Supplementary Data

Supplementary Materials and Methods

Plasmid construction and manipulation

The coding sequence of *YCF1* was cloned in the yeast vectors, p415GPD and p416GPD (7). Green fluorescent protein (GFP) or hemagglutinin (HA)-fused *YCF1* were constructed by PCR-based generation of an *Not*I restriction enzyme site before the stop codon followed by an in-frame insertion of *Not*I-flanked GFP or HA sequences. Site-directed amino-acid substitutions of cysteine to alanine or serine were conducted by the primer overlap extension method (5). Expression constructs of SOD1 of yeast *Saccharomyces cerevisiae* and *Caenorhabditis elegans* were kindly provided by Dr. Valeria C. Culotta (1). Plasmids were transformed into yeast using the lithium acetate method (4). Plasmids and primers used in this study are listed in Supplementary Tables S3 and S4, respectively.

Enzyme activity assays

Sod1p and Fet3p activities were measured as previously described (2, 8). For Fet3p oxidase activity assays, yeast cells were cultured in SC selection media that was supplemented with iron chelator bathophenanthrolinedisulfonate (BPS, 80 μM) and copper chelator bathocuproinedisulfonate (BCS, 50 μM) to enhance the expression of Fet3p to easily detectable levels as well as to induce copper limitation to visualize more dramatic effects with regard to Fet3p activity change. For *in vitro* copper loading to apo-Fet3p, the samples were mixed with reduced $CuCl_2$ (25 μM final concentration) (preincubation with 1 mM ascorbate for 30 min).

Metal measurement

Yeast cells grown in SC media until mid-log phase were cultured with and without supplementation of $CdCl_2$ in the media for 1 h. Cells were collected and washed twice in PBS containing 10 mM EDTA. Cell pellets were dissolved in 70% nitric acid at 60°C for 2 h and then overnight at room temperature. Cell-associated metals were measured by inductively coupled plasma mass spectrometry (ICP-MS). The instrument was operated in collision/reaction mode (He 3.5 ml/min, H_2 1.5 ml/min) with 50 ppb Ga as an internal standard. The results were normalized to cell number and presented as relative levels to those of control samples.

Western blotting analysis

Wild type, *ccs1*Δ and *glr1*Δ cells expressing HA-tagged *YCF1* were cultured in SC selection media with and without supplementation of $CdCl_2$ for 9 h. Cells were broken by vortexing with glass beads (1 min, 8 times). After removing unbroken cells and glass beads by centrifugation at 300 *g* for 3 min, the supernatant was incubated with a buffer containing 1% Triton X-100 on ice for 30 min and then cleared by centrifugation at 21,000 *g* for 20 min. Cell lysates were denatured in 1 × SDS sample buffer containing 0.1 *M* DTT for 15 min at 37°C. Samples were subjected to western blotting using anti-HA antibodies (Rockland, 600-401-384).

Determination of phosphorylation of Ycf1p

Phosphorylation-dependent slower migration of Ycf1p on SDS-polyacrylamide gel was determined as previously described (3). For dephosphorylation, samples (20 μg of protein prepared without phosphatase inhibitors) were incubated with 2 units of calf intestinal alkaline phosphatase (Roche) for 60 min at 37°C. Control samples were treated in the same manner with the addition of heat-inactivated phosphatase.

Cysteine sulfenic acid formation in Ycf1p

To detect cysteine sulfenic acid formation in Ycf1p, cell lysates were prepared as previously described (6) using the same buffer described in an earlier section (*Immnunoprecipitation)* but supplemented with 0.1 mM biotin-tagged dimedone (DCP-Bio1) (KeraFAST), 200 U/ml catalase, 5 mM iodoacetic acid, and 100 μM diethylenetriaminepentaacetic acid. Streptavidin beads (New England Biolabs; S1420S) were added to lysates overnight and washed (thrice) with 15 mM ice-cold sodium phosphate buffer. Bound proteins were eluted using PBS containing 0.1% SDS at 95°C for 10 min. Samples were subjected to western blotting analysis using anti-HA antibodies (Rockland, 600-401-384).

Detection of glutathionylated Ycf1p

Immunoprecipitated HA-epitope-tagged Ycf1p and the sucrose gradient fraction that were enriched with Ycf1p were denatured with SDS sample buffer without reducing reagent and then resolved in a 4%–20% Tris-glycine gel (BioRad). Glutathionylated proteins were detected by western blotting using anti-glutathione monoclonal antibodies (Virogen, 101-A-DyLite488). The specificity of this monoclonal antibody to GSH that covalently bound to proteins was determined by western blotting of the same samples by reducing SDS-PAGE (100 mM DTT). The blots were then stripped and re-probed with anti-HA antibodies (Rockland, 600-401-384).

Immunoprecipitation

Cells expressing either Ycf1-HA or Ycf1(C436A)-HA were grown with and without $CdCl_2$ (15 μM) for 9 h. Protein was extracted using a deoxygenized HEPES buffer (50 mM, pH 7.4) containing 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanidate, protease inhibitors (Complete mini, Roche), 1 mM PMSF, and 1% Triton X-100. Iodoacetic acid (5 mM) was added in the buffer to alkylate–SH group as indicated in each experiment. Immunoprecipitation of HA-tagged Ycf1p was conducted using a kit (Thermo Scientific) by following the manufacturer's instructions.

GSH and GSSG measurement

A GSH/GSSG assay kit (Cayman Chemical Co) measured total glutathione and oxidized glutathione (GSSG). Total GSH and GSSG concentrations were normalized to protein

concentration and expressed as nmoles of GSH and GSSG per mg of protein.

References

1. Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, and Gitlin JD. The copper chaperone for superoxide dismutase. *J Biol Chem* 272: 23469–23472, 1997.
2. de Silva D, Davis-Kaplan S, Fergestad J, and Kaplan J. Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J Biol Chem* 272: 14208–14213, 1997.
3. Eraso P, Martínez-Burgos M, Falcón-Pérez JM, Portillo F, and Mazón MJ. Ycf1-dependent cadmium detoxification by yeast requires phosphorylation of residues Ser908 and Thr911. *FEBS Lett* 577: 322–326, 2004.
4. Gietz RD, Schiestl RH, Willems AR, and Woods RA. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11: 355–360, 1995.
5. Ho SN, Hunt HD, Horton RM, Pullen JK, and Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51–59, 1989.
6. Kaplan N, Urao N, Furuta E, Kim SJ, Razvi M, Nakamura Y, McKinney RD, Poole LB, Fukai T, and Ushio-Fukai M. Localized cysteine sulfenic acid formation by vascular endothelial growth factor: role in endothelial cell migration and angiogenesis. *Free Radic Res* 45: 1124–1135, 2011.
7. Mumberg D, Muller R, and Funk M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156: 119–122, 1995.
8. Oyanagui Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal Biochem* 142: 290–296, 1984.

Supplementary Table S1. Steady-State Metal Levels in Wild-Type Control and *atx1*Δ Cells With and Without Expression of *CaCRP1* Copper Efflux Transporter

	Cu		*Zn*		*Mg*	
	WT	*atx1*Δ	*WT*	*atx1*Δ	*WT*	*atx1*Δ
Control	45.87 ± 1.26	51.89 ± 6.94$^{\#}$	848.86 ± 48.57	1191.32 ± 235.05$^{\#\#}$	421.59 ± 24.27	391.62 ± 60.10
CaCRP1	31.29 ± 1.72*	40.26 ± 2.48*$^{\#\#}$	837.02 ± 68.31	1039.37 ± 123.09$^{\#}$	385.24 ± 14.26	396.66 ± 12.69

Cells transformed with an empty vector (Control) and CaCRP1 expression construct were cultured in SC media at the mid-log phase (OD_{600} = 0.8-1). Cell-associated metal levels were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Each datum represents the average ± SD of at least six samples. The results were normalized to cell numbers and presented as nanogram metal per OD_{600} = 1 cells. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with empty vector expressing control cells by Student's t-test. $^{\#}$ and $^{\#\#}$ indicate $p < 0.05$ and $p < 0.01$, respectively, compared with wild-type (WT) cells by Student's *t*-test.

SUPPLEMENTARY TABLE S2. STEADY-STATE METAL LEVELS IN WILD-TYPE CONTROL AND *atx1*Δ CELLS WITH AND WITHOUT EXPRESSION OF *PCA1* CADMIUM EFFLUX TRANSPORTER

	Cd		*Zn*		*Cu*	
	WT	*atx1*Δ	*WT*	*atx1*Δ	*WT*	*atx1*Δ
Control	84.05 ± 25.52	276.88 ± 86.26$^{\#\#}$	424.13 ± 73.84	471.23 ± 108.53	75.52 ± 10.04	105.08 ± 20.39$^{\#}$
PCA1	20.33 ± 2.95**	73.87 ± 25.17**$^{\#\#}$	414.54 ± 27.84	444.64 ± 93.87	70.54 ± 15.24	95.04 ± 23.81$^{\#}$

Cells transformed with an empty vector (Control) and *PCA1* expression construct were cultured in SC media at the mid-log phase ($OD_{600} = 0.8–1$) and then co-cultured with cadmium (5 μM $CdCl_2$) for 1 h. Cell-associated metal levels were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Each datum represents the average ± SD of at least twelve samples. The results were normalized to cell numbers and presented as nanogram metal per $OD_{600} = 1$ cells. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with empty-vector expressing control cells by Student's *t*-test. $^{\#}$ and $^{\#\#}$ indicate $p < 0.05$ and $p < 0.01$, respectively, compared with WT cells by Student's *t*-test.

SUPPLEMENTARY TABLE S3. PLASMIDS USED IN THIS STUDY

Plasmid	*Description*	*Source*
p415GPD	Empty vector	(5)
p416GPD	Empty vector	(5)
pDA089	P416GPD CaCRP1	(2)
pDA245	p415GPD Pca1(393-1216)	(1)
pWW197	p415GPD Ycf1-GFP	This study
pWW198	p415GPD Ycf1-HA	This study
pWW209	p416GPD Ycf1-HA	This study
pRS315	Empty vector	(6)
pLJ256	pRS315 wSOD1	(3)
pLS108	pRS315 SOD1	(3)
pFA6a-His3MX6	Gene knockout cassette	(4)
pFA6a-HphMX4	Gene knockout cassette	(4)
pWW206	p415GPD Ycf1-HA C10A, C13A	This study
pWW233	p415GPD Ycf1-HA C31A	This study
pWW234	p415GPD Ycf1-HA C57A	This study
pWW235	p415GPD Ycf1-HA C180A	This study
pWW236	p415GPD Ycf1-HA C280A	This study
pWW237	p415GPD Ycf1-HA C436A	This study
pWW238	p415GPD Ycf1-HA C527A	This study
pWW239	p415GPD Ycf1-HA C545A C546A	This study
pWW240	p415GPD Ycf1-HA C946A	This study
pWW241	p415GPD Ycf1-HA C952A	This study
pWW242	p415GPD Ycf1-HA C1017A	This study
pWW243	p415GPD Ycf1-HA C1086A	This study
pWW244	p415GPD Ycf1-HA C1157A	This study
pWW245	p415GPD Ycf1-HA C1506A	This study
pWW246	p415GPD Ycf1-HA C729A	This study
pWW247	p415GPD Ycf1-HA C436A, C1086A	This study
pWW248	P415GPD Ycf1- GFP C436A	This study
pWW249	P415GPD Ycf1- GFP C1086A	This study
pWW250	P415GPD Ycf1-GFP C436A C1086A	This study
pWW251	P415GPD Ycf1-HA C436S	This study
pWW252	P415GPD Ycf1-HA C1086S	This study
pWW253	P415GPD Ycf1-HA C436S C1086S	This study
pWW266	p415GPD Ycf1-HA C660A	This study
pWW267	p415GPD Ycf1-HA C675A	This study
pWW268	p415GPD Ycf1-HA C729T	This study
pWW270	p415GPD Ycf1-HA C280A C436S	This study
pWW271	p415GPD Ycf1-HA C280A C436S C952A C1086S C1157A	This study
pWW492	p415GPD Ycf1-GFP C280A C436S C952A C1086S C1157A	This study
pWW272	p415GPD Ycf1-HA C57A C280A C436S C952A C1086S C1157A	This study
pWW273	p415GPD Ycf1-HA C57A C180A C280A C436S C952A C1086S C1157A	This study
pWW274	p415GPD Ycf1-HA C729A	This study
pWW275	p415GPD Ycf1-HA C660A C675A C729A	This study
pWW280	p415GPD Ycf1-HA C57A C180A C280A C436S C952A C1086S C1157A C1419A	This study
pWW281	p415GPD Ycf1-HA C1A C57A C180A C280A C436S C952A C1086S C1157A C1419A	This study
pWW282	p415GPD Ycf1-HA C280A C436S C660A C675A 729A C952A C1086S C1157A	This study
pWW283	p415GPD Ycf1-HA C57A C280A C436S C660A C675A 729A C952A C1086S C1157A	This study
pWW493	p416GPD Zrc1	This study
pWW494	p416GPD Zrc1-GFP	This study
pNS051	p415GPD Δ272 Ycf1 N-HA	This study

1. Adle DJ, Sinani D, Kim H, and Lee J. A cadmium-transporting P1B-type ATPase in yeast *Saccharomyces cerevisiae*. *J Biol Chem* 282: 947–955, 2007.

2. Adle DJ and Lee J. Expressional control of a cadmium-transporting P1B-type ATPase by a metal sensing degradation signal. *J Biol Chem* 283: 31460–31468, 2008.

3. Jensen LT and Culotta VC. Activation of Cu, Zn superoxide dismutases from *Caenorhabditis elegans* does not require the copper chaperone CCS. *J Biol Chem* 280: 41373–41379, 2005.

4. Longtine MS, McKenzie A III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, and Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14: 953–961, 1998.

5. Mumberg D, Muller R, and Funk M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156: 119–122, 1995.

6. Sikorski RS and Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19–27, 1989.

Supplementary Table S4. Primers Used in This Study

Name	*Sequence*	*Purpose*
oDA086	GCTCTAGAATGGCTGGTAATCTTGTTTCATGG	Ycf1 cloning (XbaI, Pst1)
oDA087	TAATTTCTGCAGTTGTTCCAGC	
oDA088	GGAACAACTGCAGAAATTAAATG	Ycf1 cloning (Pst1, XhoI)
oDA089	CCGCTCGAGTTAATTTTCATTGACCAAACC	
oDA152	CCGCTCGAGTTAGCGGCCGCCATTTTCATTGACCAAACCAGCC	Ycf1 3′ containing NotI
oWW074	CGGCTCGAGTTAGCGGCCGCCCGCATAGTCAGGAACATCGTA TGGGTAATTTTCATTGACCAAACCAGC	Ycf1 3′ HA-epitope tagging
oWW112	GACCAGTGCTGAACCAATACC	Ycf1 internal primer
oDA090	AATAAATTAGGGGTATCGTACTACCGTAAAGAACAAGAAAC GGATCCCGGGTTAATTAA	Ycf1 knockout using His3 cassete
oDA091	TACCAGATTGTGCGGGACAGGTTTTTATTAGTTTCACAGTGAA TTCGAGCTCGTTTAAAC	
oDA107	GATCATCTGCTATGTGGAAGC	Confirm Ycf1 knockout
oDA108	GCTATGCAGCCAGTATCTCG	
oWW081	CTAGTCTAGAATGGCTGGTAATCTTGTTTCATGGGCCG CCAAGCTCGCTAGATCTCCTGAAGGGTTTG	Ycf1 C10A and C13A
oWW100	GGAAATTTGACCgcTATTGTTGGTAAAG	Ycf1 C660A
oWW101	CTTTACCAACAATAgcGGTCAAATTTCC	
oWW102	CAGCTCTATTGTCAgcCATGTTAGGTG	Ycf1 C675A
oWW103	CACCTAACATGgcTGACAATAGAGCTG	
oWW104	CGAAAAAACGATCAAGGCCgcTGCGTTAACTATTGATCTTG	Ycf1 C729A
oWW105	CAAGATCAATAGTTAACGCAgcGGCCTTGATCGTTTTTTCG	
oWW106	CAAAGACAATTATTAgcTCTTGCAAGAGC	Ycf1 C1419A
oWW107	GCTCTTGCAAGAgcTAATAATTGTCTTTG	
oWW124	CTTTTACGGTGACTTTACTCAAgcCTTCATCGACGGTGTGATCC	Ycf1 C31A
oWW125	GGATCACACCGTCGATGAAGgcTTGAGTAAAGTCACCGTAAAAG	
oWW126	CAGAGATTTAGTTAACCTTgcCAAGAAAAAACACTCTGGCATC	Ycf1 C57A
oWW127	GATGCCAGAGTGTTTTTTCTTGgcAAGGTTAACTAAATCTCTG	
oWW128	CGTTATTCCAAGTAATAACAgcTGCCAGTATCCTGTTACTTG	Ycf1 C180A
oWW129	CAAGTAACAGGATACTGGCAgcTGTTATTACTTGGAATAACG	
oWW130	CTTCATTATCATGGGCTATAgcCAGAACTTTTGGATCTAAAATG	Ycf1 C280A
oWW131	CATTTTAGATCCAAAAGTTCTGgcTATAGCCCATGATAATGAAG	
oWW132	GGCCTTTTCAAATCATTATTgcCTTATATTCTCTGTATAAG	Ycf1 C436A
oWW133	CTTATACAGAGAATATAAGgcAATAATGATTTGAAAAGGCC	
oWW134	AATCTTACAAAACTAGGAgcTTATATGGCTGTGACAAG	Ycf1 C527A
oWW135	CTTGTCACAGCCATATAAgcTCCTAGTTTTGTAAGATT	
oWW136	AATATAGTACCATTCCTTGTTTCAgcTGCTACCTTTGCTGTATTT GTTTATAC	Ycf1 C545A C546A
oWW137	GTATAAACAAATACAGCAAAGGTAgcAgcTGAAACAAGGAATG GTACTATATT	
oWW138	CCTAGAGTACGCTAAAGCTgcCAACCCGAAAAGCGTTTGTG	Ycf1 C946A
oWW139	CACAAACGCTTTTCGGGTTGgcAGCTTTAGCGTACTCTAGG	
oWW140	CTTGCAACCCGAAAAGCGTTgcTGTATTCATATTGTTTATTG	Ycf1 C952A
oWW141	CAATAAACAATATGAATACAgcAACGCTTTTCGGGTTGCAA	
oWW142	CAATCGTTCTCTGGGTTTTTgcTACCATTCATGCCTCCAAATA	Ycf1 C1017A
oWW143	TATTTGGAGGCATGAATGGTAgcAAAAACCCAGAGAACGATTG	
oWW144	CATTCACTATTACGGTTATCgcTGCGACGACATGGCAATTTATC	Ycf1 C1086A
oWW145	GATAAATTGCCATGTCGTCGCAgcGATAACCGTAATAGTGAATG	
oWW146	GGTTTTCCCACATTAATCAAgcCCGCATTGATAATAACATGAG	Ycf1 C1157A
oWW147	CTCATGTTATTATCAATGCGGgcTTGATTAATGTGGGAAAACC	
oWW148	CAAATCATTGTTCTATTCACTGgcCATGGAGGCTGGTTTGGTCAATG	Ycf1 C1506A
oWW149	CATTGACCAAACCAGCCTCCATGgcCAGTGAATAGAACAATGATTTA	
oWW166	CAGGGCCTTTTCAAATCATTATTtcCTTATATTCTCTGTATAAGTTA	Ycf1 C436S
oWW167	CAACTTATACAGAGAATATAAGgaAATAATGATTTGAAAAGGCCCTG	
oWW200	CTTTTACGGTGACTTTACTCAAtcCTTCATCGACGGTGTGATCC	Ycf1 C31S
oWW201	GGATCACACCGTCGATGAAGgaTTGAGTAAAGTCACCGTAAAAG	
oWW246	GAGCTGACACTTATTTACTTAATGATCCTTTGGCAGCTGTTG	Ycf1 D777N
oWW247	CAACAGCTGCCAAAGGATCATTAAGTAAATAAGTGTCAGCTC	
oWW269	GCGAATTCATGATCACCGGTAAAGAATTGAGAATCAT	Zrc1 cloning
oWW270	CGCTCGAGTTAGCGGCCGCCCAGGCAATTGGAAGTATTGCAGTTTAC	
oNS165	TGCTCTAGAATGTACCCATACGATGTTCCTGACTAT GCGccttcattatcatgggc	Ycf1(Δ272) N-terminal HA epitope tagging
oDA087	TAATTTCTGCAGTTGTTCCAGC	Ycf1 internal primer