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SUPPLEMENTAL INFORMATION

Targeted proteomics identify metabolism-dependent interactors of yeast cytochrome c peroxidase: Implications in stress response and heme trafficking

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Table S1: CCP activity of GST-holoCcp1[a]

Protein [b]	CCP activity [a]	Percent activity [c]
Wild-type Ccp1	1.70 ± 0.010	100 ± 0.00
GST-holoCcp1	0.96 ± 0.020	56.4 ± 4.86
GST-apoCcp1	0.06 ± 0.008	3.50 ± 0.30

[a] One unit of CCP activity catalyzes the oxidation by H_2O_2 of 1 μmol of horse heart ferrocytochrome c per min per mg Ccp1.

[b] Recombinant Ccp1, GST-apoCcp1 and GST-holoCcp1 (1 μM) were assayed for CCP activity as described previously. For the assay, a stock solution of Cyc^{III} was ~90% reduced with sodium dithionite and the Cyc^{II} concentration determined spectrophotometrically (ε_{550} = 7.6 mM^{-1} cm^{-1}).[1]

[c] The activity of each fraction was ratioed by the activity of wild-type Ccp1 to give percent activity. Results are presented as averages ± SD from three independent experiments (*n*=3).

Table S2: Interactors in the GST pulldowns from 1 and 7 d yeast extracts (*n*=6)[a]

Accession number [a]	Protein ID [b]	Protein description [c]	Sequence coverage (%) [d]	Unique peptides [e]	Subcellular location [f]	Subcellular fraction [g]
YGR254W*	ENO1	Enolase 1	57.4 ± 10.6	11	C, M, PM	P10, S10
YHR174W*	ENO2	Enolase 2	56.1 ± 11.2	16	V, M, PM	P10, S10
YBR072W**	HSP12	Heat shock protein 12	54.1 ± 14.5	20	C, PM, N	P10, S10
YAL005C*	SSA1: HSP70	Heat shock protein 12	49.4 ± 14.4	14	C, PM, N	P10, S10
YLL024C*	SSA2: HSP70	Heat shock protein 70	45.0 ± 7.5	13	C, M, V	P10, S10
YDL229W*	SSB1/SSB2	Heat shock protein 70	34.0 ± 4.4	6	PM	P10
YNL055C*	POR1	Porin	35.7 ± 22.8	22	M	P10
YLR150W	STM1	Suppressor of TOM1	49.6 ± 8.5	11	C	S10
YPL237W	SUI3	Suppressor of initiator codon	48.6 ± 9.5	11	Multi-eIF complex	P10
YMR146C	TIF34	Translation initiation factor	52.2 ± 7.8	15	Multi-eIF complex	P10
YDR429C	TIF35	Translation inititation factor	30.7± 1.3	4	Multi-eIF complex	P10
YDL014W	NOP1	Nucleolar protein	36.6 ± 6.7	8	N	S10
YLR197W	NOP56	Nucleolar protein	42.0 ± 14.6	16	N	S10
YBR009C	HHF1	Histone H4	43.8 ± 4.9	6	N	S10
YJL131C	AIM23	Altered inheritance rate of mitochondria	40.5 ± 5.4	8	M	P10
YJR055W	HIT1	High temperature growth	40.1 ± 20.6	23	C, N	S10
YLL039C**	UBI4	Ubiquitin	39.6 ± 3.7	6	C	S10
YLR074C	BUD20	BUD site selection	31.6 ±14.5	23	C, N	S10
YNL178W*	RPS3	40S ribosomal subunit	53. 7± 3.4	11	R	P10, S10
YNL096C	RPS7B	40S ribosomal subunit	32.8 ± 4.4	18	R	P10, S10
YER102W	RPS8B	40S ribosomal subunit	32.6 ± 18.7	11	R	P10, S10
YDR064W	RPS13	40S ribosomal subunit	48.1 ± 3.0	14	R	P10, S10
YMR143W*	RPS16A	40S ribosomal subunit	55.3 ± 5.4	11	R	P10, S10
YNL302C*	RPS19B [j]	40S ribosomal subunit	59.7 ± 4.7	11	R	P10, S10
YML026C	RPS18B	40S ribosomal subunit	55.2 ± 9.7	13	R, M	P10
YCR031C	RPS14A	40S ribosomal subunit	50.4 ± 16.6	20	R	P10, S10
YHL015W*	RPS20	40S ribosomal subunit	40.6 ± 14.9	25	R	P10, S10
YIL069C	RPS24B	40S ribosomal subunit	48.4 ± 4.5	18	R	P10, S10
YLR061W	RPL22A	60S ribosomal subunit	57.4 ± 9.5	19	R	P10, S10
YDR012W	RPL4B	60S ribosomal subunit	53.3 ± 3.4	17	R	P10, S10
YDR418W	RPL12B	60S ribosomal subunit	32.5 ± 4.8	6	R	P10, S10
YHL001W	RPL14B	60S ribosomal subunit	34.0 ± 1.2	4	R	P10, S10
YJL177W YKL180W	RPL17B/A	60S ribosomal subunit	47.6 ± 12.6	9	R	P10, S10
YMR242C	RPL20A	60S ribosomal subunit	36.9 ± 4.3	6	R	P10, S10
YOL127W	RPL25	60S ribosomal subunit	33.3 ± 2.3	5	R	P10, S10
YDR471W	RPL27B	60S ribosomal subunit	34.6 ± 6.6	7	R	P10, S10
YGL030W	RPL30	60S ribosomal subunit	34.5 ± 8.5	8	R	P10, S10
YAL036C	RBG1	Ribosome interacting GTPase	42.7 ± 6.7	5	C	S10

[a-f] See corresponding footnotes to Table 1 of the main text. Interactors in **red font** and **blue font** were pulled down by GST only in **1 d cells** or **7 day cells**, respectively. Interactors in black were pulled down by GST in both 1 d cells and 7 d cells.

*,**Interactors of GST-Ccp1 also: *both 1 d and 7 d cells or in **7 d cells only.

Table S3: Reported Ccp1 interactors collated from the *Saccharomyces* Genome Database (SGD)[a]

Accession number [b]	Short name [c]	Protein description [d]	Interaction type [e]	Assay [f]	Exp format [g]	Refs
YKR066C	CCP1	Cytochrome c peroxidase	Physical	Affinity-capture Co-purification	MC HTS	1,2
YBL009W	ALK2	Protein kinase	Genetic	Negative genetic	HTS	3
YNR001C	CIT1	Citrate synthase	Physical	Co-fractionation	MC	4
YJR048W[h]	CYC1	Cytochrome c isoform 1	Physical	Co-crystal structure Affinity -capture Co-purification Reconstituted complex	MC	2,5,6
YOR065W	CYT1	Cytochrome c1	Physical	Reconstituted complex	MC	7,8
YDL160C	DHH1	Cytoplasmic DExD/H-box helicase	Physical	Affinity capture	HTS	9
YER019W	ISC1	Inositol phosphosphingolipid phospholipase C	Genetic	Positive genetic	HTS	10
YPL004C	LSP1	Eisosome core component	Physical	Two-hybrid	HTS	11
YKL195W	MIA40	Import and assembly protein in mitochondrial IMS	Physical	Co-purification	MC	12
YKR016W	MIC60	Component of the MICOS complex	Physical	Affinity capture	HTS	13
YCR077C	PAT1	Deadenylation-dependent mRNA-decapping factor	Physical	Affinity capture	HTS	9
YGR101W	PCP1	Rhomboid mitochondrial serine protease	Physical	Biochemical activity	MC	14
YHL034C	SBP1	Protein that binds eIF4G	Physical	Affinity capture	HTS	9
YHR206W	SKN7	Nuclear transcription factor	Genetic	Phenotypic suppression	MC	15
YCL037C	SRO9	Cytoplasmic RNA-binding protein;	Physical	Affinity capture	HTS	16
YNL209W[h]	SSB2	HSP70		Affinity capture	HTS	17
YHR005C-A	TIM10	Forms a complex with tim9p	Physical	Co-purification	MC	12
YNL299W	TRF5	Non-canonical poly(A) polymerase	Physical	Affinity capture	MC	18
YML028W[h]	TSA1	Thioredoxin peroxidase	Genetic	Synthetic growth defect	HTS	19
YLL039C[h]	UBI4	Ubquitin	Physical	Co-purification	HTS	20
YGR019W	UGA1	Gamma-aminobutyrate (GABA) transaminase	Physical	Negative genetic	HTS	10
YCR007C	--------	Unknown	Genetic	Two-hybrid	HTS	11

[a] Ccp1 interactors found at http://www.yeastgenome.org.

[b] The GenBank (http://www.ncbi.nlm.nih.gov/genbank/) identifier assigned to each gene sequence

[c] Four-letter/number yeast notation for protein

[d] Protein name/description from given reference(s) and/or SGD website

[e] Interaction type from given reference(s)

[f] Type of assay used to probe interactions

[g] Experimental format, MC- manually curated; HTS- high through put screen

[h] Proteins identified in this study are highlighted in grey

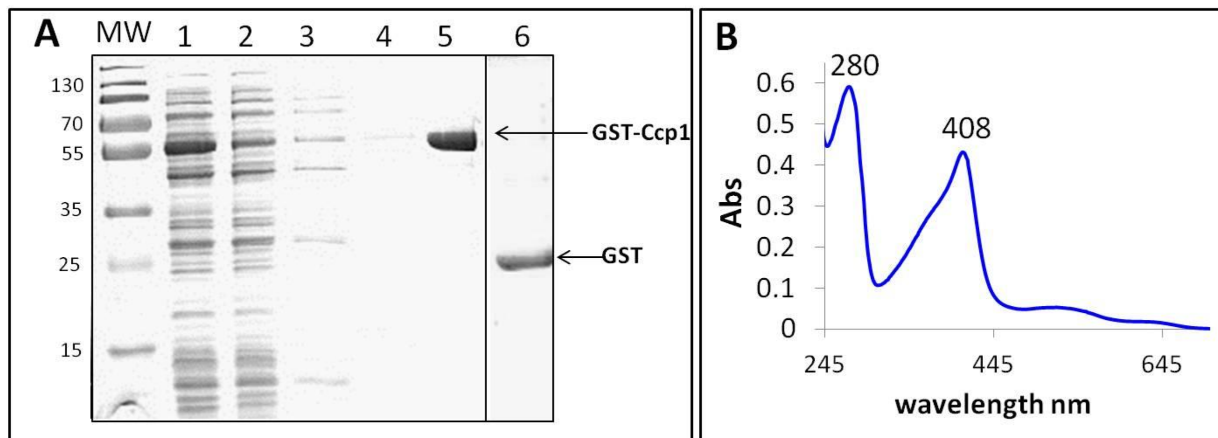


Figure S1: SD-PAGE analysis of recombinant GST-Ccp1 purification.

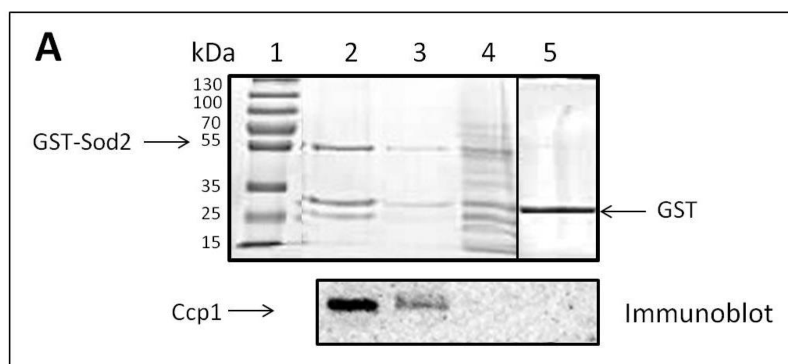
(A) Coomassie-stained 12% SDS-PAGE gel showing the proteins in: (lane 1) the soluble fraction from *E. coli* BL21(DE3) cells transfected with the pGEX-2T-Ccp1 plasmid; (lane 2) the supernatant (batch mode) of the Glutathione Sepharose 4B resin; (lanes 3, 4) washings from the resin; (lane 5) the GST-Ccp1 fusion protein (MW ~60 kDa) eluted with 50 mM reduced GSH at pH 8.0; and (lane 6) the GST protein (MW ~30 kDa) eluted with 50 mM reduced GSH at pH 8.0 from resin added to a lysate of *E. coli* harboring the empty pGEX-4T plasmid. The markers are in lane MW and their molecular weights in kDa are indicated on the left. The GSH eluate from the Glutathione Sepharose 4B resin was decomplexified by 1D SDS-PAGE under reducing conditions on 8 cm x 5.8 cm x 1 mm 6% stacking and 12% resolving gel for 1 h at 120 V. The gel was Coomassie stained for 15 min and destained overnight at 4°C with 25 mM ammonium bicarbonate (pH 8.0)/50% acetonitrile. (B) The room temperature UV-vis spectrum of 4 µM GST-Ccp1 in 20 mM KPi buffer (pH 7.5) with 500 mM NaCl in a 1-cm pathlength cuvette following reconstitution with heme (see *Materials and Methods* of the main text). The 408/280 absorbance ratio is 0.77± 0.03 (*n*=3), which agrees, within experimental error, with the calculated ratio of 0.79 for fully heme-loaded GST-Ccp1.

Screening for Ccp1 as an interactor of Sod2 in 1 d yeast cells expressing the GST-Sod2 fusion

Yeast cells transformed with the GAL1-10 UAS vector containing the GST-Sod2 coding sequence (GE Dharmacon) were grown overnight in 20 mL of synthetic complete medium with 2% raffinose but minus uracil (SR-ura) as the plasmid is URA+. Raffinose was used as a sugar since glucose represses GAL1 expression. Cells were pelleted at 2,000 x g for 5 min, washed 2X with sterile water, resuspended in 500 mL of SG-ura (4% galactose) or YPG (1% yeast extract, 2% peptone, 4% galactose) to induce GST-Sod2 expression, pelleted after 12 h at 2,000 x g, snap frozen and stored at -80 °C. The GST-Sod2 pulldown assays were performed as reported[2] using the MagneGST Pull-Down System kit (Promega). Briefly, cells were resuspended in binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 10 mM β-mercaptoethanol and 1 mM PMSF)[2], broken by vortexing for 5 x 15 s with an equal volume of 0.5 mm acid-washed glass beads in the same buffer. Unbroken cells and cell debris were removed by centrifugation at 2,000 x g for 5 min and the lysate was cleared by centrifugation at 13,000 x g for 10 min. The supernatant, which corresponds to the soluble protein extract (2 mg) was added to a

200 µL slurry of the GSH-linked magnetic beads with end-over-end rotation for 1 h at 4°C. The control GST bait purified from *E. coli* BL21(DE3) cells (see main text) was bound to a 200 µL slurry of GSH-linked magnetic beads and added to the soluble protein extract (2 mg) from wild-type cells prepared as described above for the GST-Sod2-expressing strain, and pulldowns were carried out as outlined in the *Materials and Methods* of the main text. The proteins separated by 1D 12% SDS-PAGE were transferred to a methanol-soaked polyvinylidene fluoride (PVDF, BioRad) membrane at 100 mA for 3 h at room temperature. After blocking for 1 h with 5% (w/v) skim milk in TBST (50 mM Tris, 150 mM NaCl and 0.05% v/v Tween 20, pH 7.6), the membrane was incubated for 2 h with rabbit anti-Ccp1 serum (1:10,000 dilution) (kindly provided by Professor David Goodin, University of California, Davis), washed 3 times with TBST, and probed for 1 h with goat anti-rabbit HRP conjugated secondary antibody (1:20,000, Biorad). Blots were visualized using the Super Signal West Pico Enhanced Chemiluminscence (ECL) kit from Thermo Fisher in an AlphaImager (ProteinSimple).

Figure S2A (top panel) establishes the overexpression of the GST-Sod2 and GST bait proteins in the yeast strains. The presence of Ccp1 in the GST-Sod1 pulldown from 1 d cells but not in the corresponding GST control pulldown was demonstrated by Western blotting against anti-Ccp1 (Figure S2A, bottom panel) and confirmed by LC-MS/MS (Figure S2B).



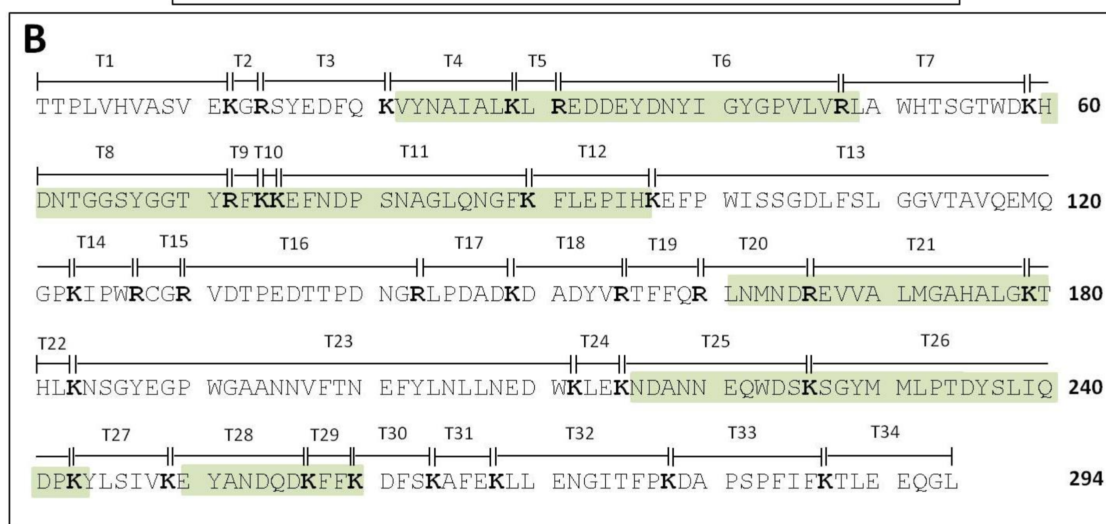


Figure S2: Identification of Ccp1 in a GST-Sod2 pulldown from a soluble protein extract of 1 d yeast.

(A) Top panel: Silver-stained 12% SDS PAGE of the GST-Sod2 bait protein and prey proteins from 1 d yeast overexpressing GST-Sod2. The molecular weight markers (kDa) are in lane 1 and proteins pulled down by the GST-Sod2 bait in a two-step elution from the GSH magnetic beads are in lanes 2, 3. Lane 4 shows the proteins released from the GSH magnetic beads after boiling in SDS loading buffer. Proteins pulled down by the GST control bait from 1 d wild-type yeast are in lane 5 and arrows indicate the bait proteins. Experimental details are provided in the text above. **Bottom panel:** Anti-Ccp1 immunoblot analysis confirming that Ccp1 is present in lanes 2 and 3 close to the 35 kDa marker. **(B)** Ccp1 sequence with the trypsin cleavage sites (**K**, **R**) bolded. Nine unique Ccp1 peptides were detected by MS in the GST-Sod2 pulldowns and the Ccp1 sequence covered (~33%) by MS/MS in two independent experiments (*n*=2) is highlighted in green. Details of the mass spectrometric analysis are given in the *Materials and Methods* of the main text.

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TDH3    1  MVRVAINGFGRIGRLVMRIALSRPNVEVVALNDPFITNDYAAYMFKYDSTHGRYAGEVSH
TDH2    1  MVRVAINGFGRIGRLVMRIALQRKNVEVVALNDPFISNDYSAYMFKYDSTHGRYAGEVSH
TDH1    1  MIRIAINGFGRIGRLVLRLALQRKDIEVVAVNDPFISNDYAAYMVKYDSTHGRYKGTVSH

TDH3   61  DDKHIIVDGKKIATYQERDPANLPWGSSNVDIAIDSTGVFKELDTAQKHIDAGAKKVVIT
TDH2   61  DDKHIIVDGHKIATFQERDPANLPWASLNIDIAIDSTGVFKELDTAQKHIDAGAKKVVIT
TDH1   61  DDKHIIIDGVKIATYQERDPANLPWGSLKIDVAVDSTGVFKELDTAQKHIDAGAKKVVIT

TDH3  121  APSSTAPMFVMGVNEEKYTSDLKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSLT
TDH2  121  APSSTAPMFVMGVNEEKYTSDLKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSMT
TDH1  121  APSSSAPMFVVGVNHTKYTPDKKIVSNASCTTNCLAPLAKVINDAFGIEEGLMTTVHSMT

TDH3  181  ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV
TDH2  181  ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV
TDH1  181  ATQKTVDGPSHKDWRGGRTASGNIIPSSTGAAKAVGKVLPELQGKLTGMAFRVPTVDVSV

TDH3  241  VDLTVKLNKETTYDEIKKVVKAAAEGKLKGVLGYTEDAVVSSDFLGDSHSSIFDASAGIQ
TDH2  241  VDLTVKLNKETTYDEIKKVVKAAAEGKLKGVLGYTEDAVVSSDFLGDSNSSIFDAAAGIQ
TDH1  241  VDLTVKLEKEATYDQIKKAVKAAAEGPMKGVLGYTEDAVVSSDFLGDTHASIFDASAGIQ

TDH3  301  LSPKFVKLVSWYDNEYGYSTRVVDLVEHVAKA
TDH2  301  LSPKFVKLVSWYDNEYGYSTRVVDLVEHVAKA
TDH1  301  LSPKFVKLISWYDNEYGYSARVVDLIEYVAKA
```

Figure S3: Sequence alignment of the TDH3/2/1 isoforms of yeast GAPDH.

Identical, similar and dissimilar residues are indicated by filled-in black boxes, black-outlined boxes and white boxes, respectively. The alignment was carried out using clustalW2 from Embl (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and ESPript was used to generate the alignment image (http://espript.ibcp.fr/ESPript/ESPript/). The red, blue and green line localizes the isoform-specific sequence in panels A, B and C, respectively, of Figure 5 of the main text.

References

1 T. Yonetani, *J Biol Chem*, 1965, **240**, 4509–4514.

2 J. Mok, H. Im and M. Snyder, *Nat Protoc*, 2009, **4**, 1820–1827.