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Loss of the mitochondrial lipid cardiolipin leads to decreased glutathione synthesis

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Abstract

Previous studies demonstrated that loss of CL in the yeast mutant *crd1* leads to perturbation of mitochondrial iron-sulfur (Fe-S) cluster biogenesis, resulting in decreased activity of mitochondrial and cytosolic Fe-S-requiring enzymes, including aconitase and sulfite reductase. In the current study, we show that *crd1* cells exhibit decreased levels of glutamate and cysteine and are deficient in the essential antioxidant, glutathione, a tripeptide of glutamate, cysteine, and glycine. Glutathione is the most abundant non-protein thiol essential for maintaining intracellular redox potential in almost all eukaryotes, including yeast. Consistent with glutathione deficiency, the growth defect of *crd1* cells at elevated temperature was rescued by supplementation of glutathione or glutamate and cysteine. Sensitivity to the oxidants iron (FeSO₄) and hydrogen peroxide (H₂O₂), was rescued by supplementation of glutathione. The decreased intracellular glutathione concentration in *crd1* was restored by supplementation of glutamate and cysteine, but not by overexpressing *YAP1*, an activator of expression of glutathione biosynthetic enzymes. These findings show for the first time that CL plays a critical role in regulating intracellular glutathione metabolism.

Keywords

Cardiolipin; Fe-S cluster; Glutathione; Mitochondria; Reactive oxygen species (ROS); Barth syndrome

1. Introduction

Cardiolipin (CL) is a unique dimeric phospholipid that is almost exclusively present in mitochondrial membranes and plays a key role in mitochondrial function. Perturbation of CL metabolism in humans leads to Barth syndrome (BTHS), a severe genetic disorder that results in cardio- and skeletal myopathy, neutropenia, and numerous metabolic abnormalities

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Declaration of competing interest

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[3]. Although the defect in BTHS is known to result from mutations in the CL remodeling enzyme tafazzin [4], the link between CL deficiency and the pathology in BTHS is not understood. Recent findings indicate that metabolic dysregulation is an important mechanism underlying the pathology in BTHS [5,6]. Studies using yeast CL mutants have provided insight into the role of CL in mitochondrial function and energy metabolism. In addition to the now well-established role of CL in oxidative phosphorylation [7–9], more recent studies using the yeast model have determined that CL is required for acetyl-CoA synthesis [10] and for the biogenesis of Fe–S clusters [11], which are incorporated as co-factors into iron-dependent enzymes [14]. These include enzymes required for mitochondrial bioenergetics, the TCA cycle, and amino acid biosynthesis. Among the latter are the enzymes aconitase (Aco1), glutamate synthase (Glt1), sulfite reductase (two subunits each of Met5 and Met10), dihydroxyacid dehydratase (Ilv3), isopropylmalate isomerase (Leu1), and homoaconitase (Lys4) [15]. Therefore, decreased Fe–S biogenesis in the mitochondria is expected to disrupt amino acid synthesis, leading to severe metabolic perturbation.

The yeast CL synthase mutant *crd1*, which cannot synthesize CL, exhibits decreased activity of mitochondrial and cytosolic Fe–S enzymes, including aconitase and sulfite reductase, among others [11]. Aconitase catalyzes the conversion of citrate to isocitrate, which is subsequently decarboxylated to α -ketoglutarate by isocitrate dehydrogenase [16,17]. In addition to its role in the TCA cycle, α -ketoglutarate is a substrate for the synthesis of glutamate [19]. In the sulfur biosynthetic pathway, sulfate taken up from the surroundings is converted to sulfite and subsequently reduced by sulfite reductase to sulfide, which combines with *O*-acetylhomoserine to form homocysteine [20]. Homocysteine can be interconverted to methionine or to cystathionine, which is used to generate cysteine [20–22]. Deficiencies in Fe–S enzymes aconitase and sulfite reductase lead to auxotrophy for glutamate and cysteine/methionine, respectively [23,24].

Depletion of glutamate and cysteine is expected to decrease the synthesis of glutathione, a tripeptide of cysteine, glutamate, and glycine [25–27]. The synthesis of glutathione is initiated by the conversion of glutamate and cysteine to γ -glutamylcysteine, catalyzed by Gsh1 (glutamate-cysteine ligase). Gsh2 (glutathione synthase) then catalyzes the ligation of glycine to the dipeptide [28,29]. Deletion of *GSH1* leads to glutathione auxotrophy, while cells lacking *GSH2* are able to grow in minimal medium lacking glutathione [30,31]. Expression of *GSH1* and *GSH2* in response to oxidative stress is under the regulatory control of the transcription factor Yap1 [32–35]. Activation of Yap1 by oxidative stress and temperature shock, or by overexpression of the *YAP1* gene, augments the expression of *GSH1* and *GSH2* and elevates GSH levels [33–35].

Glutathione is the most abundant intracellular antioxidant that plays a critical role in maintaining redox potential and protects the intracellular environment against reactive oxygen species (ROS), xenobiotics, heavy metals, and temperature-mediated stress [36–39]. Glutathione acts as a co-factor for antioxidant enzymes such as glutathione peroxidase, which utilize glutathione to neutralize ROS, including hydrogen peroxide (H₂O₂) [31]. The antioxidant function of glutathione depends on the active thiol group (-SH) [31]. Glutathione donates electrons necessary to reduce the ROS and, in turn, is oxidized to glutathione disulfide (GSSG). Depletion of glutathione leads to increased sensitivity to oxidants such as

H₂O₂, while disruption of GSH synthesis by deletion of *GSH1* leads to growth arrest in synthetic medium lacking GSH, cysteine, or reducing agent, dithiothreitol (DTT) [28–31]. Therefore, to maintain vital cellular functions, intracellular glutathione levels must be rigorously maintained, either by *de novo* synthesis or uptake from the growth medium.

In this study, we show that CL-deficient *crd1* cells exhibit decreased levels of glutamate and cysteine and decreased synthesis of glutathione. Glutathione levels, as well as defective growth in response to elevated temperature, are rescued by supplementation of glutamate and cysteine. Sensitivity to the oxidants iron (FeSO₄) and hydrogen peroxide (H₂O₂), was rescued by supplementation of glutathione. These findings suggest that decreased activities of Fe-S-requiring enzymes in CL-deficient cells result in defects in the glutamate and cysteine biosynthetic pathways, leading to deficiencies in these amino acids and decreased synthesis of glutathione. This is the first demonstration of a link between the mitochondrial lipid CL and synthesis of the antioxidant glutathione.

2. Materials and methods

2.1. Yeast strains and growth media

The yeast strains used in this study are listed in Table 1. Synthetic defined (SD) medium contained adenine (20.25 mg/L), arginine (20 mg/L), histidine (20 mg/L), leucine (60 mg/L), lysine (20 mg/L), methionine (20 mg/L), threonine (300 mg/L), tryptophan (20 mg/L), uracil (20 mg/L), yeast nitrogen base without amino acids (Difco, Detroit, MI), and either (fermentative) glucose (2%) or (respiratory-fermentative) galactose (2%) as a carbon source. SD dropout medium contained all of the above-mentioned ingredients except for the indicated amino acid. For growth experiments on excess iron, 1 μ M CuSO₄ was used, and FeSO₄ was solubilized in 0.1 N HCl, filter-sterilized, and added to the culture medium at the indicated concentration. GSH was prepared in distilled water, filter-sterilized, and added to the culture medium at the indicated concentration.

2.2. Biochemical assays

Cell extracts were prepared by resuspending cells in 500 μ L of TNTEG buffer (10 mM Tris-Cl pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 10% (vol/vol) glycerol, 0.5% (vol/vol) Triton X-100) and subjecting them to mechanical breakage with glass beads. Cell debris and unbroken cells were separated by low speed centrifugation (2000g for 5 min at 4 °C). The obtained supernatant was further centrifuged at 13,000 g for 10 min and the resulting supernatant was transferred to a new tube. Total protein concentration was determined with a Bradford assay kit (BioRad) using BSA as the standard. Enzyme assays were performed in whole-cell extracts as follows: Aconitase was assayed by the aconitase-isocitrate dehydrogenase coupled assay in which NADPH formation was monitored at A₃₄₀ [40]. Isopropylmalate isomerase was assayed by monitoring formation of the UV-absorbing double bond of isopropylmalate at A₂₃₅ due to dehydration of 3-isopropylmalate [40]. For the isopropylmalate isomerase assay, because the parental strains carry the *leu2* null mutation, they were transformed with a low/single copy pRS415 plasmid that expresses *LEU2*. Statistical significance for both assays was determined by two-tailed Student's *t*-test.

2.3. Amino acid analysis

Amino acid analyses were performed at the Protein Chemistry Laboratory, Texas A&M University, College Station, Texas. Total cell extracts of WT and *crd1* cells, standards, and control were passed through a 5000 MWCU filter (Vivaspin 500) to remove cellular proteins and large peptides. The filtrates (in duplicates) were combined with internal standards [normaline (Sigma #N7502) and sarcosine (Sigma #S7672)] and dithiodipropionic acid (100 mM) to convert cysteine and cystine to Cys MPA (S-(-2-carboxyethylthio-Cys)). This mixture was heated to 150 °C for 45 min with vapor-phase 6 N HCl. Cys-MPA in all standards, controls, and samples was quantified using an Agilent 1260 HPLC analyzer. Alanine levels in the standards, control, and samples were quantified/normalized to the cysteine levels.

Agilent 1260 HPLC analysis was performed by pre-column derivatization of the amino acids with o-phthalaldehyde (OPA, Agilent #5061–3335) and 9-fluoromethyl-chloroformate (FMOC, Agilent #5061–3337) after addition of 0.4 N borate buffer (Agilent #5061–3339) to bring to pH 10 for optimum derivatization. OPA reacts with primary amino acids and FMOC with secondary amino acids (proline). Both reagents react rapidly and quantitatively and give highly UV-absorbing isoindole derivatives. The derivatized amino acids were separated by reverse phase HPLC and detected by UV absorbance (primary at 338/390 nm and secondary at 266/324 nm) with a variable wavelength detector (G1365D). The assay was calibrated by a standard (Agilent #5061–3331), which undergoes the same treatment as the samples and control.

2.4. Glutathione levels

Total cell extracts from WT and *crd1* cells grown at 35 °C to log phase were deproteinized with 5% 5-sulfosalicylic acid solution, centrifuged to remove precipitated proteins, and then assayed for total glutathione (GSH + GSSG). Total glutathione concentrations were measured using a kinetic assay (Sigma-Aldrich #CS0260) in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to TNB, and the GSSG formed is recycled by glutathione reductase and NADPH. Statistical significance was determined by two-tailed paired Student's *t*-test.

3. Results

3.1. Decreased glutamate and cysteine in *crd1* cells

To test the prediction that deficiencies in mitochondrial and cytosolic Fe–S enzymes in *crd1* cells lead to glutamate and cysteine auxotrophies that contribute to growth defects, the effect of supplementation of these amino acids on growth of the CL mutant at elevated temperature was determined. Supplementation with cysteine (0.03%) and glutamate (0.01%) together restored growth of the mutant at elevated temperature to wild type levels (Fig. 1A). Cysteine alone partially rescued growth of *crd1* (Fig. 1C), but glutamate alone did not (Fig. 1B). This is consistent with previous reports suggesting that cysteine is the limiting substrate for intracellular glutathione synthesis [25,41]. The growth phenotype of *crd1* is similar to that of *gsh1*, which is unable to grow in minimal medium in the absence of GSH, cysteine, or DTT. Free amino acid levels were assayed to determine if the growth defect of *crd1* at

elevated temperature reflected deficiencies in intracellular glutamate and cysteine. HPLC analysis indicated that the intracellular levels of glutamate and cysteine are reduced in *crd1* cells by 35.8% and 21.21%, respectively (Table 2). In addition, CL-deficient cells showed decreased levels of leucine, aspartate, glutamine, and proline, and elevated levels of serine, histidine, threonine, alanine, valine, methionine, isoleucine, and lysine. While the cellular consequences that result in elevated levels of multiple amino acids in the CL mutant are not clear, decreased levels of aspartate, glutamine, and proline are likely due to defective Fe-S biogenesis. Our previous study has shown that the CL mutant exhibits decreased activity of Aco1 [11], which catalyzes the conversion of citrate to isocitrate, a critical intermediate of the TCA cycle. Isocitrate is converted to α -ketoglutarate, a key intermediate for glutamate biosynthesis, which can be subsequently interconverted to glutamine and proline. Oxaloacetate, another intermediate of the TCA cycle, is essential for aspartate biosynthesis. As the parental strains carry the *leu2* null mutation, growth medium was supplemented with leucine (60 mg/L). Decreased leucine levels in *crd1* suggest increased catabolism or decreased uptake, however the underlying cause for this is unclear.

3.2. Decreased glutathione in *crd1* cells

Because glutamate and cysteine are essential precursors for the synthesis of glutathione, we predicted that deficiencies in these amino acids would lead to decreased glutathione. In agreement with this, total cellular glutathione levels [GSH (reduced) + GSSG (oxidized)] were decreased by $35.74 \pm 7.57\%$ in *crd1* cells compared to WT (Fig. 2A). Supplementation of both glutamate and cysteine increased intracellular glutathione to near-wild type levels. Growth of *crd1* cells at elevated temperature was restored by glutathione supplementation (Fig. 2B). The glutathione mutant, *gsh1*, does not exhibit a growth deficiency in complex fermentable medium (*e.g.* YPD); however, the mutant exhibits growth arrest in synthetic medium lacking glutathione, cysteine, or DTT [30,32,42]. This suggests that an intracellular reducing environment is essential for the growth of *gsh1* in synthetic fermentable medium. Based on this, we examined how deletion of *GSH1* may affect the growth of *crd1*. We noted that perturbation of GSH synthesis further exacerbated the growth of *crd1* cells, such that *crd1* exhibited lethality with *gsh1* in synthetic fermentable medium (Fig. 2C).

If glutathione deficiency in *crd1* is due to decreased glutathione biosynthetic enzymes, the deficiency might be rescued by overexpression of *YAP1*, which encodes the transcription factor that regulates the expression of *GSH1* and *GSH2* [28,29,33]. *GSH1* encodes glutamate-cysteine ligase, a rate-limiting enzyme that catalyzes the formation of γ -glutamylcysteine from glutamic acid and cysteine; *GSH2* encodes glutathione synthase, which catalyzes the ligation of glycine with the dipeptide, producing glutathione. Data in Fig. 2D show that overexpression of *YAP1* did not rescue the growth of *crd1* in galactose medium (respiratory-fermentative) at elevated temperature, suggesting that the decrease in glutathione levels in *crd1* is most likely not due to deficiency of glutathione biosynthetic enzymes.

3.3. Supplementation with glutathione rescued the growth defect of CL-mutant cells in the presence of oxidants

The *gsh1* mutant exhibits hypersensitivity to oxidants, including H₂O₂ [30,43]. This phenotype is also characteristic of CL-deficient cells, which exhibit increased oxidative stress in respiratory (ethanol) and respiratory-fermentative (galactose) growth conditions, and are sensitive to ethanol, iron, and H₂O₂ [11,44]. To address the possibility that glutathione deficiency in *crd1* cells may account for sensitivity to oxidants, the effect of glutathione supplementation on growth of *crd1* cells in the presence of FeSO₄ or H₂O₂ was determined. Glutathione supplementation significantly improved growth of *crd1* cells in the presence of iron and H₂O₂ (Fig. 3).

3.4. Decreased activities of mitochondrial and cytosolic Fe–S enzymes in the *crd1* mutant are not due to decreased glutathione levels

The data discussed above suggest that decreased activities of Fe–S enzymes in the CL mutant lead to glutamate and cysteine deficiencies and, as a result, decreased synthesis of glutathione. However, an alternative explanation for decreased glutathione synthesis is that increased levels of oxidants in the CL mutant damage Fe–S enzymes required for synthesis of glutamate and cysteine [45,46]. To distinguish between these possibilities, activities of aconitase (Aco1) and isopropylmalate isomerase (Leu1) were assayed in WT and *crd1* cells grown in the presence or absence of 1 mM GSH. As the parental strains carry the *leu2* null mutation, both WT and *crd1* were transformed with a low/single copy pRS415 plasmid containing the *LEU2* gene. As seen in Fig. 4, Aco1 and Leu1 activities were decreased in the *crd1* mutant by $62.2 \pm 8.9\%$ and $49.97 \pm 7.07\%$, respectively, compared to WT, as previously observed [11]. GSH supplementation to *crd1* cells did not restore enzyme activities to WT levels, suggesting that oxidative damage is most likely not the cause of decreased activities of mitochondrial and cytosolic Fe–S enzymes.

Taken together, these data suggest that CL deficiency leads to decreased activities of Fe–S enzymes required for the synthesis of glutamate and cysteine, resulting in decreased intracellular concentrations of these amino acids and decreased synthesis of glutathione. Supplementation with glutamate and cysteine, or with glutathione, rescued the growth defect of *crd1* cells at elevated temperature and sensitivity to the oxidants was rescued by supplementation of glutathione.

4. Discussion

In this study, we show for the first time that the mitochondrial lipid CL is essential for the synthesis of glutathione. We previously determined that CL deficiency results in decreased activity of mitochondrial and cytosolic enzymes that require Fe–S cofactors [11]. These include Fe-S-requiring enzymes that play a role in glutamate and cysteine biosynthesis. In the current report, we show that the CL mutant *crd1* exhibits deficiencies in glutamate and cysteine and decreased levels of glutathione. Mutant phenotypes of decreased growth at elevated temperature and sensitivity to oxidants are rescued by supplementation with glutamate and cysteine or with glutathione.

To maintain glutathione homeostasis, yeast cells take up glutathione or glutathione precursors from the growth medium or synthesize it through the *de novo* pathway. In synthetic medium, which lacks glutathione, yeast cells depend entirely on *de novo* synthesis from glutamate, cysteine, and glycine. Therefore, the most likely explanation for decreased growth of the *crd1* mutant is that glutathione synthesis is decreased due to a deficiency of glutamate and cysteine (Table 2). Overexpression of *YAP1*, which leads to increased glutathione biosynthetic enzymes, did not restore growth of the CL mutant at elevated temperature (Fig. 2D) or improve resistance of *crd1* to iron and H₂O₂ [11], suggesting that decreased growth of the mutant is not a result of a deficiency in glutathione biosynthetic enzymes. Previous studies suggested that PE and CL share overlapping functions [2,47], and slightly higher PE levels were observed in *crd1* (from 17.71% to 19.58% of total phospholipids) [2]. Therefore, it is possible that PE partially compensates for the CL deficiency of *crd1* in regard to glutathione synthesis.

The mechanism underlying perturbation of Fe–S biogenesis in CL-deficient cells is not understood. The current study suggests that oxidative damage is not the cause of decreased activities of mitochondrial and cytosolic Fe–S enzymes, as supplementing *crd1* cells with GSH did not restore activities of aconitase (Aco1) or isopropylmalate isomerase (Leu1) (Fig. 4A and B). Previous studies have shown that the presence of CL in mitochondrial membranes is critical for efficient mitochondrial protein import, which is defective in the CL mutant [1,48,49]. Therefore, one possible explanation for decreased Fe–S biogenesis in *crd1* is that import of one or more proteins involved in the assimilation of mitochondrial Fe–S clusters is decreased. Collectively, these findings suggest that GSH deficiency in the CL mutant is due to decreased Fe–S biogenesis in the mitochondria and cytosol, resulting in depletion of GSH precursors glutamate and cysteine (Fig. 5).

The finding that CL deficiency deleteriously impacts amino acid and glutathione synthesis has implications for understanding metabolic dysfunction in Barth syndrome, in which CL synthesis is perturbed. Barth syndrome is characterized by wide disparities in clinical presentation, even among patients with identical tafazzin mutations, suggesting that physiological modifiers affect the clinical outcome [50]. Interestingly, abnormalities in the TCA cycle in CL-deficient yeast cells necessitate anaplerotic pathways to replenish TCA cycle metabolites, suggesting that these pathways may be modifiers of the Barth syndrome phenotype [51]. Similarly, amino acid and glutathione metabolism may serve as potential modifiers of the Barth syndrome phenotype and may identify avenues for rescue of metabolic deficiencies.

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Abbreviations:

CL	cardiolipin
BTHS	Barth syndrome
Fe-S	iron-sulfur
WT	wild type
SD	synthetic defined
YPD	yeast extract, peptone, and dextrose
YP	yeast extract and peptone
Aco1	aconitase
Leu1	isopropylmalate isomerase
SR	sulfite reductase
FeSO₄	ferrous sulfate
H₂O₂	hydrogen peroxide
ROS	reactive oxygen species
DTT	dithiothreitol
TCA	tricarboxylic acid cycle
GSH	reduced glutathione
GSSG	glutathione disulfide
qPCR	quantitative real-time PCR

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Fig. 1.

Supplementation of glutamate and cysteine restores the growth of *crd1* at elevated temperature. Cells were precultured in SD galactose overnight, serially diluted, and spotted on SD galactose plates containing the indicated amounts of either glutamate and cysteine together (A), glutamate alone (B), or cysteine alone (C). Plates were incubated at 37 °C for 4 days before images were taken.

Fig. 2.

Cardiolipin-deficient cells exhibit decreased glutathione levels, and growth of *crd1Δ* at elevated temperature is rescued by supplementation with GSH. (A) *crd1Δ* cells show decreased levels of glutathione (GSH + GSSG). Cells were precultured in YPD overnight at 30 °C, washed with distilled water and inoculated in SD galactose medium lacking glutamate and methionine/cysteine at 35 °C, and grown to the logarithmic phase, either in the presence or absence of glutamate and cysteine. Total glutathione levels were measured as described in materials and methods. Error bars represent mean $\pm$ SD ($n = 6$, two-tailed paired Student's t -test, $*p < 0.005$). (B) GSH supplementation rescued growth of *crd1Δ*. Cells were precultured in SD galactose overnight, serially diluted, spotted on SD galactose plates containing the indicated amounts of GSH, and incubated at 37 °C for 4 days. (C) *crd1Δ* exhibits synthetic lethality with *gsh1Δ* in fermentable medium. Cells were precultured in YPD overnight, washed with distilled water, serially diluted, and spotted on YPD (left-

panel) or SD glucose (right-panel), and incubated at 30 °C. (D) Overexpression of *YAP1* does not rescue growth of *crd1* . Cells were precultured in SD galactose overnight, serially diluted, spotted on SD galactose plates lacking leucine, and incubated at 30 °C (left-panel) and 36 °C (right-panel) for 4–5 days.

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Fig. 3. Iron and hydrogen peroxide sensitivity in *crd1* is rescued by GSH. Cells were precultured in YPD overnight, washed with distilled water, serially diluted, and spotted on SD galactose plates containing (A) 0.35 mM H₂O₂ or (B) 7 mM FeSO₄, supplemented with 1 mM GSH as indicated, and incubated at 30 °C for 5 days.

Fig. 4.

Decreased activities of mitochondrial and cytosolic Fe–S enzymes in the *crd1* mutant are not rescued by supplementation with GSH. (A) For the aconitase assay (Aco1), cells were grown in SD galactose medium lacking glutamate and methionine/cysteine. (B) For the isopropylmalate isomerase assay (Leu1), WT and *crd1* were transformed with a low/single copy pRS415 plasmid containing the *LEU2* gene and grown in SD galactose medium lacking glutamate, methionine/cysteine, and leucine. Fe–S enzymes were assayed as described in materials and methods. Error bars represent mean $\pm$ SD ($n = 4$, two-tailed paired Student's t -test, $*p < .005$).

Fig. 5.

Perturbation of GSH metabolism in *crd1*. In the proposed model, loss of CL leads to decreased mitochondrial and cytosolic Fe-S biogenesis resulting in reduced activities of Fe-S enzymes, including aconitase (Aco1) in the mitochondria and sulfite reductase (SR) in the cytosol. Decreased activity of Aco1 leads to reduced levels of α -ketoglutarate, a key substrate for glutamate biosynthesis. Decreased activity of SR leads to a reduction in intracellular cysteine levels. Decreased levels of both glutamate and cysteine result in reduced biosynthesis of glutathione, a tripeptide of glutamate, cysteine, and glycine, which subsequently causes increased intracellular oxidative stress in *crd1*.

Table 1

Yeast strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
FGY3	<i>MATa, ura3-52, lys2-801, ade2-101, trp1- 1, his3- 200, leu2- 1</i>	[1]
FGY2	<i>MATa, ura3-52, lys2-801, ade2-101, trp1- 1, his3- 200, leu2- 1, crd1 ::URA3</i>	[1]
BY4742	<i>MATa, his 3 1, leu 2 0, lys 2 0, ura 3 0</i>	Invitrogen
VGY1	<i>MATa, his 301, leu 200, lys 200, ura 300, crd1 ::URA3</i>	[2]
<i>gsh1</i>	<i>MATa, his 301, leu 200, met 1500, ura 300, gsh1 ::KanMX4</i>	Invitrogen
VGY1 gsh1	<i>MATa, his 301, leu 200, met 1500, ura 300, gsh1 ::KanMX4, crd1 ::URA3</i>	This study
YEp351	High-copy number plasmid, <i>LEU2</i> nutritional marker	[12]
pRS415	Centromere plasmid (low/single copy), <i>LEU2</i> nutritional marker	[13]
YEp351- <i>YAP1</i>	Derivative of YEp351, expresses <i>YAP1</i> from the native promoter	[18]

Table 2

Free amino acid analysis in CL mutant *crd1*. Cells were precultured in YPD overnight at 30 °C, cells were rinsed in water and inoculated in synthetic galactose medium lacking glutamate and methionine/cysteine at 35 °C and grown to the logarithmic phase. Cells were then rinsed 3 times in PBS (pH 7.4) to remove residual medium, and total amino acid levels were determined by HPLC. Data shown are mean $\pm$ S.D. (n = 4).

Amino acid	WT nmol (SD)	<i>crd1</i> nmol (SD)
ASP	2.6 $\pm$ 0.85	1.2 $\pm$ 0
GLU	25.3 $\pm$ 3.39	16.25 $\pm$ 0.07
CYS	1.65 $\pm$ 0.21	1.3 $\pm$ 0
SER	1 $\pm$ 0	1.25 $\pm$ 0.07
GLN	9.55 $\pm$ 0.49	3.8 $\pm$ 0.57
HIS	2.1 $\pm$ 0	2.65 $\pm$ 0.07
GLY	1.65 $\pm$ 0.35	2.45 $\pm$ 0.07
THR	32.05 $\pm$ 0.64	41.5 $\pm$ 1.56
ALA	5.35 $\pm$ 0.07	6.15 $\pm$ 0.07
ARG	6.3 $\pm$ 0.85	6.65 $\pm$ 0.21
TYR	0.65 $\pm$ 0.21	0.65 $\pm$ 0.07
VAL	1.2 $\pm$ 0.14	1.75 $\pm$ 0.07
MET	0.3 $\pm$ 0	0.4 $\pm$ 0
TRP	0.35 $\pm$ 0.07	0.3 $\pm$ 0.14
PHE	0.35 $\pm$ 0.07	0.35 $\pm$ 0.07
ILE	2.85 $\pm$ 0.35	4.7 $\pm$ 0
LEU	1.2 $\pm$ 0.28	0.2 $\pm$ 0
LYS	5.35 $\pm$ 0.21	8.45 $\pm$ 0.49
PRO	1.7 $\pm$ 0.14	1.05 $\pm$ 0.07

SD = standard deviation.