Loss of Cardiolipin Leads to Perturbation of Mitochondrial and Cellular Iron Homeostasis

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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 crd1Δ. 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 crd1Δ mutant. We further show that crd1Δ 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 ATMI or YAP1 did not rescue the Fe-S defects in crd1Δ. 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 crd1Δ, 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 (Fe2+) and sulfide (S2−) ions does not occur spontaneously in living cells, as trose; Aft1, activator of ferrous transport; qPCR, quantitative real time PCR; ROS, reactive oxygen species.
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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, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::KanMX4 | This study |
| FGY3 isu1Δ | MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::KanMX4 | This study |
| FGY2 isu1Δ | MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::KanMX4 | This study |
| FGY3 isu2Δ | MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::KanMX4 | This study |
| FGY2 isu2Δ | MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::KanMX4 | This study |
| pCM182  | Low copy number plasmid, TRP1 nutritional marker | 111 |
| pCM182-ISU1 | Derivative of pCM182, expresses ISU1 from the Tet-Off promoter | 112 |
| pSr415  | Low copy number plasmid, LEU2 nutritional marker | 112 |
| YEp351  | High copy number plasmid, LEU2 nutritional marker | 42 |
| YEp351-YAPI | Derivative of YEp351, expresses YAPI 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 (Fe2+) 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 are matured by the cytosolic machinery into 4Fe-4S clusters (29–30). The mitochondrial Fe-S export machinery includes the ABC transporter Atm1 in the inner membrane and the sulphydryl 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 crd1Δ cells exhibit perturbations in iron homeostasis, including increased expression of the iron uptake genes, elevated mitochondrial iron levels, and growth sensitivity to both FeSO4 supplementation and the ROS-inducing agent H2O2. 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, crd1Δ cells exhibit a synthetic genetic interaction with the Fe-S scaffolding protein Isu1. Additionally, the iron homeostasis defects in crd1Δ 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 YAPI, which regulates expression of antioxidant genes (38–40). Overexpression of ATM1 and YAPI 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) glyc erol (3%) plus ethanol (0.65%) or (respiratory-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 sulfur, 1 μM CuSO4 was used, and FeSO4 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-GGAAAACACACCGGATCCCATATTTAACC-3) and PstI-tagged primer ISU1-PstIR (5-GATCTTGTCTCGACGCGGTATTTCTT-3). Similarly, a 2098-bp sequence containing the entire open reading frame of ATM1 was amplified using NotI-tagged primer ATM1-NotIF (5-TTGTAGATGCAGCGCCAACTGCAATTAACC-3) and PstI-tagged primer ATM1-PstIR (5-TACATGTCTCGACGAAATATTTATCTTACGAGCG-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
transformed into yeast strains using the yeast one-step transformation protocol (41). A high copy number Yep351-YAPI overexpression plasmid was a kind gift from W. Scott Moye-Rowley (University of Iowa) (42).

**Microarray Analysis**—Yeast cells were grown to the 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 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 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 A340 (50). Sulfite reductase was assayed by monitoring methylene blue formation at A670 from sulfide produced by NADP-dependent sulfite reduction (19, 50). Isopropylmalate isomerase was assayed by monitoring formation of isopropylmalate at A235 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 leu2Δ 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% HNO₃ by boiling for 2 min and then diluted to 30% HNO₃. 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 crd1Δ, which completely lacks CL (52–54). The microarray analysis revealed increased expression of genes involved in iron homeostasis in the crd1Δ 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 crd1Δ. 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

### TABLE 2

| Gene | Primer | Sequence |
|------|--------|----------|
| ACT1 | Forward | TCGTGCCTGCTCCTCCCATCTATCG |
|      | Reverse | CAGATCCAGAGGTGCGCAAGAG |
| AFT1 | Forward | ATGCATCTAAAGCCCATGTC |
|      | Reverse | ACTGGGTTTGTCCCTAGTTT |
| FIT1 | Forward | CACCTCCTCTTGTACGTAAGCC |
|      | Reverse | CAGATGCGCATCCTCCATGAAG |
| FIT2 | Forward | ATCTGCTTTCCTTTGACGTTT |
|      | Reverse | TGAGCTTGAAACCTGTCTT |
| FIT3 | Forward | GTCACTACCTCTTACGACGCCC |
|      | Reverse | GCACCCATCAAACCTATGAC |
| FET1 | Forward | CACTCTGGGTATCTGGGTTTT |
|      | Reverse | GGAGACCATATGACTGCGG |
| FTR1 | Forward | ATTGCTCATGTCCTGCTGGTC |
|      | Reverse | GTGTCCTAAGGAGTATCAAT |
| ARN1 | Forward | CCACCGATCTGTTAAAAGAG |
|      | Reverse | GGGACATACATGATGCTGGA |
| ARN2 | Forward | TGGAGGACCTTGGCTGGTTA |
|      | Reverse | GGCCATAGAAGGATCAATG |
| ARN3 | Forward | TCGATTACGCGGAAAGCTAAC |
|      | Reverse | GGAATACAAGCTAGCGGCA |

**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 A600 was recorded as a reporter of decyubiquinone reduction (50). Ubiquinol-cytochrome c oxidoreductase activity was assayed by monitoring reduction of cytochrome c at A550 (51).

Cell extracts were prepared by resuspending cells in 500 μl of TNTE 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 A340 (50). Sulfite reductase was assayed by monitoring methylene blue formation at A670 from sulfide produced by NADP-dependent sulfite reduction (19, 50). Isopropylmalate isomerase was assayed by monitoring formation of isopropylmalate at A235 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 leu2Δ 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% HNO₃ by boiling for 2 min and then diluted to 30% HNO₃. Iron content was determined using an inductively coupled plasma-optical emission spectrometer.
levels of AFT1, FIT1–3, FET3, FTR1, and ARN1–3 were up-regulated more than 3-fold in crd1Δ. Up-regulation of \( AFT1 \) and the iron regulon genes in the \( \text{crd1}\Delta \) 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 \( \text{crd1}\Delta \)—We quantified mitochondrial iron using inductively coupled plasma-optical emission spectroscopy and found that iron levels in the \( \text{crd1}\Delta \) mutant were significantly increased by 33% relative to WT levels (Fig. 2). This result suggested that the iron regulon in \( \text{crd1}\Delta \) 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, \( \text{crd1}\Delta \) cells showed growth sensitivity to 5 and 10 mM FeSO\(_4\). This sensitivity to iron supplementation was observed when \( \text{crd1}\Delta \) cells were grown in galactose (respiratory) and ethanol (respiratory) but not in glucose (fermentable) media (Fig. 3A). 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, \( \text{crd1}\Delta \) cells exhibited increased sensitivity to the ROS-inducing agent H\(_2\)O\(_2\) (Fig. 3D). 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 \( \text{crd1}\Delta \)—We explored the possibility that the iron homeostasis defects in the \( \text{crd1}\Delta \) 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 dehydrogenase, ubiquinol-cytochrome \( c \) oxidoreductase, and aconitate in \( \text{crd1}\Delta \). As seen in Table 3, these enzyme activities were decreased by \(-36, 45, \) and 78%, respectively, in the \( \text{crd1}\Delta \) mutant. In addition, cytochrome \( c \) oxidase activity was decreased by 30% in the \( \text{crd1}\Delta \) mutant (data not shown), consistent with previous studies (3, 67–69). The decreased activities of mitochondrial Fe-S proteins in \( \text{crd1}\Delta \) are not due to reduced transcription of \( \text{SDH2, RIP1, and ACO1 (supplemental Table 1)} \). These results indicate that CL is required for the activ-
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TABLE 3
Decreased mitochondrial and cytosolic Fe-S enzyme activities in crd1Δ

| Mitochondrial Fe-S enzymes | % activity in crd1Δ relative to WT |
|----------------------------|----------------------------------|
| Succinate dehydrogenase    | 63.4 ± 9.5%                      |
| Ubiquinol cytochrome c reductase | 54.2 ± 13.8%                  |
| Aconitase*                 | 22.2 ± 13.9%                     |

| Cytosolic Fe-S enzymes | % activity in crd1Δ relative to WT |
|------------------------|-----------------------------------|
| Sulfite reductase      | 54.2 ± 1.8%                       |
| Isopropylmalate isomerase | 51.8 ± 9.3%                     |

* 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.

** These cells were grown at 34 °C.

activity 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 crd1Δ. 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. 4A, crd1Δ was auxotrophic for methionine at elevated temperature. The crd1Δ 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. 4B). 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, crd1Δ 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 isu1Δ and isu2Δ 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 crd1Δisu1Δ double mutant showed a synthetic growth defect in galactose media, but crd1Δisu2Δ grew normally (Fig. 5A). Isu1 is a more abundant scaffolding protein than Isu2 (82), which likely accounts for the more severe phenotypic defect of the crd1Δisu1Δ mutant. To confirm that genetic defects observed in the crd1Δisu1Δ double mutant are due to deletion of ISU1, we re-introduced ISU1 under the control of the Tet-Off pro-moter on a low copy plasmid, and this overexpression of ISU1 in the crd1Δisu1Δ double mutant reversed the growth defect (Fig. 5B). The genetic interaction between crd1Δ and isu1Δ is consistent with perturbation of Fe-S biogenesis in crd1Δ 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 isu1Δ mutation.

Increased Expression of ATM1 Does Not Rescue Iron Defects in crd1Δ—The inner membrane protein Atm1, which is involved in the export of Fe-S co-factors from mitochondria, is
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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 *crd1Δ* cells might be expected to restore the elevated iron regulon to WT levels. However, overexpression of *ATM1* in *crd1Δ* 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 crd1Δ*—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 *crd1Δ* (84). Therefore, we addressed the possibility that the iron-associated growth defects in *crd1Δ* cells may result from increased ROS. To do so, we determined the effect on *crd1Δ* 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 *H₂O₂*, 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 *crd1Δ* did not alleviate methionine auxotrophy or growth sensitivity to iron and *H₂O₂*. Thus, there is no evidence that the iron-related growth phenotypes in *crd1Δ* 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 *crd1Δ* with the mitochondrial Fe-S scaffolding mutant *isu1Δ*. 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 *crd1Δ* did not alleviate methionine auxotrophy or growth sensitivity to iron and *H₂O₂* (Fig. 7). Furthermore, we observed elevated expression of the iron regulon genes even in fermentative growth conditions, during which
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protein carbonylation in CL mutants is not increased (84). In addition, expression of antioxidant genes is not increased during these growth conditions in crd1Δ (supplemental Table 1). Therefore, there is no evidence that ROS contributes to the observed iron phenotypes of crd1Δ 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 aconitate, which are reduced in crd1Δ (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 aconitate 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 activation of Fet3, which may activate the iron regulon (92, 93).

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 crd1Δ 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.

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 aconitate 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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