ccp1-GFP S2 (5.90 ± 0.4) and P10 (13.2 ± 1.1) fractions.

[c] Results are those from three independent cultures (n=3) given as averages ± SD.

[d] ND – Not detected

Table S3. Normalized CCP activity of subcellular fractions from wild-type, *cta1*Δ and *ctt1*Δ yeast [a-c]

Strain/Cell age	S2 fraction	P10 fraction	S10 fraction
WT 2-day	1.00 ± 0.05	1.00 ± 0.10	ND
***cta1*Δ 2-day**	1.08 ± 0.05	1.07 ± 0.09	ND
ctt1Δ 2-day	0.98 ± 0.05	0.95 ± 0.05	ND
WT 7-day	1.07 ± 0.05	0.38 ± 0.01	ND
***cta1*Δ 7-day**	0.88 ± 0.20	0.89 ± 0.03	ND
***ctt1*Δ 7-day**	1.01 ± 0.05	0.40 ± 0.04	ND

[a] See footnotes a-d of Table S1. Note that CCP activities were ratioed by the relative Ccp1 levels in Fig. 4*B*.

Specific activity (μmol/min/mg total protein) is normalized to that of 2-day WT S2 (5.70 ± 0.6) and P10 (13.8 ± 1.4) fractions.

Table S4. Percent heme transfer from Ccp1 or hyperoxidized Ccp1* to apoMb

Reagents[#]	Heme content Mb (μM)	Heme content Ccp1 (μM)	% heme transfer
Ccp1	--------	20 ± 0.0	0.0 ± 0.0
Hyperoxidized Ccp1	--------	20 ± 0.0	0.0 ± 0.0
Ccp1 + apoMb	5.7 ± 0.06	14 ± 0.74	32 ± 9.0
hyperoxidized Ccp1 + apoMb	12 ± 0.39	7.9 ± 1.8	64 ± 8.0

*Recombinant Ccp1 (20 μM) was hyperoxidized with 200 μM H_2O_2 for 60 min in 20 mM KPi (pH 7.5) at 4° C.

[#]Ccp1 or hyperoxidized Ccp1 (20 μM) and 60 μM apoMb were incubated in the same buffer at 30 °C for 60 min with gentle stirring. Following their separation by anion exchange, the heme-loading of Mb and Ccp1 was determined spectrophotometrically (Fig. S2).

Table S5. Relative peak areas of the oxidized forms of tryptic peptide EVVALMGAHALGK [a]

Residue oxidized	Obs mass[b] (u)	Calc mass[c] (u)	Error (ppm)[c]	% peak area $100(P_{ox}/P_{tot})$[d]			
				[Ccp1]:[H_2O_2]			
				1:0	1:1	1:5	1:10
None	1295.7211	1295.7140	5.48	91 ± 0.1	66 ± 10.2	18 ± 1.0	9.2 ± 1.4
Met +16	1311.7108	1311.7089	1.44	8.8 ± 11.1	30 ± 2.9	47 ± 5.7	48 ± 3.1
Met +32	1327.7091	1327.7038	3.99	0.3 ± 0.0	---	---	0.5 ± 0.3
His +16	1311.7050	1311.7089	2.97	---	3.2 ± 2.5	28 ± 2.0	37 ± 3.2
Met +16, His +16	1327.7064	1327.7038	1.95	---	---	6.8 ± 4.0	4.9 ± 0.5

[a] Ccp1 (5 µM) was reacted with 1, 5, and 10 molar equiv of H_2O_2 for 1 h at 4 °C in 20 mM KPi (pH 7.5) with 100 µM DTPA prior to tryptic digestion and LC-MS/MS analysis (see SI *Materials and Methods*).

[b] The precursor ions selected for MS/MS analysis were filtered using a mass exclusion threshold of 10 ppm.

[c] Peptide masses were calculated with Proteome Discoverer and oxidized Met (+16, +32) and His (+16). The error in ppm is given by 10^6 (Obs mass–Calc mass)/Calc mass.

[d] The integrated peak area in the extracted ion chromatogram (XIC) from MS1 of the peptide with the indicated oxidation (P_{ox}) was divided by the sum of the peak areas of all detected forms of the peptide (P_{tot}). The percent oxidation of His175 or Met172 is estimated from $100(P_{ox}/P_{tot})$.

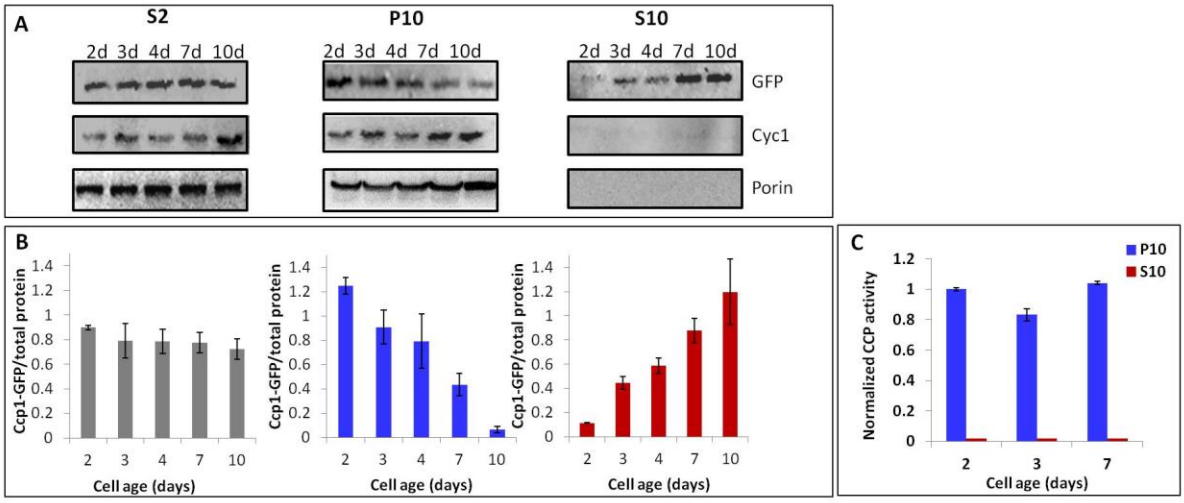

Fig. S1. Ccp1-GFP exits mitochondria as yeast begin respiring and extramitochondrial Ccp1-GFP does not possess CCP activity. (A) Immunoblot analysis of equal volumes of denucleated (S2), mitochondrial (P10), and cytosolic (S10) fractions vs cell age. Porin and Cyc1 are mitochondrial outer membrane and IMS markers, respectively. **(B)** The GFP signals in panel A were quantified and normalized to the sum of the integrated intensity of all Coomassie bands in the same lane. **(C)** Normalized CCP activity in mitochondrial (P10) and cytosolic (S10) fractions. Specific activity was ratioed by the Ccp1 protein levels in panel B, and normalized to the level for 2-day cells (Table S3). Results in panel A are representative of three independent cultures (n = 3) and averages ± SD are plotted in panels B and C.

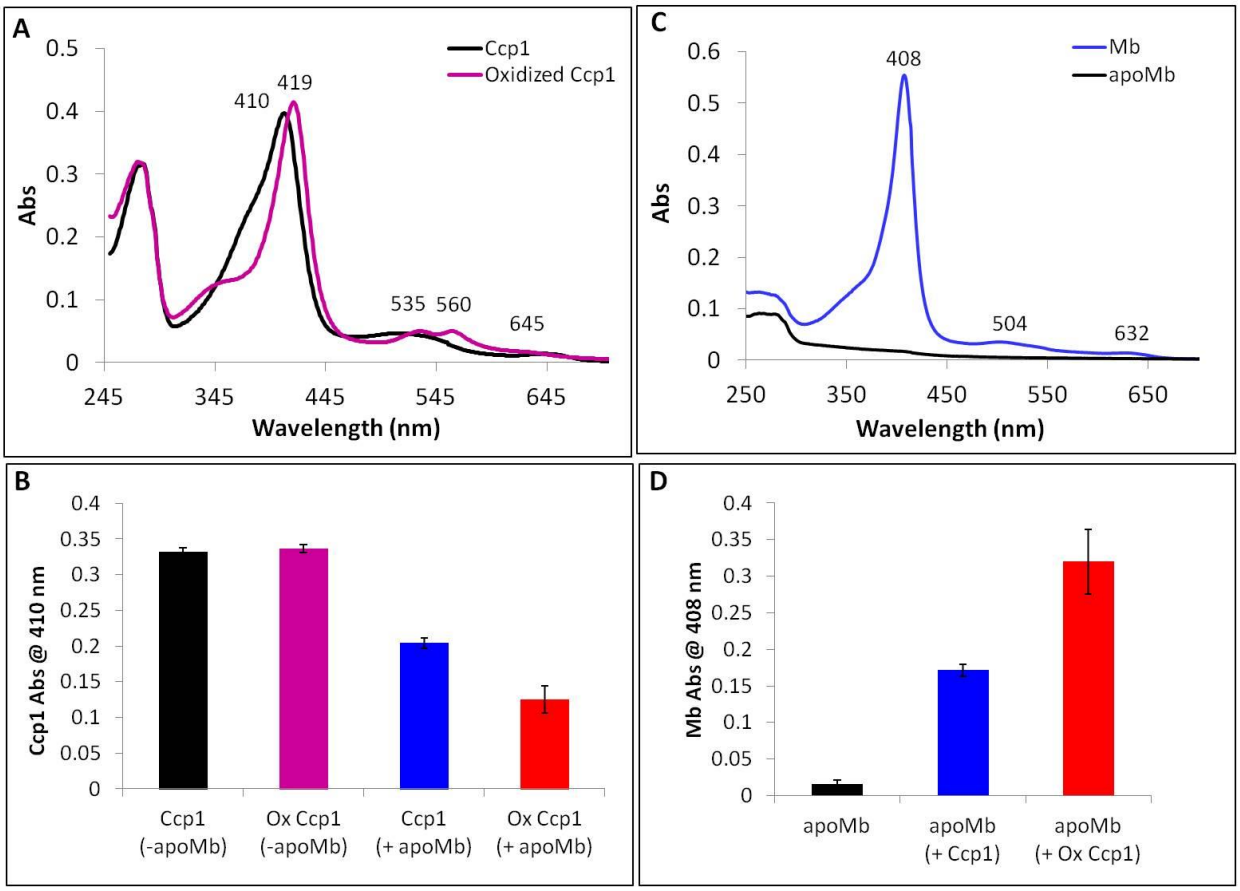

Fig. S2. Ccp1 hyperoxidation by H_2O_2 increases heme transfer to apoMb. Ccp1 or hyperoxidized Ccp1 (20 µM) was incubated with 60 µM apoMb in 20 mM KPi (pH 7.5) at 30 °C for 60 min. The proteins were separated by anion exchange, diluted to 0.5–3.0 µM and their absorption spectra were recorded in a 1-cm quartz cuvette. Spectrum of **(A)** Ccp1 and hyperoxidized Ccp1 after 60 min incubation (minus apoMb), and of **(B)** apoMb and reconstituted Mb. **(C)** Soret absorbance of Ccp1 and hyperoxidized Ccp1 after 60 min incubation ± apoMb. Note that the Fe^{IV} heme of Ccp1 decayed to Fe^{III} heme during anion-exchange chromatography so the Soret absorbance of the latter (ε_{410}=98 cm^{-1} mM^{-1}) was used to monitor the heme loading of Ccp1. **(D)** Soret absorbance of Mb (ε_{408}=188 cm^{-1} mM^{-1}) after 60 incubation ± Ccp1 or hyperoxidized Ccp1. The percent heme transfer calculated from the absorbance data in panels C and D is summarized in Table 1 of the main text for three independent experiments (*n* = 3).

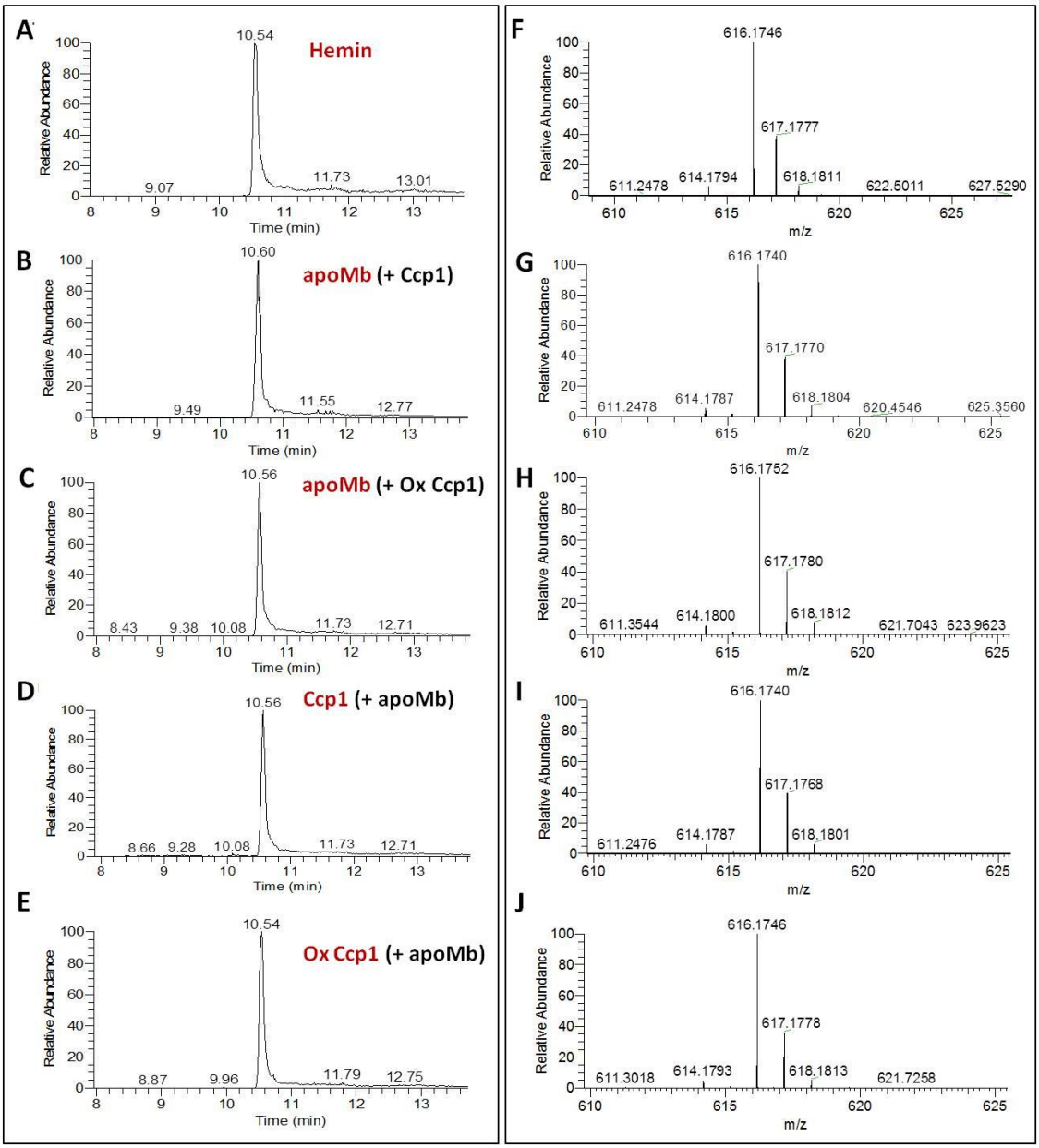

Fig. S3. Heme from hyperoxidized Ccp1 is not oxidized. Following their separation by anion-exchange chromatography, Ccp1, hyperoxidized Ccp1 (Ox Ccp1) and Mb from the heme-transfer experiments (Fig. S2) were diluted 10-fold to 0.5 µM protein with 2% acetonitrile/0.1% formic acid, and 5 µL of each sample was analyzed by LC-MS (SI *Methods and Materials*). **(A-E)** Chromatograms of the hemin that dissociated from the indicated polypeptide (red font) on the LC column at pH 4.0 (the protein partner in the heme-transfer reaction is indicated in brackets). The average hemin retention time on the C4 column is 10.56 ± 0.03 min. **(F-J)** The corresponding hemin mass spectra show the isotopic distribution expected for $FeC_{34}H_{22}N_4O_4$ (calc monoisotopic mass: 616.1773 u). Ccp1-derived hemin has a mass of 616.1745 ± 0.0005 vs 616.1746 u for authentic hemin (spectrum F), which serves as an external standard.

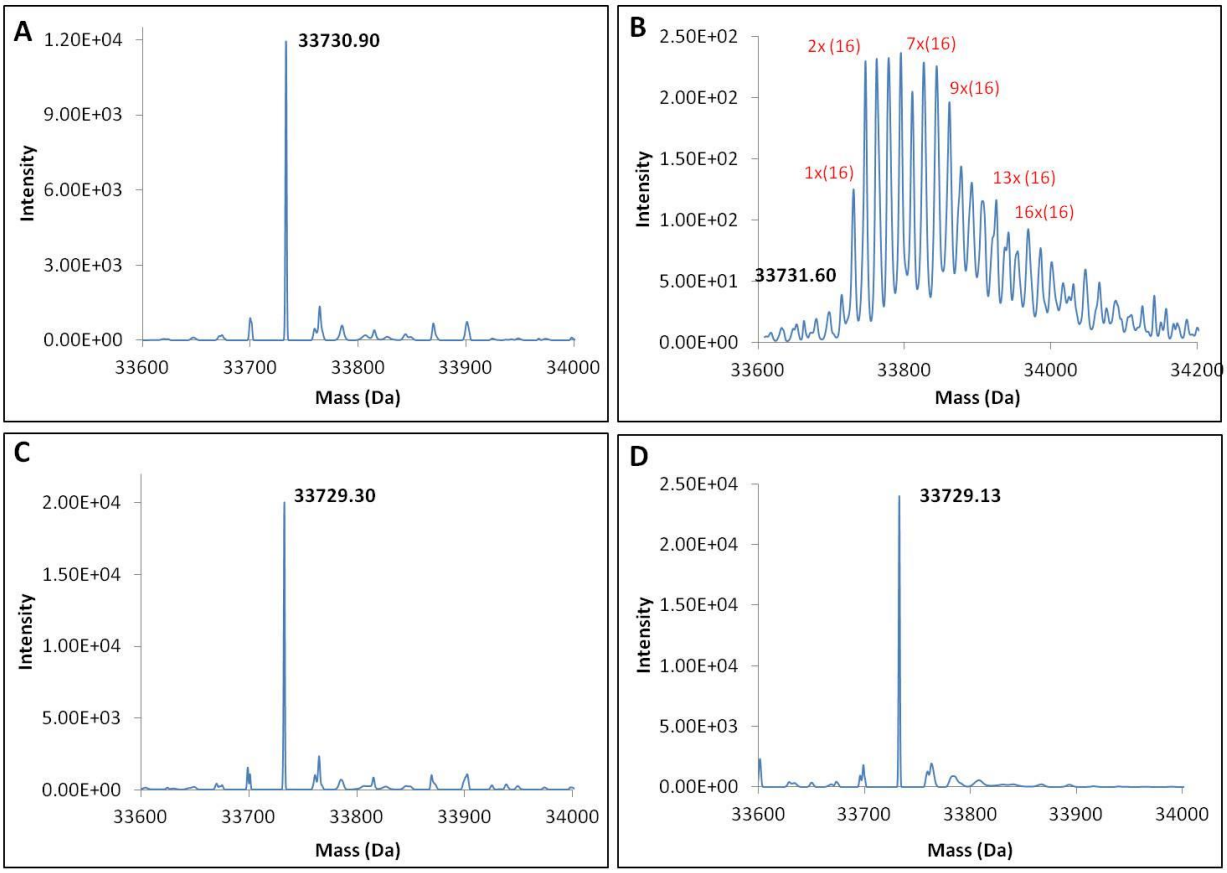

Fig. S4. H_2O_2 oxidizes holoCcp1 but not apoCcp1. Recombinant holo- or apoCcp1 (5 μM) was incubated with 50 μM H_2O_2 in 20 mM KPi (pH 7.5)/100 μM DTPA at 4 °C for 60 min, 0.1 nM catalase was added to remove any remaining H_2O_2 and samples were further incubated at 30 °C for 60 min before recording the mass spectra of the intact proteins as described in the SI *Materials and Methods*. Deconvolved mass spectrum of **(A)** holoCcp1 (control, no H_2O_2 treatment), **(B)** hyperoxidized Ccp1 with the number of oxygen adducts (+16) indicated in red font, **(C)** apoCcp1 (control, no H_2O_2 treatment), and **(D)** H_2O_2-treated apoCcp1. The masses corresponding to the unmodified Ccp1 polypeptide indicated on the peaks in panels A, C and D agree with the calculated mass of 33730.33 u within the accuracy of the QToF3 mass spectrometer, which was mass calibrated using horse heart Mb as a standard (Obs mass 16952.10 u; Calc mass 16951.49 u; 36 ppm error).

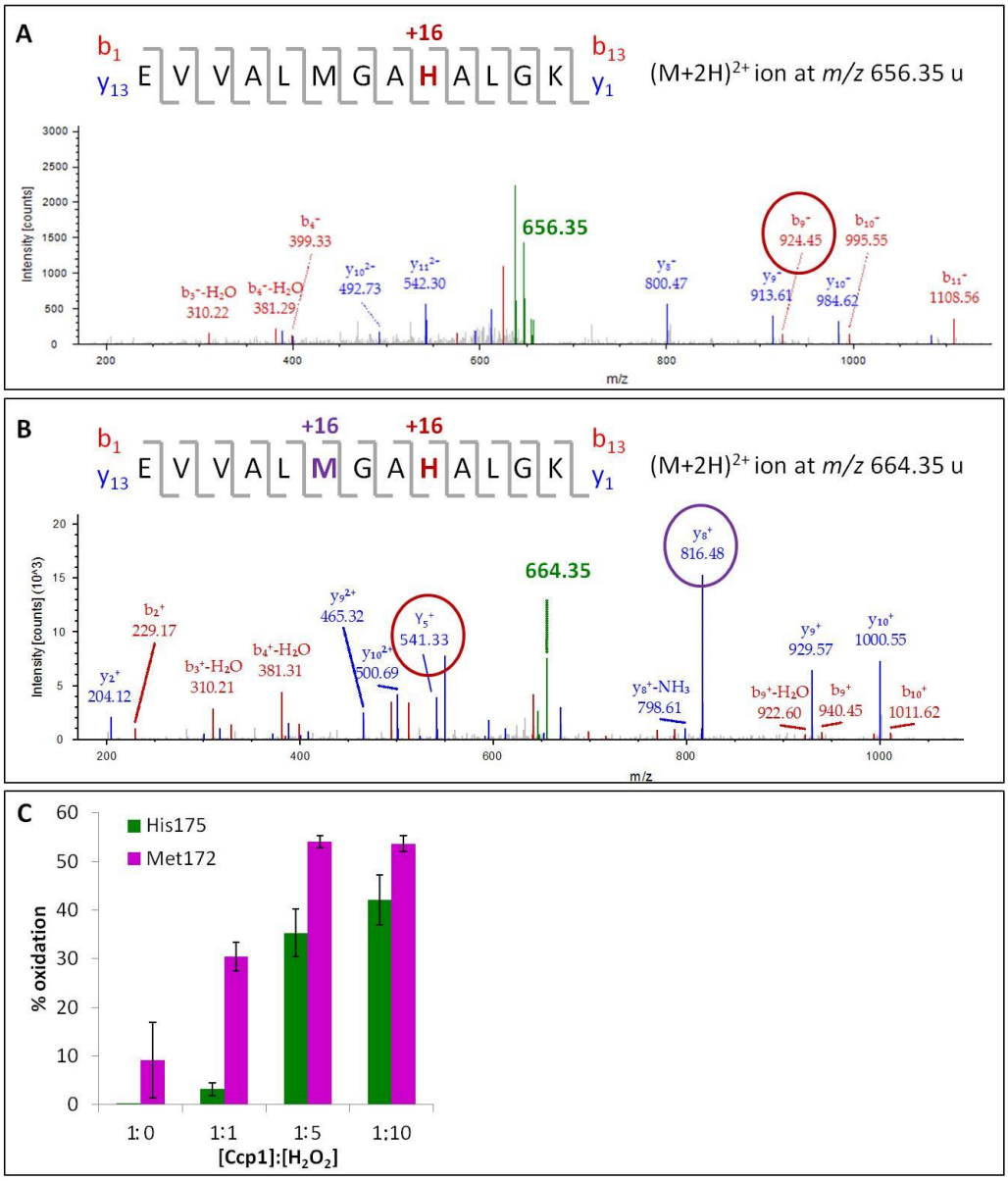

Fig. S5. H_2O_2 oxidizes His175, the proximal Fe ligand, in recombinant Ccp1.

Recombinant Ccp1 (5 µM) was hyperoxidized with 50 µM H_2O_2 for 60 min at room temperature, diluted into 50 mM Tris (pH 7.4)/100 µM DTPA to 1 µM protein, digested with trypsin and the peptides were analyzed by LC-MS as described in the SI *Materials and Methods*. MS/MS spectrum of the $(M+2H)^{2+}$ precursor ion of the EVVALMGAHALGK peptide oxidized at **(A)** His175 (+16) and **(B)** His175 (+16) plus Met172 (+16). The precursor ions at m/z 656.35 and 664.35 (green) were fragmented by CID (30 V) to give b_n (red) and y_n sequence ions (blue). The b_9^+ ion bearing oxo-His175 at its C-terminus, and the y_5^+ and y_8^+ ions bearing oxidized His175 and oxidized Met172 at their N-termini are circled in panels A and B, respectively. **(C)** Percent oxidized Met172 and His175 increases with amount of H_2O_2 added to Ccp1 (data from Table S5). Interestingly, Met172 appears to compete with His175 as an electron donor to

H_2O_2 since < 10% of peptide EVVAL**M**GA**H**ALGK is oxidized at both residues (Table S5). We speculate that switching between Met172 and His175 as a donor controls heme labilization in Ccp1 in vivo but a better understanding of this process requires further detailed examination of hyperoxidized Ccp1.

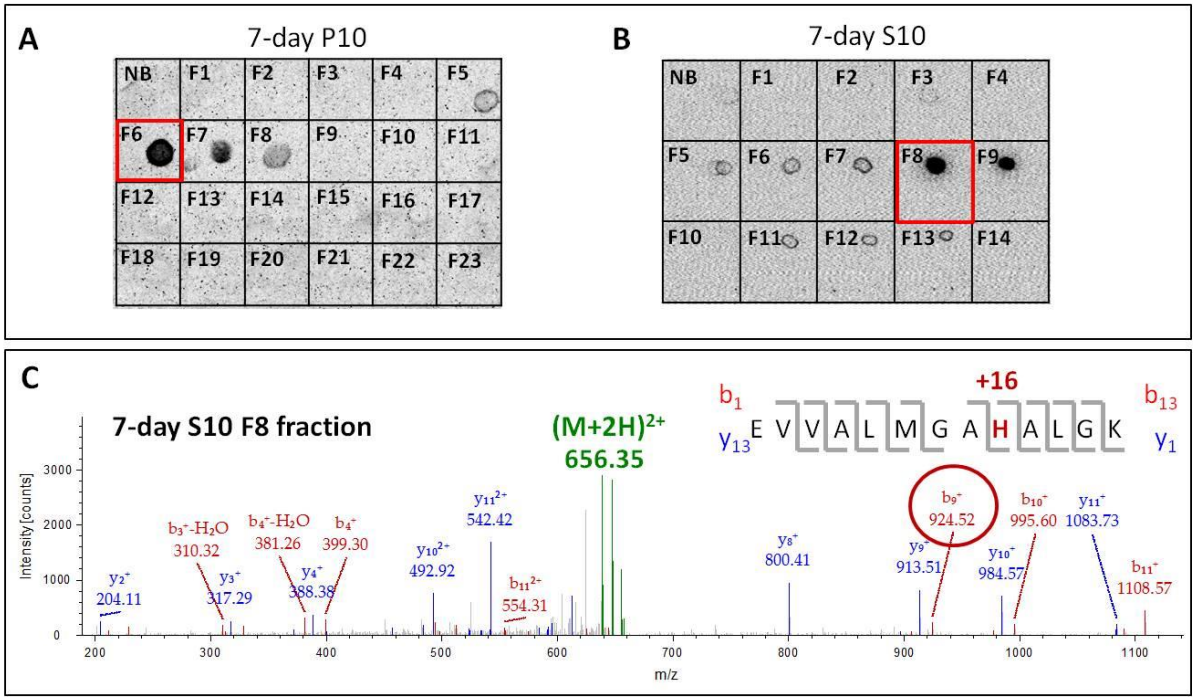

Fig. S6. LC-MS/MS analysis of extramitochondrial Ccp1 isolated from 7-day cells reveals His175 oxidation. Ccp1 was isolated by anion-exchange chromatography from **(A)** mitochondrial (P10) and **(B)** extramitochondrial (S10) subcellular fractions from 7-day cells and the fractions dot blotted onto PVDF membranes and probed with anti-Ccp1. The main Ccp1-containing fractions (red boxes) were analyzed by reducing SDS-PAGE, gel bands were excised, the proteins were digested with trypsin and the peptides were analyzed by LC-MS/MS. **(C)** The $(M+2H)^{2+}$ precursor ion at m/z 656.35 (green) of the oxidized EVVALMGAHALGK peptide (+16) from extramitochondrial fraction 8 was fragmented by CID (30 V) to give MS/MS spectrum shown with b_n (red) and y_n sequence ions (blue). The circled b_9^+ ion bears oxo-His175 at its C-terminus, identifying His175 as the site of oxidation. Results are representative of those from three independent cultures (n=3) and further experimental details are provided in the SI *Materials and Methods*. The percentages of oxidized His175 in S10 and P10 estimated from peptide peak areas in the LC-MS spectra are plotted in Fig. 5B of the main text.

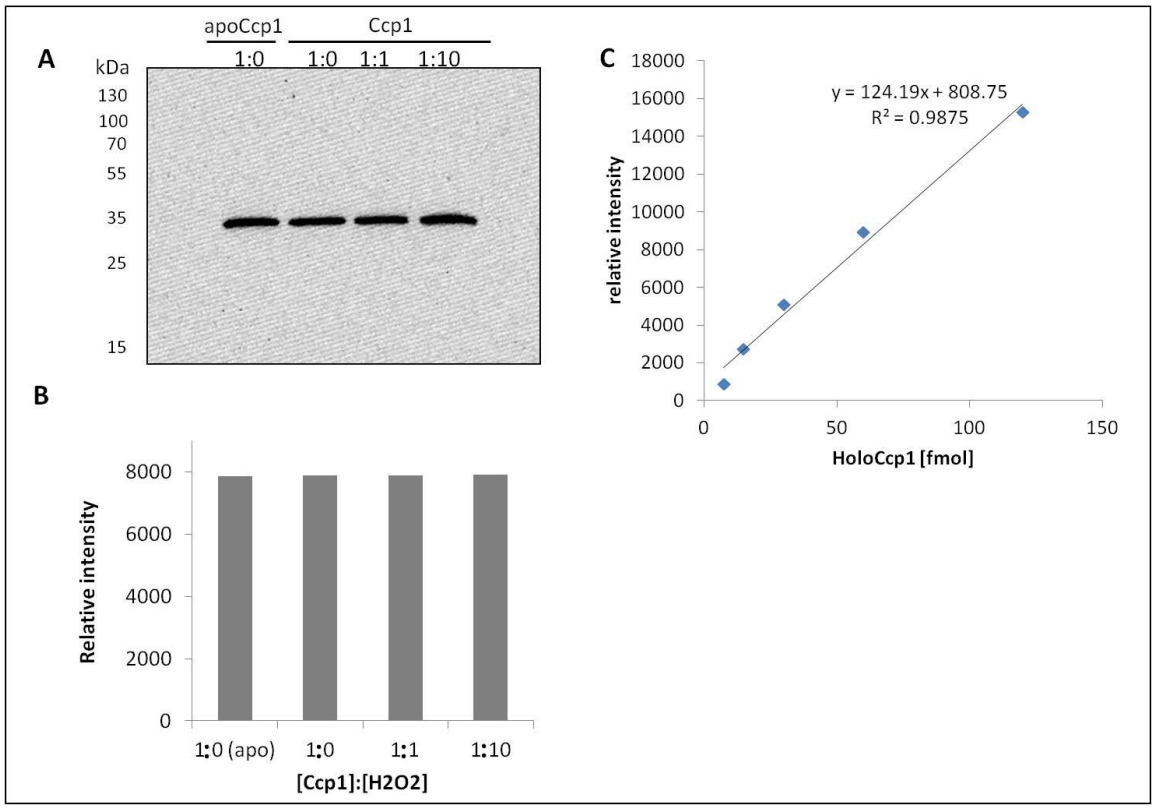

Fig. S7. The anti-Ccp1 antibody detects the heme free (apo), heme loaded (holoCcp1) and oxidized forms of Ccp1. (A) Immunoblot analysis of 60 fmol of apoCcp1, holoCcp1 (Ccp1), and holoCcp1 treated with 1 (CpdI) and 10 molar equiv (hyperoxidized) of H_2O_2 as indicated by the [Ccp1]:[H_2O_2] ratios. **(B)** The Ccp1 signals in panel A were quantified and normalized to the sum of the Coomassie bands in the same lane. **(C)** Standard curve prepared from a representative immunoblot showing the anti-Ccp1 response is linear between 4-120 fmol of recombinant holoCcp1.

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