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Loss of Cardiolipin Leads to Perturbation of Mitochondrial and Cellular Iron Homeostasis*[S]

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Background: Cardiolipin (CL) deficiency causes multiple defects affecting mitochondrial bioenergetics.
Results: CL deficiency leads to defective mitochondrial Fe-S biogenesis, causing decreased activity of several mitochondrial and cytosolic Fe-S proteins and perturbation of iron homeostasis.
Conclusion: CL is an important regulator of mitochondrial and cellular iron homeostasis.
Significance: Mitochondrial iron homeostasis may be an important physiological modifier that contributes to the clinical phenotypes observed in Barth syndrome patients.

Cardiolipin (CL) is the signature phospholipid of mitochondrial membranes, where it is synthesized locally and plays a critical role in mitochondrial bioenergetic functions. The importance of CL in human health is underscored by the observation that perturbation of CL biosynthesis causes the severe genetic disorder Barth syndrome. To fully understand the cellular response to the loss of CL, we carried out genome-wide expression profiling of the yeast CL mutant *crd1*Δ. Our results show that the loss of CL in this mutant leads to increased expression of iron uptake genes accompanied by elevated levels of mitochondrial iron and increased sensitivity to iron and hydrogen peroxide. Previous studies have shown that increased mitochondrial iron levels result from perturbations in iron-sulfur (Fe-S) cluster biogenesis. Consistent with an Fe-S defect, deletion of *ISU1*, one of two *ISU* genes that encode the mitochondrial Fe-S scaffolding protein essential for the synthesis of Fe-S clusters, led to synthetic growth defects with the *crd1*Δ mutant. We further show that *crd1*Δ cells have reduced activities of mitochondrial Fe-S enzymes (aconitase, succinate dehydrogenase, and ubiquinol-cytochrome *c* oxidoreductase), as well as cytosolic Fe-S enzymes (sulfite reductase and isopropylmalate isomerase). Increased expression of *ATM1* or *YAP1* did not rescue the Fe-S defects in *crd1*Δ. These findings show for the first time that CL is required for Fe-S biogenesis to maintain mitochondrial and cellular iron homeostasis.

Cardiolipin (CL)[3] is a structurally and functionally unique phospholipid that is almost exclusively present in mitochondrial membranes (1, 2). The presence of CL is critical for maintaining mitochondrial function, structure, and membrane fluidity. Perturbation of CL synthesis alters mitochondrial bioenergetics, resulting in reduced membrane potential, inefficient coupling of respiration, and decreased ATP synthesis (3–6). In the inner membrane, CL is tightly associated with several proteins in respiratory complexes I, III, and IV (7). CL is essential for the stability of respiratory chain supercomplexes that, in yeast, are composed of dimeric ubiquinol-cytochrome *c* oxidoreductase (complex III) and one or two complexes of cytochrome *c* oxidase (complex IV) (8–10). Perturbation of CL synthesis due to mutations in the CL remodeling enzyme tafazzin causes the severe human genetic disorder known as Barth syndrome (BTHS) (11). Tafazzin (Taz1) deficiency in yeast leads to biochemical and bioenergetic defects similar to those seen in BTHS patients (12–15).

Although perturbation of CL synthesis due to loss of tafazzin leads to cardio- and skeletal myopathy, neutropenia, and growth retardation in BTHS (16), the clinical presentation of this disorder is highly variable, ranging from neonatal death to lack of clinical symptoms (16, 17). To gain insight into CL functions that might explain the pathology and variable phenotypes observed in BTHS, we carried out a genome-wide expression analysis in the yeast CL mutant *crd1*Δ, which lacks CL synthase. The most striking alterations in gene expression were observed in iron uptake genes. These genes encode components of the yeast high and low affinity iron uptake systems, collectively referred to as the iron regulon (18). Because the gene expression analyses indicating elevated expression of the iron regulon were carried out in iron-replete conditions, we hypothesized that CL might be required for mitochondrial Fe-S biogenesis and/or export of mitochondrial Fe-S co-factors to the cytosol, two processes known to induce up-regulation of the iron regulon (19, 20).

The assembly of Fe-S clusters from ferrous (Fe^{2+}) and sulfide (S^{2-}) ions does not occur spontaneously in living cells, as

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[S] This article contains supplemental Table 1.
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[3] The abbreviations used are: CL, cardiolipin; BTHS, Barth syndrome; Fe-S, iron-sulfur; SD, synthetic defined; YPD, yeast extract, peptone, and dextrose; Aft1, activator of ferrous transport; qPCR, quantitative real time PCR; ROS, reactive oxygen species.

TABLE 1
Yeast strains and plasmids used in this study

Strain	Genotype	Source or Ref.
FGY3	MATa, *ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1*	52
FGY2	MATa, *ura 3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::URA3*	52
FGY3 *isu1*Δ	MATa, *ura 3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, isu1Δ::KanMX4*	This study
FGY2 *isu1*Δ	MATa, *ura 3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::URA3, isu1Δ::KanMX4*	This study
FGY3 *isu2*Δ	MATa, *ura 3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, isu2Δ::KanMX4*	This study
FGY2 *isu2*Δ	MATa, *ura 3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::URA3, isu2Δ::KanMX4*	This study
pCM182	Low copy number plasmid, *TRP1* nutritional marker	ATCC[regs] no. 87656
pCM182-*ISU1*	Derivative of pCM182, expresses *ISU1* from the Tet-Off promoter	This study
pRS415	Low copy number plasmid, *LEU2* nutritional marker	111
YEp351	High copy number plasmid, *LEU2* nutritional marker	112
YEp351-*YAP1*	Derivative of YEp351, expresses *YAP1* from the native promoter	42

unchaperoned iron and sulfur are toxic. Rather, cells utilize a complex Fe-S assembly and transport process that is highly conserved from yeast to humans (21). The assembly of Fe-S clusters in the mitochondria begins on the highly conserved scaffolding protein Isu1 and its homolog Isu2 (22). The Nfs1-Isd11 complex delivers sulfur (23, 24), and Yfh1 donates iron (Fe^{2+}) to the Isu scaffold (25). This process also includes ferredoxin (Arh1) and ferredoxin reductase (Yah1), which provide electrons for the reduction of sulfur to sulfide (26, 27). The Fe-S clusters assembled on the scaffold are transferred to the recipient apoproteins, in a process that is assisted by several proteins localized in the mitochondrial matrix (21).

Mitochondria also possess Fe-S export machinery, which transports an unknown Fe-S component from the mitochondria that is matured by the cytosolic machinery into 4Fe-4S clusters (28–30). The mitochondrial Fe-S export machinery includes the ABC transporter Atm1 in the inner membrane and the sulfhydryl oxidase Erv1 in the intermembrane space. Perturbations in mitochondrial Fe-S assembly or Fe-S export machinery are known to induce expression of the Aft1/Aft2-regulated iron uptake genes, leading to increased mitochondrial iron levels. Defects in mitochondrial Fe-S assembly lead to decreased maturation of both mitochondrial and cytosolic Fe-S proteins (20, 28, 31–34). In addition, excess mitochondrial iron causes oxidative damage to Fe-S clusters due to the formation of reactive oxygen species (ROS) (35–37).

In this study, we show that *crd1*Δ cells exhibit perturbations in iron homeostasis, including increased expression of the iron uptake genes, elevated mitochondrial iron levels, and growth sensitivity to both $FeSO_4$ supplementation and the ROS-inducing agent H_2O_2. We further demonstrate that the loss of CL leads to decreased activities of both mitochondrial and cytosolic Fe-S enzymes, suggesting that the mechanism underlying altered iron homeostasis is perturbation of Fe-S biogenesis. Consistent with this conclusion, *crd1*Δ cells exhibit a synthetic genetic interaction with the Fe-S scaffolding protein Isu1. Additionally, the iron homeostasis defects in *crd1*Δ are not rescued by overexpression of *ATM1*, the major component of mitochondrial Fe-S export machinery, which is activated by CL (36), nor is it rescued by overexpression of *YAP1*, which regulates expression of antioxidant genes (38–40). Overexpression of *ATM1* and *YAP1* might reasonably be expected to overcome defective Fe-S cluster export from mitochondria. However, overexpression did not rescue the mutant defects, suggesting that the loss of CL affects the process of mitochondrial Fe-S biogenesis. This study is the first to demonstrate that CL is required for Fe-S cluster biogenesis and for the maintenance of mitochondrial and cellular iron homeostasis.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—The yeast *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Synthetic defined (SD) medium contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (20 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), and uracil (20 mg/liter), yeast nitrogen base without amino acids (Difco), and carbon source (fermentative) glucose (2%) or (respiratory) glycerol (3%) plus ethanol (0.65%) or (respiro-fermentative) galactose (2%). SD-drop out medium contained all of the above-mentioned ingredients except for the indicated amino acid. For growth experiments on excess iron, 1 μM $CuSO_4$ was used, and $FeSO_4$ was solubilized in 0.1 N HCl, filter-sterilized, and added to the culture medium at the indicated concentration. Complex media (YPD or YP-gal) contained yeast extract (1%), peptone (2%), and either glucose (2%) or galactose (2%) as indicated.

Deletion mutants were constructed by replacing the entire open reading frame of the target gene with the *KanMX4* cassette by homologous recombination. The *KanMX4* cassette was amplified from the pUG6 plasmid using primers consisting of 51 nucleotides identical to the target gene flanking regions at the 5′ end and 21 nucleotides for the amplification of the *KanMX4* gene at the 3′ end. The PCR product was transformed by electroporation into cells, and transformants were selected on YPD media containing G418 (300 μg/ml). Disruption of the target gene was confirmed by PCR using primers against the target gene coding sequences.

Plasmid Construction and Cloning—To construct the *ISU1*-overexpressing plasmid, a 538-bp sequence containing the entire open reading frame of *ISU1* was amplified from yeast genomic DNA using BamHI-tagged primer *ISU1*-BamHIF (5-GGAAAACACAACGGATCCCACATATTTAACC-3) and PstI-tagged primer *ISU1*-PstIR (5-GATCTTGTTCTGCAGC-CGGTTATCTTCTT-3). Similarly, a 2098-bp sequence containing the entire open reading frame of *ATM1* was amplified using NotI-tagged primer *ATM1*-NotIF (5-TTGATAGAT-GCGGCCGCAACCTGCAAATG-3) and PstI-tagged primer *ATM1*-PstIR (5-TACATGTCTGCAGCAATATTTACTTAC-GAGCG-3). The PCR products were ligated to pCM182 (a low copy number plasmid with selectable marker *TRP1*) downstream of the Tet-Off promoter. All the plasmids were amplified and extracted using standard protocols. The plasmids were

TABLE 2
Primers used for qPCR analyses

Gene	Primer	Sequence
ACT1	Forward	TCGTGCTGTCTTCCCATCTATCG
	Reverse	CGAATTGAGAGTTGCCCCAGAAG
AFT1	Forward	ATGCATCTAAAAGGCCATGC
	Reverse	ACTGGCTTTTCGGTTTCCTT
FIT1	Forward	CCACCTCCTCTGAGTCAAGC
	Reverse	CAGATTGGGCATCCCTAGAA
FIT2	Forward	ACAAAGGTTGTCACCGAAGG
	Reverse	TGAACCTGAACCGTTTGTCA
FIT3	Forward	GCTACATCCTCTAGCACCGC
	Reverse	GCACCCATCAAACCAGTACC
FET3	Forward	ACAGTTTCGATCCGGACAAC
	Reverse	CACCTGGGTTATCGGCTTTA
FTR1	Forward	GATTCAACCTTGCCAGTGGT
	Reverse	ATTGTCCAGTTCTGGGTTGC
ARN1	Forward	CTCGCGATCCTGTTAAGGAG
	Reverse	GGGAGACCATATGAGTCGGA
ARN2	Forward	TGTGGGACTTGTCGGTGTTA
	Reverse	GGGCCATGAAGGTATCAATG
ARN3	Forward	TGGATTAGCGGGAACGTAAC
	Reverse	GGAATACAAGCTAGCGGCAG

transformed into yeast strains using the yeast one-step transformation protocol (41). A high copy number Yep351-*YAP1* overexpression plasmid was a kind gift from W. Scott Moye-Rowley (University of Iowa) (42).

Microarray Analysis—Yeast cells were grown to the early stationary phase in YPD, and total RNA was isolated by hot phenol extraction (43). RNA was further purified using an RNeasy kit from Qiagen. Yeast 6.4k microarray slides containing 6240 different yeast expressed sequence tags (double-spotted) were purchased from University Health Network (Toronto, Canada). Synthesis of Cy3- or Cy5-labeled cRNA and hybridization were performed using SlideHyb 1 buffer (Ambion) at the Research Technology Support Facility at Michigan State University (East Lansing, MI). The glass slides were scanned with an Affymetrix 428 array scanner and quantified using GenePix Pro 3.0 software (Axon). Array normalization and statistical analysis were performed using the "limma: Linear Models for Microarray Data" library module (version 2.2.0) of the R statistical package (version 2.2.0) (44–48). Slide intensity data were normalized using the global loess method. The least squares method was used for the linear model fit utilizing the Benjamini and Hochberg method to control the false discovery rate. Each experiment was repeated once with switched Cy3 and Cy5 labeling. The average of the four signal log ratios of each gene was computed and converted to a fold change. The raw data can be downloaded from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov, GPL3464).

Quantitative PCR (qPCR) Analysis—Yeast cultures (10 ml) were grown to the logarithmic growth phase; cells were harvested, and total RNA was isolated using the RNeasy Plus mini kit from Qiagen. The cDNAs were synthesized with Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics), and quantitative PCRs were performed in a 25-μl volume using Brilliant™ SYBR® Green QPCR master mix (Stratagene) in a 96-well plate. Duplicates were included for each reaction. The primers used for qPCR are listed in Table 2. *ACT1* was used as the internal control, and the RNA level of the gene of interest was normalized to *ACT1* levels. PCRs were initiated at 95 °C for 10 min for denaturation followed by 40 cycles consisting of 30 s at 95 °C and 60 s at 55 °C.

Biochemical Assays and Measurement of Mitochondrial Metal Ion Content—Mitochondria were isolated from cell lysates prepared as described previously (49). Briefly, spheroplasts created by lyticase were ruptured by Dounce homogenization, and mitochondria were isolated by differential centrifugation. Total protein concentration was determined with the Bradford assay kit (Pierce) with BSA as the standard. The following assays were performed in isolated mitochondria. Succinate dehydrogenase activity was assayed by determining succinate-dependent reduction of 2,6-dichlorophenolindophenol. The absorbance decrease at A_{600} was recorded as a reporter of decylubiquinone reduction (50). Ubiquinol-cytochrome *c* oxidoreductase activity was assayed by monitoring reduction of cytochrome *c* at A_{550} (51).

Cell extracts were prepared by resuspending cells in 500 μl of TNTEG buffer (10 mM Tris-Cl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 10% v/v glycerol, 0.5% v/v Triton X-100) and subjecting them to mechanical breakage with glass beads. Cell debris and unbroken cells were separated by low speed centrifugation (2000 × *g* 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 as mentioned above. The following enzyme assays were performed in whole-cell extracts. Aconitase was assayed by the aconitase-isocitrate dehydrogenase-coupled assay, in which NADPH formation was monitored at A_{340} (50). Sulfite reductase was assayed by monitoring methylene blue formation at A_{670} from sulfide produced by NADPH-dependent sulfite reduction (19, 50). Isopropylmalate isomerase was assayed by monitoring formation of isopropylmalate at A_{235} from dehydration of 3-isopropylmalate (50). Statistical significance of all enzyme assay results was determined by an analysis of variance and Bonferroni's post hoc test in KaleidaGraph. In the isopropylmalate isomerase assay, because the parental strains carry the *leu2*Δ null mutation, cells were transformed with a low/single copy pRS415 plasmid containing the *LEU2* marker.

For the measurement of mitochondrial iron content, mitochondria were further purified via ultracentrifugation through a discontinuous Histodenz (Sigma) gradient (14 and 22%). Mitochondria (0.25 mg of mitochondrial protein) were digested in 70% HNO_3 by boiling for 2 min and then diluted to 30% HNO_3. Iron content was determined using an inductively coupled plasma-optical emission spectrometer.

RESULTS

Loss of CL Leads to Increased Expression of Iron Uptake Genes—To understand the cellular response to CL deficiency, we performed a genome-wide microarray analysis in cells of the CL synthase mutant *crd1*Δ, which completely lacks CL (52–54). The microarray analysis revealed increased expression of genes involved in iron homeostasis in the *crd1*Δ mutant (supplemental Table 1). To confirm the effect of loss of CL on expression of the iron regulon genes, we carried out quantitative PCR analysis of the Aft1-regulated iron uptake genes in *crd1*Δ. Cells were grown in SD respiratory media to the logarithmic growth phase, and RNA was extracted for mRNA quantitation, as described under "Experimental Procedures." As seen in Fig. 1, the mRNA

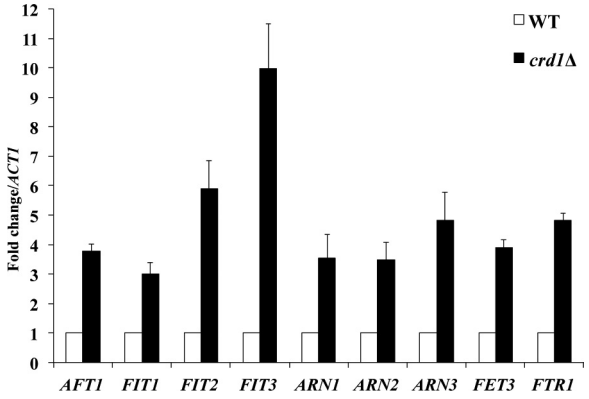

FIGURE 1. **Increased expression of the iron regulon in *crd1*Δ.** The mRNA levels of iron regulon genes were quantified by qPCR from cells grown in SD glycerol-ethanol at 30 °C to the logarithmic phase. Values are reported as fold change in expression over WT. Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.E. ($n = 6$).

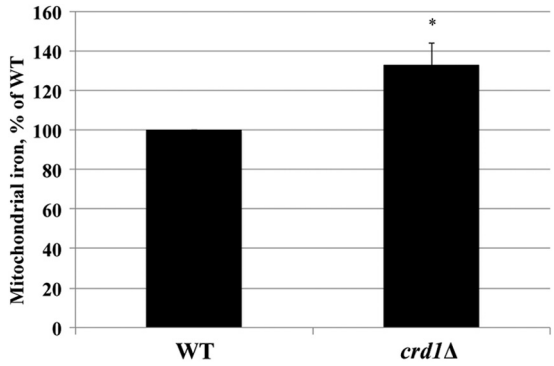

FIGURE 2. **Increased mitochondrial iron levels in *crd1*Δ.** Cells were grown in YP-galactose at 30 °C to the logarithmic phase, and mitochondrial iron levels were determined by an inductively coupled plasma-optical emission spectrometer. Data shown are mean ± S.E. ($n = 9$). The *asterisk* indicates a significant difference relative to WT.

levels of *AFT1*, *FIT1–3*, *FET3*, *FTR1*, and *ARN1–3* were up-regulated more than 3-fold in *crd1*Δ. Up-regulation of *AFT1* and the iron regulon genes in the *crd1*Δ mutant suggested either deficient cellular iron levels or perturbation of mitochondrial Fe-S cluster biogenesis, and/or export of extra-mitochondrial Fe-S co-factors are perturbed in this mutant (20, 28, 55).

*Perturbation of Iron Homeostasis in crd1*Δ—We quantified mitochondrial iron using inductively coupled plasma-optical emission spectroscopy and found that iron levels in the *crd1*Δ mutant were significantly increased by 33% relative to WT levels (Fig. 2). This result suggested that the iron regulon in *crd1*Δ is up-regulated for a reason other than low cellular iron levels. Previous studies have reported that mutations in yeast genes involved in Fe-S cluster synthesis or in the export of Fe-S co-factors lead to elevated mitochondrial iron levels (20, 32, 34, 56–58).

Perturbation of mitochondrial Fe-S biogenesis leads to growth sensitivity in the presence of $FeSO_4$ (59–62). As seen in Fig. 3, *B* and *C*, *crd1*Δ cells showed growth sensitivity to 5 and 10 mM $FeSO_4$. This sensitivity to iron supplementation was observed when *crd1*Δ cells were grown in galactose (respiro-fermentative) and ethanol (respiratory) but not in glucose (fermentable) media (Fig. 3*A*). This is most likely because cells have a greater demand for iron in respiratory and respiro-fermentative media, so as to synthesize heme and Fe-S containing proteins involved in oxidative phosphorylation (63, 64).

Increased mitochondrial iron levels cause hypersensitivity to oxidative stress (32, 65, 66), which may be reflected in sensitivity to ROS-inducing agents. Consistent with increased oxidative stress, *crd1*Δ cells exhibited increased sensitivity to the ROS-inducing agent H_2O_2 (Fig. 3*D*). In summary, the absence of CL leads to increased mitochondrial iron levels as well as sensitivity to iron supplementation and oxidative stress, consistent with perturbation of iron homeostasis.

*Fe-S Deficiencies in crd1*Δ—We explored the possibility that the iron homeostasis defects in the *crd1*Δ mutant resulted from perturbation of Fe-S biogenesis. Perturbation of mitochondrial Fe-S assembly has been shown to cause decreased activity of mitochondrial proteins containing Fe-S clusters (26, 31, 32). To this end, we assayed the activities of the Fe-S enzymes succinate

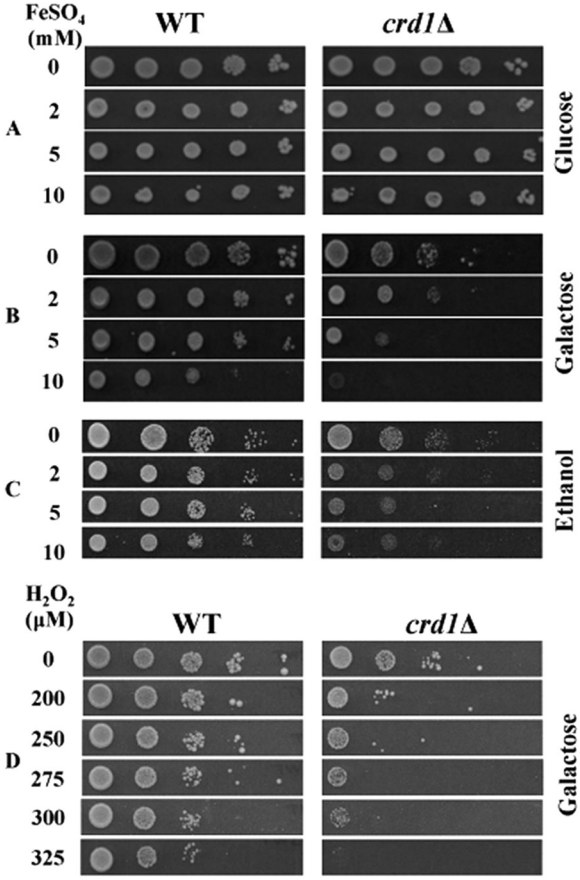

FIGURE 3. **Sensitivity of *crd1*Δ to iron and H_2O_2.** Cells were precultured in YPD overnight, serially diluted, spotted on SD plates containing glucose + $FeSO_4$ (*A*), galactose + $FeSO_4$ (*B*), ethanol + $FeSO_4$ (*C*), and galactose + H_2O_2 (*D*), and incubated at 30 °C for 5 days.

dehydrogenase, ubiquinol-cytochrome *c* oxidoreductase, and aconitase in *crd1*Δ. As seen in Table 3, these enzyme activities were decreased by ~36, 45, and 78%, respectively, in the *crd1*Δ mutant. In addition, cytochrome *c* oxidase activity was decreased by 30% in the *crd1*Δ mutant (data not shown), consistent with previous studies (3, 67–69). The decreased activities of mitochondrial Fe-S proteins in *crd1*Δ are not due to reduced transcription of *SDH2*, *RIP1*, and *ACO1* (supplemental Table 1). These results indicate that CL is required for the activ-

TABLE 3

Decreased mitochondrial and cytosolic Fe-S enzyme activities in *crd1*Δ

Cells were grown in galactose medium, and activities of Fe-S enzymes were assayed as described under "Experimental Procedures." Data shown are mean ± S.D. ($n \geq 4$).

	% activity in *crd1*Δ relative to WT
Mitochondrial Fe-S enzymes	
Succinate dehydrogenase	63.4 ± 9.5%
Ubiquinol cytochrome *c* oxidoreductase	54.2 ± 13.8%
Aconitase[a]	22.2 ± 13.9%
Cytosolic Fe-S enzymes	
Sulfite reductase	54.2 ± 1.8%
Isopropylmalate isomerase[b]	51.8 ± 9.3%

[a] These cells were grown at 35 °C. For sulfite reductase and isopropylmalate isomerase assays, cells were grown in media lacking methionine and cysteine or leucine, respectively.
[b] These cells were grown at 34 °C.

ity of mitochondrial Fe-S proteins present in the inner membrane and matrix.

Mitochondrial Fe-S cluster biogenesis is also required for the maturation of cytosolic Fe-S proteins, as cytosolic Fe-S assembly depends on Fe-S co-factors synthesized in the mitochondria (26, 28, 31, 32). To determine the impact of CL deficiency on the activities of cytosolic Fe-S proteins, we measured the activities of sulfite reductase, which catalyzes the conversion of sulfite to sulfide, and isopropylmalate isomerase, which catalyzes the inter-conversion of α-isopropyl malate and β-isopropyl malate (70–73). Sulfite reductase and isopropylmalate isomerase each contain a 4Fe-4S cluster (74–76). As seen in Table 3, sulfite reductase activity was decreased by ~46% in *crd1*Δ. Because sulfite reductase is required for the synthesis of methionine and cysteine, a decrease in activity would be expected to lead to methionine auxotrophy. As seen in Fig. 4*A*, *crd1*Δ was auxotrophic for methionine at elevated temperature. The *crd1*Δ mutant also exhibited an ~49% decrease in activity of the leucine biosynthetic pathway enzyme isopropylmalate isomerase (Table 3) along with leucine auxotrophy at elevated temperature (Fig. 4*B*). These results indicate that the loss of CL also affects activity of Fe-S proteins in the cytosol. Taken together, these experiments indicate that activities of both mitochondrial and cytosolic Fe-S enzymes are affected by CL deficiency.

Genetic Interaction between CRD1 and ISU1—If CL is required for the biogenesis of Fe-S clusters in the mitochondria, *crd1*Δ would be expected to be sensitive to further perturbation of Fe-S biogenesis. Most of the genes involved in Fe-S cluster assembly, including *NFS1*, *ISD11*, *YAH1*, and *ARH1*, are required for viability (34, 77–79). *YFH1* deletion mutants are viable in some genetic backgrounds but exhibit severe growth defects (80, 81). However, *ISU1* and *ISU2* both encode the mitochondrial Fe-S scaffolding protein and have overlapping functions. Single mutants *isu1*Δ and *isu2*Δ do not show growth defects, as the presence of either Isu1 or Isu2 is sufficient for survival, but deletion of both genes is lethal (31, 66). The *crd1*Δ*isu1*Δ double mutant showed a synthetic growth defect in galactose media, but *crd1*Δ*isu2*Δ grew normally (Fig. 5*A*). Isu1 is a more abundant scaffolding protein than Isu2 (82), which likely accounts for the more severe phenotypic defect of the *crd1*Δ*isu1*Δ mutant. To confirm that genetic defects observed in the *crd1*Δ*isu1*Δ double mutant are due to deletion of *ISU1*, we re-introduced *ISU1* under the control of the Tet-Off promoter on a low copy plasmid, and this overexpression of *ISU1* in the *crd1*Δ*isu1*Δ double mutant reversed the growth defect (Fig. 5*B*). The genetic interaction between *crd1*Δ and *isu1*Δ is consistent with perturbation of Fe-S biogenesis in *crd1*Δ and suggests that decreased Fe-S biogenesis resulting from CL deficiency is exacerbated by further loss of the Fe-S scaffold in the presence of the *isu1*Δ mutation.

*Increased Expression of ATM1 Does Not Rescue Iron Defects in crd1*Δ—The inner membrane protein Atm1, which is involved in the export of Fe-S co-factors from mitochondria, is

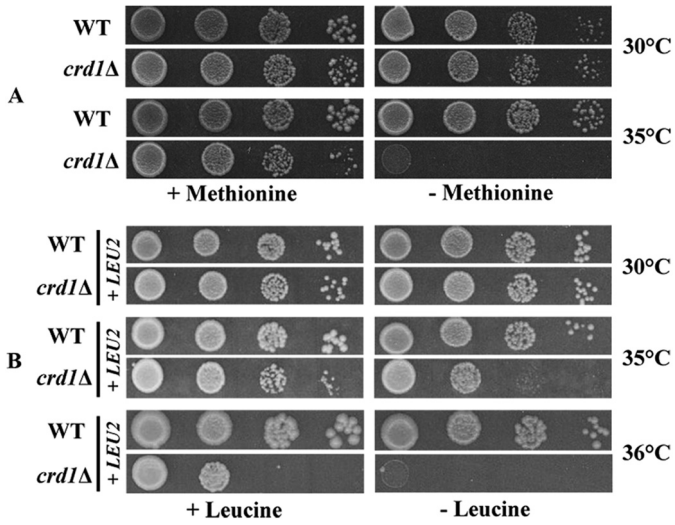

FIGURE 4. **Methionine and leucine auxotrophy in *crd1*Δ.** *A*, cells were precultured overnight in galactose medium, serially diluted, spotted on SD galactose media plates lacking methionine, and incubated for 3–5 days. *B*, cells were precultured overnight in galactose medium lacking leucine, serially diluted, spotted on SD glucose plates lacking leucine, and incubated for 3–5 days.

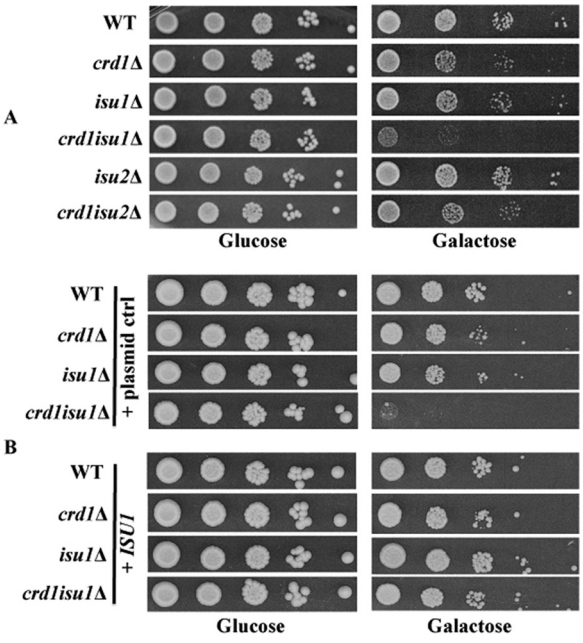

FIGURE 5. **Genetic interaction between *crd1*Δ and *isu1*Δ.** *A*, cells were precultured overnight in YPD, serially diluted, spotted on SD glucose or galactose plates, and incubated at 30 °C for 3–5 days. *B*, cells were precultured overnight in SD glucose lacking tryptophan, serially diluted, spotted on SD glucose or galactose plates lacking tryptophan, and incubated for 3–5 days.

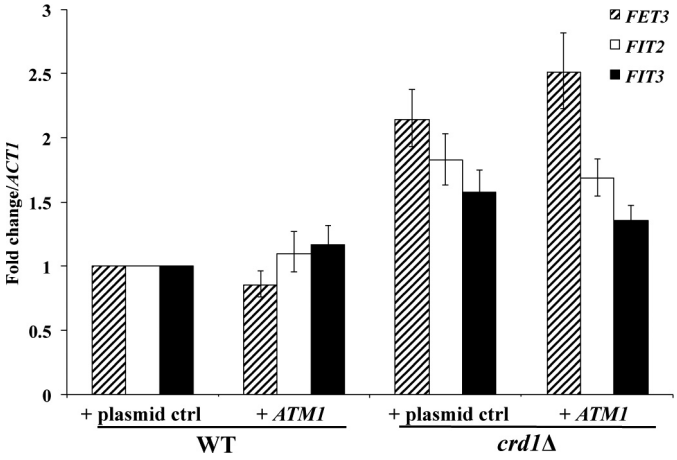

FIGURE 6. **Overexpression of *ATM1* in *crd1*Δ does not restore wild type expression of iron-uptake genes.** The mRNA levels of *FET3, FIT2,* and *FIT3* were quantified by qPCR in cells grown in SD glycerol-ethanol at 30 °C to the logarithmic phase. Values are reported as fold change in expression over WT. Expression was normalized to the mRNA levels of the internal control *ACT1*. Data shown are mean ± S.E. ($n = 6$).

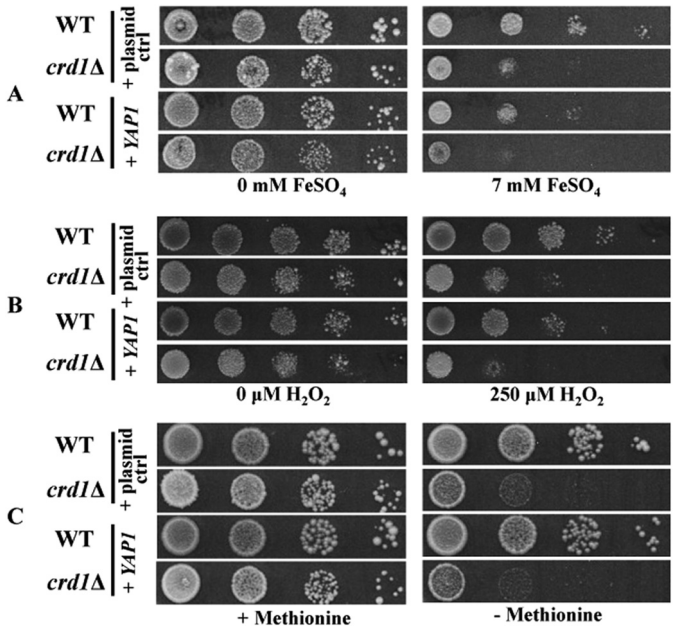

FIGURE 7. **Overexpression of *YAP1* in *crd1*Δ does not alleviate the iron-related growth phenotypes.** Cells were precultured in SD media overnight, serially diluted and spotted on SD galactose plates supplemented with $FeSO_4$ (*top panels*), supplemented with H_2O_2 (*middle panels*), and lacking methionine (*bottom panels*). Plates were incubated for 5 days.

activated by CL (83); the *in vitro* activity of Atm1 is ~50% lower in the absence of CL. If up-regulation of the iron regulon resulted from reduced Atm1 activity, then increasing Atm1 levels in *crd1*Δ cells might be expected to restore the elevated iron regulon to WT levels. However, overexpression of *ATM1* in *crd1*Δ cells did not restore the expression of *FET3*, *FIT2*, and *FIT3* to WT levels (Fig. 6). In addition, others have shown that loss of *ATM1* does not affect the activities of aconitase and succinate dehydrogenase (28). Therefore, it is not likely that Fe-S defects in the CL mutant result from Atm1 deficiency.

*Increased Expression of Antioxidant Genes Does Not Rescue Iron Defects in crd1*Δ—Published studies have shown that Fe-S clusters in proteins such as aconitase are particularly sensitive to degradation by superoxide (35, 37). In a previous study, we showed that the loss of CL leads to decreased stability of respiratory supercomplexes (9), which is expected to cause increased ROS formation. Consistent with this, protein carbonylation, a sensitive marker of intracellular ROS, was significantly increased in *crd1*Δ (84). Therefore, we addressed the possibility that the iron-associated growth defects in *crd1*Δ cells may result from increased ROS. To do so, we determined the effect on *crd1*Δ cells of increasing antioxidant production via overexpression of *YAP1*, which regulates expression of a number of antioxidant genes required for tolerance to oxidants (39, 85). In response to H_2O_2, Yap1 positively regulates genes that affect glutathione metabolism (*GSH1*, *GLR1*, and *ZWF1*), catalase (*CTT1*), cytosolic thioredoxins (*TRR1* and *TRX2*), glutathione peroxidases (*GPX1* and *GPX2*), and superoxide dismutases (*SOD1* and *SOD2*) (39, 40, 86, 87). As seen in Fig. 7, overexpression of *YAP1* in *crd1*Δ did not alleviate methionine auxotrophy or growth sensitivity to iron and H_2O_2. Thus, there is no evidence that the iron-related growth phenotypes in *crd1*Δ arise from oxidative stress.

DISCUSSION

In this study, we show for the first time that CL deficiency leads to altered mitochondrial and cellular iron homeostasis, as seen in increased expression of the iron regulon genes, elevated mitochondrial iron levels, and sensitivity to iron supplementation and ROS-inducing agents. Our findings indicate that the most likely mechanism underlying the iron homeostasis defects is that of perturbation of Fe-S biogenesis, as is evident from decreased activities of both mitochondrial and cytosolic Fe-S enzymes, concomitant auxotrophies for the amino acid products of these enzymes, and synthetic interaction of *crd1*Δ with the mitochondrial Fe-S scaffolding mutant *isu1*Δ. The observed decrease in Fe-S enzyme activity is not likely to result solely from a loss of direct enzyme activation by CL in the mitochondrial inner membrane. Although the mitochondrial enzymes succinate dehydrogenase and ubiquinol-cytochrome *c* oxidoreductase are membrane-bound and may be activated by CL, aconitase is a matrix enzyme that is unlikely to be directly regulated by CL. Furthermore, we observed reduced activity of cytosolic Fe-S enzymes sulfite reductase and isopropylmalate isomerase, which are not in contact with the mitochondrial membrane.

How does CL deficiency cause perturbation of mitochondrial Fe-S biogenesis? Several potential mechanisms can be ruled out. First, the Fe-S defects are most likely not due to decreased Atm1 activity, as overexpression of *ATM1* did not rescue the iron regulon defects (Fig. 6). Furthermore, as previous studies indicated that aconitase and succinate dehydrogenase activities are not affected by decreased expression of *ATM1,* it is unlikely that decreased activities of aconitase and succinate dehydrogenase observed in the CL mutant result from Atm1 deficiency (28). A second potential mechanism, disruption of Fe-S clusters by increased ROS in the CL mutant, is also unlikely, as overexpression of *YAP1* in *crd1*Δ did not alleviate methionine auxotrophy or growth sensitivity to iron and H_2O_2 (Fig. 7). Furthermore, we observed elevated expression of the iron regulon genes even in fermentative growth conditions, during which

protein carbonylation in CL mutants is not increased (84). In addition, expression of antioxidant genes is not increased during these growth conditions in *crd1*Δ (supplemental Table 1). Therefore, there is no evidence that ROS contributes to the observed iron phenotypes of *crd1*Δ cells.

A third possibility, perturbation of glutathione (GSH) metabolism, is also not a likely cause of Fe-S defects in the CL mutant. GSH plays a critical role in maintaining an intracellular reducing environment and regulates cellular iron homeostasis (88, 89). Although perturbation of GSH metabolism does lead to elevated mitochondrial iron levels and decreased cytosolic Fe-S biogenesis, depletion of GSH does not affect activities of the mitochondrial Fe-S proteins succinate dehydrogenase and aconitase, which are reduced in *crd1*Δ (88). However, depletion of GSH, a tripeptide of glutamate, cysteine, and glycine, is a predicted outcome of Fe-S deficiency. First, the glutamate precursor α-ketoglutarate is likely to be depleted as a result of aconitase deficiency. Second, decreased activity of the Fe-S enzyme glutamate synthase would lead to a decrease in the conversion of glutamine and α-ketoglutarate to glutamate (90, 91). Third, decreased activity of sulfite reductase is expected to affect synthesis of methionine, the sulfur donor for synthesis of cysteine. Therefore, it is probable that glutathione deficiency is a downstream effect of Fe-S defects in CL-deficient cells.

Previous studies have indicated that yeast cells exhibit iron deficiency resulting from defective vacuolar protein sorting or activation of Fet3, which may activate the iron regulon (92, 93). However, defective vacuolar function is not a likely cause of up-regulation of the iron regulon in *crd1*Δ cells because, in this study, mitochondria from *crd1*Δ cells contain elevated, rather than decreased, levels of iron.

The most likely explanation for perturbation of Fe-S biogenesis in CL-deficient cells is that alterations in the mitochondrial membrane perturb the stability and integrity of the protein complexes that drive mitochondrial protein import (94, 95). We have shown that *crd1*Δ cells exhibit defective import of precursor proteins into mitochondria (3, 94). Cells lacking CL may be compromised in the import of a protein or nutrient important for Fe-S biogenesis in the matrix. Recent studies have shown that Zim17, a heat-shock protein, interacts with both Ssc1 and PAM to promote their activities (96, 97). Mitochondria from the *ZIM17* mutant exhibit decreased protein import due to aggregation of Pam16, Ssc1, and Ssq1 proteins (96–99). Aggregation of both Ssc1 and Ssq1 results in decreased Fe-S biogenesis, leading to up-regulation of the Aft1-controlled iron regulon. The loss of CL may affect mitochondrial import or processing of Fe-S biosynthetic proteins or, alternatively, affect nutrient import through inner membrane carrier proteins. Experiments to address this mechanism are in progress.

We propose the following model for the role of CL in maintaining mitochondrial and cellular iron homeostasis (Fig. 8). CL deficiency leads to decreased mitochondrial import of Fe-S proteins resulting in defects in Fe-S cluster biogenesis and maturation of Fe-S proteins and thus reduced activities of mitochondrial and cytosolic Fe-S enzymes. The cellular response to the decrease in Fe-S biogenesis is up-regulation of the iron regulon, leading to elevated mitochondrial iron levels.

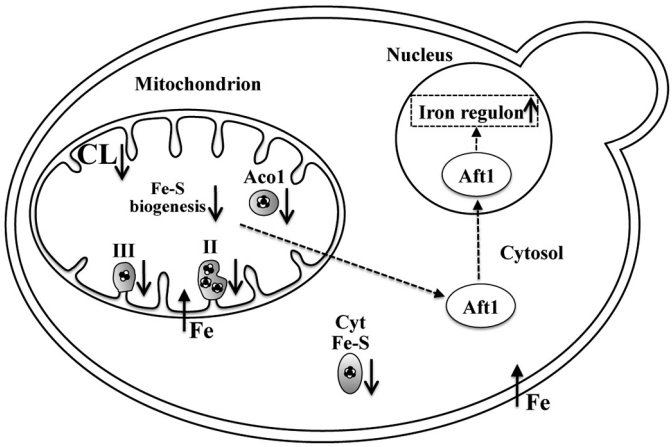

FIGURE 8. **Perturbation of Fe-S biogenesis in *crd1*Δ.** In the proposed model, loss of CL leads to decreased Fe-S biogenesis and maturation of Fe-S proteins, resulting in reduced activities of succinate dehydrogenase (II), ubiquinol-cytochrome *c* oxidoreductase (III), and aconitase (Aco1) in the mitochondria, as well as cytosolic Fe-S enzymes (Cyt Fe-S) sulfite reductase and isopropylmalate isomerase. Decreased Fe-S biogenesis is sensed by Aft1, which activates expression of the iron regulon genes, leading to increased mitochondrial iron levels.

It remains unclear how CL deficiency contributes to the observed pathology in BTHS. Interestingly, some of the clinical symptoms found in BTHS patients are also seen in patients with Fe-S biogenesis defects. Mutations in the human *ISCU* gene, which is homologous to yeast *ISU1*, lead to deficiencies in succinate dehydrogenase and aconitase in skeletal muscle, causing cardiomyopathy, lactic acidosis, muscle weakness, and exercise intolerance (100–103). Depletion of several proteins of the Fe-S biosynthetic machinery severely affects mitochondrial inner membrane structure and cristae morphology (104, 105), similar to what has been observed in the lymphoblasts of BTHS patients (106). In addition, deficiency of frataxin, which is involved in mitochondrial Fe-S biogenesis, is characterized by hypertrophic cardiomyopathy and heart failure (107). Interestingly, overexpression of frataxin leads to increased mitochondrial membrane potential, elevated ATP levels, resistance to oxidative stress, and life span extension (108, 109), defects which are characteristic of CL deficiency (7). In transgenic mice, overexpression of frataxin counteracted cardiotoxic stress, preventing cardiomyopathy and cardiac failure (110). We suggest that mitochondrial iron homeostasis may be an important physiological modifier that contributes to the phenotypes observed in BTHS patients.

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