The Role Of Cardiolipin In Iron Homeostasis And Glutathione Metabolism

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THE ROLE OF CARDIOLIPIN IN IRON HOMEOSTASIS AND GLUTATHIONE METABOLISM

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DEDICATION

To my mother and late father, my wife, and my sister, with love
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CHAPTER 1

INTRODUCTION

Parts of this chapter have been submitted for publication in the journal of Advances in Experimental Medicine and Biology, Springer (invited review).

1. Role of CL in cellular signaling – an overview

It is not unusual to find even current depictions of membranes as homogenous lipid matrices that function primarily to support the allegedly important protein molecules embedded within. This belies the fascinating discoveries in the past two decades of cellular and organelle-specific functions attributed to individual membrane lipids, and of the plethora of regulatory and signaling molecules derived from glycerolipids and sphingolipids. In this light, it is essential to elucidate the functions of specific membrane lipids and the cellular consequences of their depletion.

A phospholipid that has been the focus of considerable attention relatively recently - although it was first isolated and purified from beef heart in 1942 (Pangborn, 1942; Pangborn, 1947), is cardiolipin (CL). CL is structurally unique. In contrast to the other membrane phospholipids, in which a single glycerol backbone is acylated to two fatty acid chains, CL contains two phosphatidyl groups (linked to a glycerol backbone) and four fatty acyl chains. It is enriched in energy harvesting membranes of mitochondria, chloroplasts, bacterial plasma...
membranes, and hydrogenosomes, underscoring the importance of this lipid in energy production (Corcelli, 2009; Depalo et al., 2004; Dowhan, 1997). CL is tightly associated with mitochondrial proteins and respiratory chain complexes and is essential for their optimal activity (Fry et al., 1980; Fry and Green, 1980; Fry and Green, 1981; Lange et al., 2001a; Pfeiffer et al., 2003). In the inner membrane, CL provides structural stability to membrane proteins through hydrophobic and electrostatic interactions.

In light of its association with the respiratory apparatus, the role of CL in mitochondrial bioenergetics was not entirely unexpected. Interestingly, however, recent studies carried out primarily in yeast indicate that CL is also required for cellular functions that are not directly associated with oxidative phosphorylation. In accordance with a broad definition of a ‘bioactive lipid’ as one in which changes in levels lead to functional consequences (Hannun and Obeid, 2008), perturbation of CL composition (including CL levels, acyl species, and degree of peroxidation) leads to dramatic cellular consequences: (1) Alterations in CL levels and acyl chain composition increases the recruitment to the mitochondria of cytosolic proteins that trigger apoptosis (Kagan et al., 2005; Kuwana et al., 2002; Schug and Gottlieb, 2009). (2) Perturbation of CL synthesis or remodeling leads to increased production of reactive oxygen species (ROS), which induces aging (Cao et al., 2009; Chen et al., 2008a; Lee et al., 2006; Li et al., 2012; Li et al., 2010; Paradies et al., 2010). (3) Blocking CL synthesis in yeast at the first step of the pathway deleteriously affects cell wall biogenesis and alters the response of two signaling pathways, the protein kinase C (PKC)-Slt2 mitogen
activated protein kinase (MAPK) and the high osmolarity glycerol (HOG) pathways (Zhong et al., 2005; Zhong et al., 2007; Zhou et al., 2009). (4) The inability of yeast cells to synthesize CL leads to decreased vacuolar function and reduced V-ATPase activity, suggesting that CL mediates cross talk between mitochondria and the vacuole (Chen et al., 2008b).

2. CL biosynthesis and remodeling

One of the most intriguing aspects of CL biosynthesis is that the lipid that is initially synthesized contains primarily saturated fatty acids, while the mature CL that is essential for normal cellular function contains primarily unsaturated fatty acids. The distinct composition of acyl chains is achieved through a highly conserved pathway of synthesis and remodeling, as shown in Fig. 1.1. The first step is catalyzed by phosphatidylglycerolphosphate (PGP) synthase (Pgs1), which converts CDP-diacylglycerol (DAG) and glycerol-3-phosphate (G-3-P) to PGP (Chang et al., 1998a; Kawasaki et al., 1999). PGP is dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase (Gep4) (Osman et al., 2010; Zhang et al., 2011). The mammalian homologue of the yeast GEP4 gene was recently identified as protein tyrosine phosphatase localized in the mitochondrion 1 (Zhang et al., 2011). CL synthase (Crd1) catalyzes an irreversible condensation reaction in which the phosphatidyl group of CDP-DAG is linked to PG via cleavage of a high-energy anhydride bond to form CL (Chang et al., 1998b; Chen et al., 2006; Hostetler et al., 1971; Hostetler et al., 1972; Houtkooper et al., 2006; Jiang et al., 1997; Tamai and Greenberg, 1990; Tuller et
al., 1998). CL synthase does not show strong preference for specific fatty acyl chains (Hostetler et al., 1975; Houtkooper et al., 2006; Nowicki et al., 2005). How, then, is acyl specificity achieved? The newly synthesized CL undergoes deacylation by a CL-specific deacylase (Cld1), which is homologous to the mammalian phospholipase A₂ (Beranek et al., 2009; Mancuso et al., 2009; Mancuso et al., 2007). Cld1 removes one saturated fatty acyl chain from CL to form monolysocardiolipin (MLCL) (Beranek et al., 2009). The transacylase tafazzin (Taz1) reacylates MLCL with an unsaturated fatty acid to form mature CL (Gu et al., 2004; Xu et al., 2003b; Xu et al., 2006). Taz1 carries out a bidirectional exchange of acyl chains between CL and phosphatidylcholine (PC) to sequentially replace the fatty acyl chains from all four acyl positions of CL (Malhotra et al., 2009). The end result of this exchange is molecular symmetry of CL molecules across the eukaryotic kingdom, from yeast to humans, which is characteristic of the organism and of specific tissues and organs (Schlame et al., 2005). For example, in yeast, the mature form of CL contains oleic acid, while CL in the normal human heart is primarily tetranoleoyl-CL (L₄-CL) (Schlame et al., 2005). A deficiency of tafazzin in humans leads to a complete absence of L₄-CL, resulting in the severe cardiomyopathy observed in BTHS.

While tafazzin is the only known yeast enzyme that adds fatty acyl chains to MLCL, two other enzymes in addition to tafazzin remodel CL in mammalian cells. MLCL acyltransferase-1 (MLCLAT1), isolated and purified from pig liver mitochondria, shows specificity for linoleate (Taylor and Hatch, 2003). Thus,
Figure 1.1 Synthesis and remodeling of CL in yeast. CL synthesis begins with the conversion of CDP-diacylglycerol (CDP-DG) to phosphatidylglycerolphosphate (PGP) by PGP synthase (encoded by PGS1). PGP is dephosphorylated to phosphatidylglycerol (PG) by GEP4-encoded PGP phosphatase. CL synthase (Crd1 defined in the text for either lower or capital case) converts PG to premature CL containing primarily saturated fatty acids (FA). CL is deacetylated by CL deacylase (Cld1) to monolyso-CL (MLCL), which is reacetylated by the TAZ1-encoded enzyme tafazzin to mature CL containing primarily unsaturated fatty acids. The yeast gene names are depicted in green, while phospholipids and their intermediates are shown in red.
over-expression of MLCLAT1 in tafazzin-deficient BTHS lymphoblasts increased incorporation of linoleic acid into CL, and RNAi knockdown of MLCLAT1 in HeLa cells showed reduced linoleic acid inclusion in CL (Taylor and Hatch, 2009). The biological function of this enzyme is not clear. A second enzyme, acyl-CoA:lysoCL acyltransferase 1 (ALCAT1), identified in mouse, was initially thought to be located in the endoplasmic reticulum, but was subsequently determined to be present in the mitochondrial-associated membranes, where phospholipid traffic between the endoplasmic reticulum and the mitochondria takes place (Cao et al., 2004; Li et al., 2010). In contrast to MLCLAT1, ALCAT1 shows no specificity for linoleic acid. ALCAT1 was shown to catalyze CL remodeling to incorporate long chain polyunsaturated fatty acyl chains such as docosahexaenoic acid (DHA) (Li et al., 2010). Enhanced incorporation of polyunsaturated fatty acyl chains in CL makes it more susceptible to oxidative damage by ROS, causing early peroxidation (Hong et al., 2002; Ng et al., 2005; Watkins et al., 1998). ALCAT1 null mutant mice exhibit elevated CL levels along with increased L₄-CL (Li et al., 2012; Li et al., 2010), whereas overexpression of ALCAT1 has been shown to decrease total CL levels and increase incorporation of long chain polyunsaturated fatty acyl chains (Sparagna and Lesnefsky, 2009). These findings suggest that ALCAT1 may negatively regulate CL biosynthesis.

In light of the importance of CL in cellular function, it is not surprising that perturbation of CL synthesis leads to serious illness. The most direct example of this link is seen in BTHS, a life-threatening illness characterized by dilated cardiomyopathy and sudden death from arrhythmia (Barth et al., 1999a;
Christodoulou et al., 1994). BTHS results from mutation in the CL remodeling enzyme tafazzin (Bione et al., 1996a; Xu et al., 2003a). This leads to an abnormal CL profile characterized by decreased total CL, increased monolysoc-L, and aberrant CL acylation, most notably the loss of the predominant CL species in normal myocardium, L₄-CL (Vreken et al., 2000). How these abnormalities cause the associated pathology in BTHS is not known (Schlame et al., 2002).

CL abnormalities have also been observed in heart failure (Saini-Chohan et al., 2009; Sparagna et al., 2007). Heart failure due to dilated cardiomyopathy is the primary cause of death in diabetic patients (Garcia et al., 1974; Nichols et al., 2001). Metabolic perturbations observed in diabetic cardiomyopathy include increased utilization of fatty acid substrates, decreased utilization of glucose, and mitochondrial dysfunction (Fang et al., 2004; Poornima et al., 2006; Wang et al., 2006). However, the molecular mechanism that leads to heart failure in diabetic patients is not known. Interestingly, a decrease in CL levels and alterations in CL acyl species were found in early stages of diabetes induced by streptozotocin in mice, suggesting that mitochondrial dysfunction and cardiomyopathy may be due to alterations in CL metabolism (Han et al., 2005; Han et al., 2007). The decrease in CL levels may result from remodeling of CL fatty acyl species with DHA, which is known to cause CL peroxidation by ROS (Hong et al., 2002; Ng et al., 2005; Watkins et al., 1998). In summary, depletion of CL content and alterations in CL fatty acyl species lead to BTHS, and may also contribute to pathological conditions and metabolic perturbations in other human disorders.
3. **CL and apoptosis**

Perturbations in CL levels and acyl composition play a crucial role in regulating apoptosis, the complex process leading to programmed cell death. The role of CL in apoptosis derives from its interactions with cytochrome c (Cyt c) and with apoptotic proteins (Fig. 1.2).

**i) CL and Cyt c.** Interactions between CL and Cyt c are an important determinant of apoptosis. Cyt c, which transfers electrons from complex III to complex IV, is bound to the outer leaflet of the mitochondrion inner membrane through interactions with CL (Gorbenko, 1999; Iverson and Orrenius, 2004). The binding of Cyt c to CL is essential to anchor it to the inner membrane, and release of Cyt c to the cytosol serves as a signal to recruit apoptotic proteins to the mitochondria to initiate apoptosis (Choi et al., 2007; Petrosillo et al., 2006; Spooner and Watts, 1992; Tuominen et al., 2002). CL binds Cyt c in two different conformations - a loosely bound state that is facilitated by means of electrostatic interactions, and a tightly bound state that is mediated by hydrophobic interactions in which Cyt c is partially embedded in the inner membrane (Gorbenko, 1999; Iverson and Orrenius, 2004). Release of Cyt c from CL requires dissociation of both electrostatic and hydrophobic interactions (Ott et al., 2002). The production of ROS may alter the CL-Cyt c association (Ott et al., 2002; Petrosillo et al., 2006; Petrosillo et al., 2003). Alternatively, the peroxidation of CL by hydrogen peroxide generated in the mitochondria leads to the release of Cyt c from the tightly bound state into the intermembrane space (Belikova et al., 2006; Kagan et al., 2005).
Figure 1.2. Perturbation of CL metabolism triggers apoptosis. The binding of cytochrome c (Cyt c) to CL is essential to anchor it to the inner mitochondrial membrane, facing the intermembrane space. Peroxidation of CL (CL-OOH) by reactive oxygen species (ROS) leads to release of Cyt c to the cytosol, which serves as a signal to initiate apoptosis. Caspase-8 cleaves Bid protein to its active form, truncated Bid (t-Bid). Binding of t-Bid to CL enhances translocation of CL to the outer mitochondrial membrane, which facilitates targeting of apoptotic proteins (Bak and Bax) to the outer membrane.
ii) **Recruitment of apoptotic proteins.** An early trigger of apoptosis is the change in CL composition in the mitochondrial inner and outer membranes, followed by dissipation of the membrane potential and flipping of phosphatidylserine (PS) to the external surface of the plasma membrane (Garcia Fernandez et al., 2002; Thiagarajan and Tait, 1990). A diverse set of apoptotic proteins such as t-Bid, Bax, Bak, and caspase-8 are recruited to the mitochondrial surface of cells undergoing apoptosis in a CL-dependent manner (Gonzalvez et al., 2010; Gonzalvez et al., 2008; Li et al., 1998). Upon activation, caspase-8 migrates to the mitochondrial outer membrane in regions where CL is present. Caspase-8 is said to cleave Bid to its active form, tBid (truncated Bid). A significant amount of CL is translocated from the inner to the outer mitochondrial membrane, which likely serves as a signal for binding of the apoptotic proteins (Gonzalvez et al., 2005; Kuwana et al., 2002; Lutter et al., 2000). The binding of t-Bid to CL is thought to further increase CL transfer to the outer membranes. Alternatively, apoptotic proteins may be guided to the mitochondria by means of altering the outer membrane charge (Heit et al., 2011). By increasing the CL content, the mitochondrial outer membrane may accrue a more negative charge, which serves as a targeting signal for recruiting polycationic apoptotic proteins to the mitochondria (Aguilar et al., 1999; Lutter et al., 2000). Consistent with this, ectopic overexpression of a CL-binding protein masked the negative charge on the membrane and inhibited apoptosis (Heit et al., 2011). The recruitment to and oligomerization of Bak-Bax in the outer mitochondrial membrane is a CL dependent process, which permeabilizes the outer mitochondria to trigger Cyt c
release and progression of apoptosis (Liu et al., 2004; Sani et al., 2009). This suggests that CL-rich regions in the outer membrane serve as a key signal for targeting pro-apoptotic proteins of the Bcl2 family to bring about apoptosis (Lutter et al., 2000; Lutter et al., 2001).

**iii) Translocation of CL.** Early in apoptosis, CL translocation from the inner to the outer mitochondrial membrane may be carried out through several transport modes. First, CL is enriched at the inner and outer membrane contact sites, which are enriched in CL through interactions with mitochondrial creatine kinase (MtCK), could facilitate the transfer of CL from the inner to outer membrane (Epand et al., 2007a; Epand et al., 2007b; Maniti et al., 2009; Schlame and Augustin, 1985; Schlattner et al., 2004; Speer et al., 2005). Second, phospholipid scramblase-3 (PLS-3) has been shown to translocate CL from the inner membrane to the outer membrane during the onset of apoptosis (Liu et al., 2003a; Liu et al., 2003b; Van et al., 2007). Consistent with this, cells overexpressing PLS-3 exhibit increased apoptosis, while inactivation of PLS-3 leads to increased resistance to UV-induced apoptosis (Liu et al., 2003b). CL and Bid interactions have been shown at the contact sites, which likely contribute to mitochondrial permeabilization to induce apoptosis (Kim et al., 2004). Changes in CL content in the membrane may be mediated by Bid, as evidence suggests that Bid exhibits lipid transfer activity (Esposti et al., 2001; Sorice et al., 2004). Lymphoblastoid cells derived from BTHS and TAZ knockdown HeLa cells were more resistant to Fas-induced apoptosis (Gonzalvez et al., 2008). Specifically, reduction of mature CL caused defective activation of caspase-8, suggesting that
processing of caspase-8 on the mitochondrial membranes is CL-dependent. To summarize, CL in the mitochondria is an important mediator of apoptosis, and apoptotic proteins are directed to the mitochondria in a CL-dependent manner.

4. **CL in bioenergetics and mitochondrial dysfunction**

The relationship between CL and ROS is complex. The important role of CL in mitochondrial bioenergetics suggests that CL deficiency may lead to an increase in ROS, which is generated in the mitochondria as a byproduct of oxidative phosphorylation (Barros et al., 2003; Grant et al., 1997; Pitkanen and Robinson, 1996). CL physically interacts with proteins of the mitochondrial respiratory chain complexes and other components of the membrane and forms lipid scaffolds for tethering and stabilizing mitochondrial membrane proteins to enhance their enzymatic activities (Beyer and Klingenberg, 1985; Beyer and Nuscher, 1996; Fry and Green, 1980; Fry and Green, 1981; Joshi et al., 2009; Sedlak and Robinson, 1999). Consistent with this role in bioenergetics, mitochondria deficient in CL exhibit decreased activity of respiratory complexes and carrier proteins (Claypool, 2009), and CL deficiency leads to an increase in ROS (Chen et al., 2008a). ROS, in turn, damages CL by peroxidation of the unsaturated fatty acids.

*i) CL and supercomplexes*

For efficient substrate channeling between the individual complexes, the mitochondrial respiratory chain components are organized in supramolecular
structures called supercomplexes (Schagger and Pfeiffer, 2000). In mammalian cells, complex I is associated with two units of complex III and multiple units of complex IV. In *S. cerevisiae*, which lacks complex I, two copies of complex III are bound to either one or two units of complex IV. CL deficiency in yeast leads to destabilization of the respiratory supercomplexes, indicating that CL functions to stabilize these complexes (Pfeiffer et al., 2003; Zhang et al., 2002a; Zhang et al., 2005a). Similarly, tafazzin deficient human fibroblasts exhibit destabilization of the supercomplexes (McKenzie et al., 2006). For efficient ADP/ATP exchange, CL is also required for the association of the ADP/ATP carrier protein with the supercomplexes (Claypool et al., 2008).

**ii) CL deficiency and ROS generation.** The role of CL in the supercomplexes may be that of a proton trap, to avoid leakage of protons and enhance the membrane potential for efficient oxidative phosphorylation (Haines and Dencher, 2002; Hoch, 1998; Jiang et al., 2000). CL may also reduce leakage of electrons, which can increase the generation of ROS. Not surprisingly, defective supercomplex formation and CL deficiency lead to increased ROS production (Chen et al., 2008a; Chen et al., 2012).

Among the respiratory chain complexes, complexes I and III are prime sites for ROS generation (Barja, 1999; Grivennikova and Vinogradov, 2006; Kushnareva et al., 2002; Turrens et al., 1985). Because of the proximity of CL to these ROS generating centers, the unsaturated fatty acyl chains of CL are susceptible to damage by peroxidation. Superoxide generated by respiratory
complex III causes peroxidation of CL and alters the activity of Cyt c oxidase (Paradies et al., 2000; Paradies et al., 2001; Paradies et al., 1998). Optimal function of Cyt c oxidase, the terminal enzyme complex of the respiratory chain, is dependent on CL (Abramovitch et al., 1990; Fry et al., 1980; Powell et al., 1987; Robinson et al., 1980). Reduced activity of Cyt c oxidase from reperfused heart was restored specifically by exogenous supplementation of CL, but not by peroxidized CL or other phospholipids (Paradies et al., 1999b). In addition, reduced activity and increased ROS generation by complexes I and III were also rescued by CL supplementation (Paradies et al., 2001; Petrosillo et al., 2007). These studies indicate that peroxidi
czed CL cannot effectively carry out mitochondrial functions that are dependent on normal CL.

Peroxidation of CL by ROS is seen as the primary cause of CL mobilization to the outer leaflet of the inner membrane. Human leukemia cells treated with the apoptosis-inducing drug staurosporine rapidly underwent apoptosis along with an increase in CL content in the outer mitochondrial membrane (Garcia Fernandez et al., 2002). However, the change in CL content was preceded by increased ROS production and CL peroxidation, suggesting that perturbation of CL metabolism could be an early step in mitochondria-
induced apoptosis. Due to the high content of unsaturated fatty acyl chains, CL is particularly susceptible to peroxidation (Ferlini et al., 1999; Ushmorov et al., 1999). Peroxidation of CL alters the molecular conformation leading to formation of non-bilayer hexagonal structures, which could serve as a marker for targeting the cytosolic apoptotic machinery to the mitochondria (Aguilar et al., 1999).
iii) **CL in mitochondrial dysfunction and aging.** Under normal physiological conditions, the damaged fatty acyl chains of CL may be replaced through the remodeling process (Malhotra et al., 2009). Pathological remodeling of CL has been linked to mitochondrial dysfunction in human diseases (Han et al., 2007; Lee et al., 2006; Li et al., 2010; Saini-Chohan et al., 2009). As discussed above, loss of the CL remodeling enzyme tafazzin leads to BTHS. Recent studies have shown that ALCAT1 may be involved in the pathological remodeling of CL in cells undergoing oxidative stress. As mentioned earlier, *ALCAT1* overexpression leads to a decrease in CL levels and aberrant remodeling of CL with long chain polyunsaturated acyl chains such as DHA, which are highly susceptible to oxidation by ROS (Hong et al., 2002; Li et al., 2010; Watkins et al., 1998). The close proximity of CL to respiratory complexes in the inner membrane where ROS is generated increases exposure of these long chain unsaturated fatty acyl chains to ROS. Aberrant CL remodeling resulting from increased *ALCAT1* expression leads to the mitochondrial dysfunction seen in pathological conditions such as hyperthyroid cardiomyopathy, diabetes, and diet-induced obesity in mice (Cao et al., 2009; Li et al., 2012; Li et al., 2010; Liu et al., 2012). ALCAT1 null mice exhibit increased expression of MLCAT1 along with elevated levels of CL containing linoleic acid. These findings underscore the significance of CL remodeling and the impact of this process on mitochondrial function and ROS generation.

A decline in CL levels appears to be a primary feature of aging (Lenaz et al., 1997; Lewin and Timiras, 1984; Maftah et al., 1994; Paradies and Ruggiero,
1990; Paradies et al., 1993; Paradies et al., 1997). In aging cells, CL is pathologically remodeled with polyunsaturated fatty acyl chains such as arachidonic and docosahexaenoic acids, which are more susceptible to peroxidation than linoleic acid in normal CL (Lee et al., 2006; Sparagna and Lesnefsky, 2009). Mitochondrial CL levels, along with oxidative capacity and ATP synthesis, decrease significantly with age (Hagen et al., 1998a; Hoch, 1992; Maftah et al., 1994; Sen et al., 2007; Sen et al., 2006).

CL is required for the optimal function of several mitochondrial carrier proteins involved in the transport of essential metabolites into mitochondria (Claypool, 2009). In the heart, oxidation of pyruvate and β-oxidation of fatty acids are two major sources of ATP generation (Christe and Rodgers, 1994; Davila-Roman et al., 2002; Wisneski et al., 1985). The transport of pyruvate into mitochondria by the pyruvate carrier and the exchange of carnitine esters by the carnitine:acylcarnitine translocase are, therefore, critical for energy metabolism. Studies have demonstrated that enzymatic activities of both the mitochondrial pyruvate carrier and carnitine:acylcarnitine translocase, which are dependent on CL (Nalecz et al., 1986; Noel and Pande, 1986), are decreased in aging heart muscle (Paradies et al., 1999a; Paradies et al., 1995). Interestingly, administration of acetyl-L-carnitine in aged rats restored decreased CL levels and the activities of the mitochondrial pyruvate carrier and carnitine:acylcarnitine translocase to levels found in young rats (Paradies et al., 1999a; Paradies et al., 1995). Dietary supplementation of acetyl-L-carnitine also showed similar beneficial effects, increasing mitochondria membrane potential and, in turn,
improving physical mobility in aged rats (Hagen et al., 1998a; Hagen et al., 1998b). These findings suggest that the supply of carnitine to the mitochondria may become limited during aging, hindering energy production through β-oxidation (Maccari et al., 1990; Paradies et al., 1995). Although acetyl-L-carnitine supplementation restored CL levels and improved mitochondrial metabolic functions in aged animals, the underlying molecular mechanism remains unresolved.

5. CL and the PKC-Slt2 cell integrity pathway:

Null mutants in yeast have been characterized for each step of the CL biosynthetic pathway, and mutants blocked earlier in the pathway have more severe phenotypes. Thus, the pgs1Δ mutant, which cannot synthesize CL or the precursor PG (Fig. 1.1), exhibits severe growth defects not only in non-fermentable carbon sources, which are metabolized by respiration, but also in fermentable carbon sources, in which respiration is not required (Chang et al., 1998a; Dzugasova et al., 1998). This observation indicated that PG and/or CL are required for cellular functions apart from mitochondrial bioenergetics (Jiang et al., 1999). Genetic studies to isolate spontaneous suppressors of the pgs1Δ mutant growth defect identified a loss of function mutation of KRE5, a gene involved in cell wall biogenesis (Zhong et al., 2005). Consistent with defective cell wall biogenesis, the pgs1Δ mutant exhibited enlarged cell size characteristic of cell wall mutants, reduced levels of β-1,3-glucan as a result of decreased activity of glucan synthase, and sensitivity to cell wall perturbing agents (de Nobel et al.,
2000; Popolo et al., 1993; Zhong and Greenberg, 2005). These defects were restored by disruption of KRE5 in pgs1Δ, which increased expression of the genes FKS1 and FKS2 encoding glucan synthase (Zhong et al., 2007). These findings were in agreement with the identification of PGS1 in a screen to identify genes involved in cell wall biogenesis (Lussier et al., 1997).

Studies to gain insight into the mechanism linking CL to the cell wall focused on the PKC-Slt2 cell integrity pathway. Activation of the cell integrity pathway is triggered by signals generated from cell wall sensor proteins to Rom2, which, in turn, activates formation of the GTP-bound form of Rho1p. The activated Rho1 protein transmits a signal to Pkc1 to trigger the Mpk1/Slt2 MAPK signaling cascade, which results in dual phosphorylation of Slt2 (Heinisch et al., 1999; Levin, 2005). The dual phosphorylation of Slt2 is essential to activate transcription factors that up-regulate genes involved in cell wall remodeling, particularly in response to heat stress (de Nobel et al., 2000; Jung and Levin, 1999; Terashima et al., 2000). The pgs1Δ mutant exhibited defective activation of the PKC-Slt2 cell-integrity signaling cascade, indicated by decreased Slt2 phosphorylation levels (Zhong et al., 2007). Consistent with this, overexpression of individual genes in the PKC-Slt2 pathway rescued the growth defect of pgs1Δ at elevated temperature and improved resistance to the cell wall perturbing chemicals calcofluor white and caffeine. Interestingly, deletion of KRE5 in pgs1Δ also led to increased activation of the PKC-Slt2 cell-integrity pathway.

A mitochondrial connection to the cell wall is not new. Genome-wide screens have identified several yeast genes required for mitochondrial function
that, when mutated, affect chemical components of the cell wall (Conde et al., 2003; Lussier et al., 1997; Page et al., 2003). Furthermore, mitochondrial respiratory defects negatively impact the synthesis of cell wall components (Lussier et al., 1997; Page et al., 2003). The underlying mechanism whereby CL regulates cell wall remodeling is not known. One possibility is that CL is required for activity of one or more proteins that exhibit dual localization in the cell wall/plasma membrane and mitochondria (Velours et al., 2002). Interesting possibilities include three proteins of the PKC-Slt2 cell integrity pathway, Fks1, Zeo1 and Rho1, which are found both in the mitochondria and the plasma membrane (Green et al., 2003; Sickmann et al., 2003; Zahedi et al., 2006). Mitochondrial targeting of these proteins may be CL-dependent. Alternatively, their stability in the mitochondrial membrane may be decreased in the absence of CL.

The yeast cell wall also plays an important role in regulating replicative life span (Kaeberlein and Guarente, 2002). Consistent with this, the pgs1Δ mutant, which exhibits cell wall defects, also has a decreased replicative life span (Zhong et al., 2005; Zhou et al., 2009). Intriguingly, experiments to elucidate the mechanism linking PG/CL to defects in the cell wall, PKC/Slt2 signaling and aging led to another signaling pathway – the HOG stress response pathway.

6. **CL and the HOG stress response pathway:**

In response to stress, cells are regulated by the opposing actions of the PKC-Slt2 and HOG signaling pathways (Hahn and Thiele, 2002; Hayashi and
Maeda, 2006; Winkler et al., 2002). Heat or low osmolarity stress leads to activation of the PKC-Slt2 pathway, resulting in increased expression of the cell wall remodeling genes leading to a decrease in turgor pressure (Davenport et al., 1995; Garcia-Rodriguez et al., 2005; Hohmann, 2002). In contrast, activation of the HOG signaling pathway causes an increase in turgor pressure (Hohmann, 2002; Rep et al., 2000). Because the *pgs1Δ* mutant exhibited defective activation of the PKC-Slt2 signaling cascade, it was hypothesized that growth defects of the mutant resulted from increased turgor pressure, which may be rescued by down-regulation of the HOG pathway (Fig. 1.3) (Zhou et al., 2009). This hypothesis was supported by the finding that deletion of *SHO1*, an upstream activator of HOG signaling, rescued growth defects, increased the replicative life span, and alleviated sensitivity to cell wall perturbing agents in *pgs1Δ* (Zhou et al., 2009).

Interestingly, the mutant did not exhibit increased activation of the HOG pathway. It is possible that, in the absence of PKC-Slt2 activation, even wild type levels of HOG activation lead to turgor pressure levels that affect growth. These findings suggest that homeostasis achieved by these two signaling pathways is perturbed upon CL deficiency (Fig. 1.3).

7. **CL mediates cross-talk between mitochondria and vacuole:**

The yeast *crd1Δ* mutant, which lacks CL, was shown to have defective vacuolar function (Chen et al., 2008b). CL deficiency caused decreased V-ATPase activity and proton pumping, reduced vacuolar acidification, and enlargement of the vacuole. The yeast vacuole plays a crucial role in adjusting to
Figure 1.3. CL deficiency leads to perturbation of PKC-Slt2 and HOG signaling pathways. The PKC-Slt2 and HOG signaling pathways coordinately regulate cell wall biogenesis and intracellular turgor pressure, respectively. Under hypertonic or cold stress conditions, extracellular osmolarity is increased, causing an efflux of intracellular water to reduce the turgor pressure on the cell wall. To counteract augmented extracellular osmolarity, the HOG pathway is activated, which leads to an increase in intracellular turgor pressure. In contrast, under heat or hypotonic stress, extracellular osmolarity is decreased, which causes an influx of water inside the cell to increase intracellular turgor pressure. To counteract the increased turgor pressure, the activated PKC-Slt2 pathway induces cell wall synthesis. We hypothesize that disruption of the CL pathway by mutation of PGS1 generates a signal that is detected by regulators or components of the PKC-Slt2 pathway, which, in turn, down-regulates the pathway. Under these conditions, an increase in intracellular turgor pressure by activation of the HOG pathway is deleterious in pgs1Δ cells.
high external osmolarity and decreased turgor pressure, and in maintaining cytosolic ion concentrations (Klionsky et al., 1990; Latterich and Watson, 1993). Consistent with perturbation of intracellular osmotic balance in the crd1Δ mutant, growth and vacuolar defects were rescued by supplementation of sorbitol (Chen et al., 2008b).

In some genetic backgrounds, the crd1Δ mutant exhibits increased expression of RTG2, a critical sensor of mitochondrial dysfunction that relays metabolic defects to the nucleus via the retrograde signaling pathway (Butow and Avadhani, 2004; Liu and Butow, 2006). Consistent with overactivation of Rtg2, deletion of the RTG2 gene restored vacuolar acidification and V-ATPase activity and rescued the growth defect of the crd1Δ mutant at elevated temperature. However, deletion of the retrograde pathway activator RTG3 did not rescue the mutant, suggesting that the defects observed in crd1Δ resulted from Rtg2 functions unrelated to retrograde activation.

One possible explanation for the vacuolar defects in crd1Δ is that the loss of CL leads to intracellular osmotic imbalance, as suggested by the enlarged cell size of the mutant (Fig. 1.4). Consistent with this, deletion of the NHX1 gene (but not any of the other vacuole ion transporters) in crd1Δ restored vacuolar morphology to wild type levels (Chen et al., 2008b). Nhx1 is the Na+/H+ exchanger located in late endosomal/prevacuolar membranes, and is involved in the export of protons in exchange for cytosolic Na⁺ or K⁺ (Ali et al., 2004; Brett et al., 2005).
Figure 1.4. Proposed models to explain the role of CL in vacuolar function.

It is likely that CL is transported to the vacuole through mitophagy, the selective degradation of mitochondria via the autophagosome, which delivers its cargo to the vacuole. (A) Under normal physiological conditions, CL may provide stability to the V-ATPase, which is essential to maintain its activity. (B) CL deficiency may lead to perturbation of mitophagy, which results in decreased delivery of CL to the vacuole and, subsequently, to destabilization of the V-ATPase, decreased ATPase activity, and enlargement of the vacuole.
Another possibility is that CL may regulate vacuolar function by directly activating the V-ATPase (Fig. 1.4). While CL is predominantly found in the mitochondrial membranes, significant amounts are also detected in the vacuolar membrane, and the levels vary depending on the carbon source of the growth media (Zinser et al., 1991). How does CL, which is synthesized in the mitochondria, get to the vacuole? The most likely mechanism is via selective degradation of the mitochondria by the autophagic process known as mitophagy, which is strongly induced in yeast by nutrient starvation and during the stationary growth phase (Kanki et al., 2009; Komatsu et al., 2005; Lemasters, 2005; Shintani et al., 2002; Takeshige et al., 1992; Tal et al., 2007). CL that has integrated into the vacuolar membrane as a result of mitophagy may directly activate the V-ATPase and/or stabilize the protein. This possibility is highly speculative at this stage, as such interactions have not yet been reported.

Cross-talk between mitochondria and vacuole is further supported by a recent finding, which showed that the vacuolar pH is a determinant of mitochondrial function and aging in yeast cells (Hughes and Gottschling, 2012). Aging yeast cells exhibit a decline in vacuolar acidity, which causes mitochondrial dysfunction and a decrease in replicative life-span (Hughes and Gottschling, 2012). Consistent with this, enhancing vacuolar acidity by overexpressing VMA1 or VPH2, which encode proteins that regulate V-ATPase activity, suppressed mitochondrial dysfunction. The mechanisms underlying the interplay between vacuole and mitochondria, and the role of CL in this process, remain to be elucidated.
8. CL and iron homeostasis:

In addition to the cellular functions mentioned above, our recent studies, which are the focus of this dissertation, suggest that CL is required for maintaining mitochondrial and cytosolic iron homeostasis (Patil et al., 2012). Iron is an essential metal that is utilized for the synthesis of two crucial cellular components, heme and iron-sulfur (Fe-S) clusters (Kaplan et al., 2006; Ye and Rouault, 2010). Both heme and Fe-S clusters are incorporated into proteins that participate in cellular functions that include respiration, DNA replication, and synthesis of cholesterol and amino acids (Philpott et al., 2012; Shakoury-Elizeh et al., 2010).

Iron exists in two oxidation states; in the ferric (Fe$^{3+}$) form it is insoluble whereas in the ferrous (Fe$^{2+}$) form it is highly reactive. While sufficient iron is essential, excess iron is extremely toxic (Hentze et al., 2004). Therefore, cellular iron must be constantly chaperoned, and its acquisition and transport tightly regulated. To acquire iron, yeast employs high and low affinity iron uptake systems, which are collectively referred, the iron regulon (Kosman, 2003). Expression of the iron regulon is controlled by the iron-responsive transcription factors Aft1 and its parologue, Aft2 (Rutherford and Bird, 2004; Rutherford et al., 2003; Yamaguchi-Iwai et al., 1995). Aft1 and Aft2 are the direct sensors of mitochondrial Fe-S biogenesis, and they respond to iron deficiency or impairment of mitochondrial Fe-S clusters by up-regulating the iron regulon, which leads to an increase in iron-uptake (Hausmann et al., 2008; Kaplan et al., 2006).
Fe-S cluster biogenesis in the mitochondria occurs via a complex pathway composed of two stages (Rawat and Stemmler, 2011). First, Fe-S clusters are assembled on the Isu scaffold, process in which iron (Fe) is delivered by Yfh1 and sulfur (S) is provided by the Nfs1-Isd11 complex. Second, the 2Fe-2S clusters formed on Isu1 are transferred to apoenzymes to form holoenzymes by a process catalyzed by Ssq1/Grx5/Jac1/Mge1 proteins.

The optimal function of several mitochondrial Fe-S proteins, including aconitase and succinate dehydrogenase of the TCA cycle and respiratory complex III, depends on Fe-S biogenesis (Gerber et al., 2004; Lange et al., 2000; Muhlenhoff et al., 2002). Similarly, mitochondrial Fe-S 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 (Gerber et al., 2004; Lange et al., 2000; Muhlenhoff et al., 2002). Perturbations of mitochondrial Fe-S biogenesis also lead to defective maturation of cytosolic Fe-S proteins. The studies described in chapter two demonstrate that CL deficiency leads to perturbation of mitochondrial Fe-S biogenesis, which results in decreased activity of both mitochondrial and cytosolic Fe-S proteins.

9. Project outline:

Although disruption of CL synthesis due to mutations in tafazzin leads to BTHS, it is unclear how CL deficiency contributes to the pathology observed. As discussed above, studies primarily in yeast have enhanced our understanding of the cellular role of CL. Yet, very little is known of the biochemical processes that
are disrupted in BTHS patients. To gain insight into CL functions that might explain the pathology and the variable clinical phenotypes observed in BTHS, genome-wide expression profiling was carried out in the yeast CL mutant crd1Δ. The most intriguing finding in this study was increased expression of the iron uptake genes in the mutant, suggesting either iron deficiency or perturbation of processes involving mitochondrial Fe-S cluster biogenesis and/or export of extra-mitochondrial Fe-S co-factors.

The data presented in chapter two demonstrates that CL deficiency leads to perturbation of iron homeostasis due to defective Fe-S cluster biogenesis in the mitochondria. As an indication of this, the crd1Δ mutant exhibits elevated mitochondrial iron levels along with decreased activity of enzymes that contain Fe-S clusters. The synthetic genetic interaction of crd1Δ with the mitochondrial Fe-S scaffolding protein Isu1 suggests that the underlying mechanism for perturbation of iron homeostasis is defective synthesis of Fe-S clusters. These findings suggest that iron homeostasis could be an important physiological modifier that contributes to the phenotypes observed in BTHS patients.

Because the synthesis of several amino acids is dependent on Fe-S enzymes, perturbation of Fe-S biogenesis would be predicted to cause amino acid deficiencies. The data presented in chapter three show that a decrease in the activities of Fe-S enzymes aconitase and sulfite reductase in crd1Δ leads to auxotrophies for both glutamate and cysteine. Interestingly, these two amino acids are required for the synthesis of an essential antioxidant, glutathione (GSH), a tripeptide of glutamate, cysteine, and glycine. Consistent with
decreased synthesis of GSH, the growth defect of crd1Δ at elevated temperature is rescued by GSH supplementation. These findings suggest that the underlying mechanism for GSH deficiency in crd1Δ is defective mitochondrial Fe-S biogenesis, which results in depletion of precursors for GSH synthesis.

In order to identify the cellular functions that are perturbed in the absence of CL, a screen of suppressors of the crd1Δ mutant was carried out, which is described in chapter four. Briefly, the yeast genomic DNA library was overexpressed in crd1Δ to identify genes that restore the growth of crd1Δ at elevated temperature. In this screen, 50 putative suppressors of crd1Δ were obtained. Interestingly, one of the suppressor genes was LEU2, which encodes an enzyme involved in leucine biosynthesis, a process that is perturbed in crd1Δ (described in chapter two). This supports the finding that perturbation of leucine synthesis likely contributes to the growth defect of crd1Δ.

Although the data presented in this thesis describe new cellular functions of CL, the mechanism underlying the role played by CL in these processes remains to be answered. These questions and future studies are described in chapter five.
CHAPTER 2

LOSS OF CARDIOLIPIN LEADS TO PERTURBATION OF MITOCHONDRIAL AND CELLULAR IRON HOMEOSTASIS

The work described in this chapter has been published in the *Journal of Biological Chemistry*, **288**(3): 1696-1705, 2013.

INTRODUCTION

Cardiolipin (CL) is a structurally and functionally unique phospholipid that is almost exclusively present in mitochondrial membranes (Schlame and Ren, 2009; Schlame et al., 2000). 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 (Claypool et al., 2008; Jiang et al., 2000; Koshkin and Greenberg, 2000; Koshkin and Greenberg, 2002). In the inner membrane, CL is tightly associated with several proteins in respiratory complexes I, III, and IV (Joshi et al., 2009). CL is essential for the stability of respiratory chain supercomplexes which, in yeast, are comprised of dimeric ubiquinol-cytochrome c oxidoreductase (complex III) and one or two complexes of cytochrome c oxidase (complex IV) (Cruciat et al., 2000; Pfeiffer et al., 2003; Zhang et al., 2002b). Perturbation of CL synthesis due to mutations in the CL remodeling enzyme tafazzin causes the severe human genetic disorder known as Barth syndrome (BTHS) (Bione et al., 1996b).
Tafazzin (Taz1) deficiency in yeast leads to biochemical and bioenergetic defects similar to those seen in BTHS patients (Brandner et al., 2005; Gu et al., 2004; Ma et al., 2004; McKenzie et al., 2006).

While perturbation of CL synthesis due to loss of tafazzin leads to cardio- and skeletal myopathy, neutropenia, and growth retardation in BTHS (Barth et al., 1999b), the clinical presentation of this disorder is highly variable, ranging from neonatal death to lack of clinical symptoms (Barth et al., 1999b; Bleyl et al., 1997). In order 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 (Kosman, 2003). 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 upregulation of the iron regulon (Hausmann et al., 2008; Rutherford et al., 2005).

The assembly of Fe-S clusters from ferrous (Fe^{2+}) and sulfide (S^{2-}) ions does not occur spontaneously in living cells, as 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 (Lill and Muhlenhoff, 2006). The assembly of Fe-S clusters in the mitochondria begins on the highly conserved
scaffolding protein Isu1 and its homolog Isu2 (Muhlenhoff et al., 2003). The Nfs1-Isd11 complex delivers sulfur (Muhlenhoff et al., 2004; Zheng et al., 1994), and Yfh1 donates iron (Fe\(^{2+}\)) to the Isu scaffold (Zhang et al., 2006). This process also includes ferredoxin (Arh1) and ferredoxin reductase (Yah1), which provide electrons for the reduction of sulfur to sulfide (Lange et al., 2000; Li et al., 2001). 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 (Lill and Muhlenhoff, 2006).

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 (Kispal et al., 1999; Lange et al., 2001b; Rissler et al., 2005). 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 (Gerber et al., 2004; Hausmann et al., 2008; Kaut et al., 2000; Kispal et al., 1999; Li et al., 1999; Muhlenhoff et al., 2002). In addition, excess mitochondrial iron causes oxidative damage to Fe-S clusters due to the formation of reactive oxygen species (ROS) (Bulteau et al., 2007; Moreno-Cerneno et al., 2010; Vasquez-Vivar et al., 2000).
In the current 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 FeSO$_4$ supplementation and the ROS inducing agent H$_2$O$_2$. 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, *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 *YAP1*, which regulates expression of antioxidant genes (Inoue et al., 1999; Lee et al., 1999; Sugiyama et al., 2000). 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.
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/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), and uracil (20 mg/l), yeast nitrogen base without amino acids (Difco, Detroit, MI), 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.
Table 2.1. Yeast strains and plasmids used in this study.

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<th>Strain</th>
<th>Genotype</th>
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<td>(Wemmie et al., 1994)</td>
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</table>
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'-GGAAACACAACGGATCCACATATTTAACC-3') and PstI-tagged primer *ISU1*-PstIR (5'-GATCTTGTTCTGCAGCCGTTATCTTCTT-3'). Similarly, a 2098 bp sequence containing the entire open reading frame of *ATM1* was amplified using NotI-tagged primer *ATM1*-NotIF (5'-TTGATAGATGCGGCCGCAACCTGCAAATG-3') and PstI-tagged primer *ATM1*-PstIR (5'-TACATGTCTGCAGCAAATATTACTTACGAGCG-3'). The PCR products were ligated to pCM182 (a low-copy number plasmid with selectable marker *TRP1*) downstream of the *TET<sub>OFF</sub>* 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 (Chen et al., 1992). A high-copy number YEp351-*YAP1* overexpression plasmid was a kind gift from W. Scott Moye-Rowley (Univ. of Iowa) (Wemmie et al., 1994).

**Microarray analysis**

Yeast cells were grown to the early stationary phase in YPD, and total RNA was isolated by hot phenol extraction (Kohrer and Domdey, 1991). 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, M5G 2C4). 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) (R_Development_Core_Team, 2005; Smyth, 2005; Smyth, 2004; Smyth et al., 2005; Smyth and Speed, 2003). Slide intensity data was 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 4 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 (http://www.ncbi.nlm.nih.gov/geo, 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 PCR reactions 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 2.2. ACT1 was used as the internal control, and the RNA level of the gene of interest was normalized to ACT1 levels. PCR reactions 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 previously described (Diekert et al., 2001). Briefly, spheroplasts obtained by zymolase treatment were disrupted by Dounce homogenization, and mitochondria were isolated by differential centrifugation. Total protein concentration was determined with a Bradford assay kit (BioRad), 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-dichlorophenol-indolphenol (DCPIP). The absorbance decrease at A₆₀₀ was recorded as a reporter of decylubiquinone reduction (Pierik et al., 2009). Ubiquinol-cytochrome c oxidoreductase activity was assayed by monitoring reduction of cytochrome c at A₅₅₀ (Atkinson et al., 2011).

Cell extracts were prepared by resuspending cells in 500 µl 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
<table>
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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} (Pierik et al., 2009). Sulfite reductase was assayed by monitoring methylene blue formation at A_{670} from sulfide produced by NADPH-dependent sulfite reduction (Pierik et al., 2009; Rutherford et al., 2005). Isopropylmalate isomerase was assayed by monitoring formation of isopropylmalate at A_{235} from dehydration of 3-isopropylmalate (Pierik et al., 2009). Statistical significance of all enzyme assay results was determined by an analysis of variance (ANOVA) and Bonferroni’s posthoc 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-Aldrich) gradient (14% and 22%). Mitochondria (0.25 mg 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 (ICP-OES).
RESULTS

Loss of CL leads to increased expression of iron uptake genes:

In order 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 (Chang et al., 1998b; Jiang et al., 1997; Tuller et al., 1998). The microarray analysis revealed increased expression of genes involved in iron homeostasis in the crd1Δ mutant (Patil et al., 2012). 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 in the "Materials and Methods." As seen in Fig. 2.1, the mRNA levels of AFT1, FIT1-3, FET3, FTR1 and ARN1-3 were upregulated more than 3-fold in crd1Δ. Up-regulation of AFT1 and the iron regulon genes in the crd1Δ 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 (Chen et al., 2004; Hausmann et al., 2008; Kispal et al., 1999).

Perturbation of iron homeostasis in crd1Δ:

We quantified mitochondrial iron using ICP-OES (inductively coupled plasma-optical emission spectroscopy) and found that iron levels in the crd1Δ mutant were significantly increased by 33% relative to WT levels (Fig. 2.2). This
Figure 2.1. Increased expression of the iron regulon in crd1Δ. 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 ± SE (n=6).
Figure 2.2. Increased mitochondrial iron levels in \textit{crd1\Delta}. (This experiment was carried out in the laboratory of Dr. Dennis R. Winge, University of Utah Health Sciences Center) Cells were grown in YP-galactose at 30°C to the logarithmic phase, and mitochondrial iron levels were determined by ICP-OES. Data shown are mean ± SE (n=9). The asterisk indicates a significant difference relative to WT.
result suggested that the iron regulon in *crd1Δ* is upregulated 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 (Garland et al., 1999; Hausmann et al., 2008; Kispal et al., 1997; Knight et al., 1998; Li et al., 1999; Muhlenhoff et al., 2002).

Perturbation of mitochondrial Fe-S biogenesis leads to growth sensitivity in the presence of FeSO₄ (Foury, 1999; Foury et al., 2007; Leidgens et al., 2010; Ramazzotti et al., 2004). As seen in Fig. 2.3B-C, *crd1Δ* cells showed growth sensitivity to 5 mM and 10 mM FeSO₄. This sensitivity to iron supplementation was observed when *crd1Δ* cells were grown in galactose (respiro-fermentative) and ethanol (respiratory) but not in glucose (fermentable) media (Fig. 2.3A). This is most likely because cells have a greater demand for iron in respiratory and respiro-fermentative media, in order to synthesize heme and Fe-S containing proteins involved in oxidative phosphorylation (Garber Morales et al., 2010; Kaplan et al., 2006).

Increased mitochondrial iron levels cause hypersensitivity to oxidative stress (Babcock et al., 1997; Muhlenhoff et al., 2002; Schilke et al., 1999), which may be reflected in sensitivity to ROS inducing agents. Consistent with increased oxidative stress, *crd1Δ* cells exhibited increased sensitivity to the ROS-inducing agent H₂O₂ (Fig. 2.3D). 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.
Figure 2.3. Sensitivity of *crd1Δ* to iron and H$_2$O$_2$. Cells were precultured in YPD overnight, serially diluted, spotted on SD plates containing (A) glucose + FeSO$_4$, (B) galactose + FeSO$_4$, (C) ethanol + FeSO$_4$, and (D) galactose + H$_2$O$_2$, and incubated at 30°C for 5 days.
Fe-S deficiencies in crd1Δ:

We explored the possibility that the iron homeostasis defects in the crd1Δ 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 (Gerber et al., 2004; Lange et al., 2000; Muhlenhoff et al., 2002). To this end, we assayed the activities of the Fe-S enzymes succinate dehydrogenase, ubiquinol-cytochrome c oxidoreductase, and aconitase in crd1Δ. As seen in Table 2.3, these enzyme activities were decreased by approximately 36%, 45%, and 78% respectively, in the crd1Δ mutant. In addition, cytochrome c oxidase activity was decreased by 30% in the crd1Δ mutant (data not shown), consistent with previous studies (Fry and Green, 1980; Jiang et al., 2000; Robinson, 1993; Sedlak and Robinson, 1999). The decreased activities of mitochondrial Fe-S proteins in crd1Δ are not due to reduced transcription of SDH2, RIP1, and ACO1 (Patil et al., 2012). These results indicate that CL is required for the 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 (Gerber et al., 2004; Kispal et al., 1999; Lange et al., 2000; Muhlenhoff et al., 2002). 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 alpha-
isopropylmalate and beta-isopropylmalate (Hsu and Schimmel, 1984; Masselot and De Robichon-Szulmajster, 1975; Masselot and Surdin-Kerjan, 1977; Skala et al., 1991). Sulfite reductase and isopropylmalate isomerase each contain a 4Fe-4S cluster (Balk et al., 2005; Crane et al., 1997; Yoshimoto and Sato, 1968). As seen in Table 2.3, 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. 2.4A, crd1Δ was auxotrophic for methionine at elevated temperature. The crd1Δ mutant also exhibited a ~49% decrease in activity of the leucine biosynthetic pathway enzyme isopropylmalate isomerase (Table 2.3) along with leucine auxotrophy at elevated temperature (Fig. 2.4B). 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 (Barros and Nobrega, 1999; Li et al., 1999; Manzella et al., 1998; Wiedemann et al., 2006). YFH1 deletion mutants are viable in some genetic backgrounds, but exhibit severe
Table 2.3. Decreased mitochondrial and cytosolic Fe-S enzyme activities in *crd1Δ*. Cells were grown in galactose medium and activities of Fe-S enzymes were assayed as described in the ‘Materials and Methods’. Data shown are mean ± SD (n≥4).

<table>
<thead>
<tr>
<th>Mitochondrial Fe-S enzymes</th>
<th>% activity in <em>crd1Δ</em> relative to WT</th>
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<tbody>
<tr>
<td>Succinate dehydrogenase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.4 ± 9.5%</td>
</tr>
<tr>
<td>Ubiquinol cytochrome c oxidoreductase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>54.2 ± 13.8%</td>
</tr>
<tr>
<td>Aconitase&lt;sup&gt;a&lt;/sup&gt;</td>
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<th>Cytosolic Fe-S enzymes</th>
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</thead>
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<tr>
<td>Sulfite reductase</td>
<td>54.2 ± 1.81%</td>
</tr>
<tr>
<td>Isopropylmalate isomerase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.76 ± 9.27%</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> These cells were grown at 34°C.

<sup>c</sup> These assays were carried out in the laboratory of Dr. Dennis R. Winge, University of Utah Health Sciences Center.
Figure 2.4. Methionine and leucine auxotrophy in \textit{crd1}\textdelta. (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.
growth defects (Lesuisse et al., 2003; Zhang et al., 2005b). However, $ISU1$ and $ISU2$ both encode the mitochondrial Fe-S scaffolding protein and have overlapping functions. Single mutants $isu1\Delta$ and $isu2\Delta$ do not show growth defects, as the presence of either Isu1 or Isu2 is sufficient for survival, but deletion of both genes is lethal (Gerber et al., 2004; Schilke et al., 1999). The $crd1\Delta isu1\Delta$ double mutant showed a synthetic growth defect in galactose media, but $crd1\Delta isu2\Delta$ grew normally (Fig. 2.5A). Isu1 is a more abundant scaffolding protein than Isu2 (Andrew et al., 2008), which likely accounts for the more severe phenotypic defect of the $crd1\Delta isu1\Delta$ mutant. To confirm that genetic defects observed in the $crd1\Delta isu1\Delta$ 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, this overexpression of $ISU1$ in the $crd1\Delta isu1\Delta$ double mutant reversed the growth defect (Fig. 2.5B).

The genetic interaction between $crd1\Delta$ and $isu1\Delta$ is consistent with perturbation of Fe-S biogenesis in $crd1\Delta$ 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 $isu1\Delta$ mutation.

**Increased expression of $ATM1$ does not rescue iron defects in $crd1\Delta$:**

The inner membrane protein Atm1, which is involved in the export of Fe-S co-factors from mitochondria, is activated by CL (Kuhnke et al., 2006): the in vitro activity of Atm1 is $\sim50\%$ lower in the absence of CL. If upregulation of the iron regulon resulted from reduced Atm1 activity, then increasing Atm1 levels in
Figure 2.5. Genetic interaction between \textit{crd1\Delta} and \textit{isu1\Delta}. (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.
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. 2.6). In addition, others have shown that loss of ATM1 does not affect the activities of aconitase and succinate dehydrogenase (Kispal et al., 1999). 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 (Moreno-Cermeño et al., 2010; Vasquez-Vivar et al., 2000). In a previous study, we showed that the loss of CL leads to decreased stability of respiratory supercomplexes (Pfeiffer et al., 2003), which is expected to cause increased ROS formation. Consistent with this, protein carbonylation, a sensitive marker of intracellular ROS, was significantly increased in crd1Δ (Chen et al., 2008a). 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 (Lee et al., 1999; Wemmie et al., 1997). In response to H2O2, Yap1 positively regulates genes that affect glutathione metabolism (GSH1,
Figure 2.6. Overexpression of *ATM1* in *crd1Δ* 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 ± SE (n=6).
GLR1, ZWF1), catalase (CTT1), cytosolic thioredoxins (TRR1, TRX2), glutathione peroxidases (GPX1, GPX2), and superoxide dismutases (SOD1, SOD2) (Jamieson, 1998; Lee et al., 1999; Lucau-Danila et al., 2005; Sugiyama et al., 2000). As seen in Fig. 2.7, 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.
Figure 2.7. Overexpression of *YAP1* in *crd1Δ* does not alleviate the iron-related growth phenotypes. Cells were precultured in SD media overnight, serially diluted and spotted on SD galactose plates (A) supplemented with FeSO$_4$, (B) supplemented with H$_2$O$_2$, and (C) lacking methionine. Plates were incubated for 5 days.
Figure 2.8. Model: Perturbation of Fe-S biogenesis in crd1Δ. 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.
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. While 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. 2.6). 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 (Kispal et al., 1999). 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. 2.7). 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 (Chen et al., 2008a). In addition, expression of antioxidant genes is not increased during these growth conditions in crd1Δ (Patil et al., 2012). 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 (Kumar et al., 2011; Sipos et al., 2002). While 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 crd1Δ (Sipos et al., 2002). 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 (Shakoury-Elizeh et al., 2004; Vanoni and Curti,
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 (Davis-Kaplan et al., 2004; Radisky et al., 1997). However, defective vacuolar function is not a likely cause of upregulation of the iron regulon in crd1Δ cells because, in the current study, mitochondria from crd1Δ 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 (Kutik et al., 2008; Tamura et al., 2009). We have shown that crd1Δ cells exhibit defective import of precursor proteins into mitochondria (Jiang et al., 2000; Kutik et al., 2008). 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 (Diaz de la Loza Mdel et al., 2011; Sanjuan Szklarz et al., 2005). Mitochondria from the ZIM17 mutant exhibit decreased protein import due to aggregation of Pam16, Ssc1, and Ssq1 proteins (Burri et al., 2004; Diaz de la Loza Mdel et al., 2011; Sanjuan Szklarz et al., 2005; Yamamoto et al., 2005). Aggregation of both Ssc1 and Ssq1 result in decreased Fe-S biogenesis,
leading to upregulation 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. 2.8). 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 aconitase in skeletal muscle, causing cardiomyopathy, lactic acidosis, muscle weakness, and exercise intolerance (Hall et al., 1993; Haller et al., 1991; Kollberg et al., 2009; Mochel et al., 2008). Depletion of several proteins of the Fe-S biosynthetic machinery severely affects mitochondrial inner membrane structure and cristae morphology (Biederbick et al., 2006; Sheftel et al., 2012), similar to what has been observed in the lymphoblasts of BTHS patients (Acehan et al., 2007). In addition, deficiency of frataxin, which is involved in mitochondrial Fe-S biogenesis, is characterized by
hypertrophic cardiomyopathy and heart failure (Durr et al., 1996). Interestingly, overexpression of frataxin leads to increased mitochondrial membrane potential, elevated ATP levels, resistance to oxidative stress, and life-span extension (Ristow et al., 2000; Runko et al., 2008), defects which are characteristic of CL deficiency (Joshi et al., 2009). In transgenic mice, overexpression of frataxin counteracted cardiotoxic stress, preventing cardiomyopathy and cardiac failure (Schulz et al., 2010). We suggest that mitochondrial iron homeostasis may be an important physiological modifier that contributes to the phenotypes observed in BTHS patients.
CHAPTER 3

LOSS OF CARDIOLIPIN LEADS TO GLUTATHIONE DEFICIENCY

INTRODUCTION

In the studies discussed in the previous chapter, I demonstrated that CL deficiency leads to perturbation of iron homeostasis due to decreased Fe-S biogenesis (Patil et al., 2012). The majority of iron taken up by yeast cells is utilized for the synthesis of Fe-S clusters, which are incorporated as co-factors into iron-dependent enzymes (Kaplan et al., 2006). In addition to enzymes required for mitochondrial bioenergetics, several amino acid biosynthetic enzymes are dependent on Fe-S clusters (Lill and Muhlenhoff, 2008). In yeast, iron-dependent enzymes involved in amino acid synthesis include aconitase (Aco1), glutamate synthase (Glt1), sulfite reductase (Met5), dihydroxyacid dehydratase (Ilv3), isopropylmalate isomerase (Leu1), and homoaconitase (Lys4) (Lill and Muhlenhoff, 2008). Therefore, decreased Fe-S biogenesis is expected to disrupt amino acid synthesis, causing severe metabolic perturbations.

As discussed in chapter two, I have shown that the crd1Δ mutant exhibits decreased activity of aconitase (Patil et al., 2012). Aconitase catalyzes the conversion of citrate to isocitrate, which is converted to α-ketoglutarate by isocitrate dehydrogenase (Cupp and McAlister-Henn, 1991; Cupp and McAlister-Henn, 1992). α-ketoglutarate is an essential substrate for the synthesis of glutamate (Chen et al., 2005; Gangloff et al., 1990). A decrease in α-
ketoglutarate is expected to cause depletion of glutamate. Sulfite reductase, which catalyzes the conversion of sulfite to sulfide, is essential for the synthesis of both methionine and cysteine (Masselot and De Robichon-Szulmajster, 1975; Masselot and Surdin-Kerjan, 1977). The *crd1Δ* mutant exhibits decreased activity of sulfite reductase along with auxotrophy for methionine (Patil et al., 2012).

Depletion of these amino acids is expected to decrease the synthesis of glutathione (GSH), an essential antioxidant. GSH is synthesized in the cytosol from glutamate, cysteine, and glycine, through sequential reactions catalyzed by Gsh1 (γ-glutamyl cysteine synthase) and Gsh2 (glutathione synthase) (Inoue et al., 1998; Ohtake and Yabuuchi, 1991) (Fig. 3.1). The synthesis of GSH is under the regulatory control of the transcription factor Yap1 (Lee et al., 1999; Sugiyama et al., 2000; Wu and Moye-Rowley, 1994). 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 (Sugiyama et al., 2000; Wu and Moye-Rowley, 1994).

GSH is the major free thiol that functions to maintain a reducing environment and to combat oxidative stress (Kumar et al., 2011; Meister, 1988; Penninckx, 2002). GSH acts as a co-factor for antioxidant enzymes such as glutathione peroxidase, which utilize GSH to neutralize the reactive oxygen species (ROS) (Jamieson, 1998). The antioxidant function of GSH depends on the active thiol group (-SH) (Jamieson, 1998). GSH donates electrons necessary to reduce the ROS, and in turn, is oxidized to glutathione disulfide (GSSG). To regenerate GSH, glutathione reductase (Glr1) catalyzes the reduction of GSSG,
Figure 3.1. The GSH biosynthetic pathway and the redox cycle in yeast.

Enzymes are indicated in red, and intermediate steps are shown in blue.
and NADPH for this reaction is provided by glucose 6-phosphate dehydrogenase (Zwf1) (Grant, 2001; Izawa et al., 1998; Nogae and Johnston, 1990; Outten and Culotta, 2004; Outten et al., 2005) (Fig. 3.1). The depletion of GSH leads to increased sensitivity to oxidants such as hydrogen peroxide (H$_2$O$_2$), while elimination of GSH synthesis by the deletion of $GSH1$ leads to growth arrest (Jamieson, 1998). Therefore, to maintain vital cellular functions, intracellular GSH levels must be rigorously maintained by de novo synthesis, redox cycling, or uptake from the growth media.

Because activities of Fe-S enzymes involved in the synthesis of glutamate and cysteine are decreased, I hypothesized that growth defects observed in $crd1\Delta$ cells are due to depletion of glutamate and cysteine, which leads to GSH deficiency. Consistent with this prediction, growth of $crd1\Delta$ at elevated temperature is restored by supplementation of both glutamate and cysteine or GSH. The data presented in this study show for the first time that CL is required for the synthesis of GSH.
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), and uracil (20 mg/l), yeast nitrogen base without amino acids (Difco, Detroit, MI), and carbon source (fermentative) glucose (2%) or (respiratory) glycerol (3%) plus ethanol (1%) or (respiro-fermentative) galactose (2%). SD drop out medium contained all of the above-mentioned ingredients except for the indicated amino acid. For testing iron sensitivity, the growth media was supplemented with 1 µM CuSO₄ and FeSO₄ was solubilized in 0.1 N HCl, filter-sterilized and added to the culture medium at the indicated concentration. GSH stock (250 mM) was prepared in distilled water, filter-sterilized and diluted to achieve the desired final concentration.

Construction of double mutants

The CL mutant strain *crd1Δ::URA3 MATα* was crossed with mutants of the opposite mating type obtained from the yeast deletion collection (Invitrogen). Heterozygous diploids were selected on SD dropout media lacking methionine and lysine, sporulated, and tetrads were dissected. Synthetic interactions between CL and deletion mutants was determined by comparing growth of the double mutant to isogenic parent and wild type strains on YPD.
Table 3.1. Yeast strains and plasmids used in this study.

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<th>Strain</th>
<th>Genotype</th>
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<td>FGY3</td>
<td>MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1</td>
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<td>FGY2</td>
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<tr>
<td>glr1Δ</td>
<td>MATa, his 301, leu 200, met 1500, ura 300, glr1Δ::KanMX4, crd1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>VGY1</td>
<td>MATα, his 301, leu 200, lys 200, ura 300, zwf1Δ::KanMX4, crd1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>zwf1Δ</td>
<td>MATα, his 301, leu 200, met 1500, ura 300, zwf1Δ::KanMX4, crd1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YEpl51</td>
<td>High-copy number plasmid, LEU2 nutritional marker</td>
<td>(Hill et al., 1986)</td>
</tr>
<tr>
<td>YEpl51-YAP1</td>
<td>Derivative of YEpl51, expresses YAP1 from the native promoter</td>
<td>(Wemmie et al., 1994)</td>
</tr>
</tbody>
</table>
Mitochondria isolation

Mitochondria were isolated from cell lysates prepared as previously described (Diekert et al., 2001). Briefly, spheroplasts obtained by zymolase treatment were disrupted by Dounce homogenization, and mitochondria were isolated by differential centrifugation. Total protein concentration was determined with a Bradford assay kit (BioRad), with BSA as the standard.

SDS-PAGE and western blot analyses of mitochondrial Fe-S assembly proteins

Mitochondria isolated from cells grown to an $A_{550}$ of 1.0 were lysed in sample buffer (250 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 20% β-mercaptoethanol) by heating the lysate to 95°C for 5 mins. The lysate was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane as described in Zhong et al. (2007). The blots were probed with polyclonal primary antibodies (rabbit) against Yfh1 and Nfs1 (gifts from Dr. Andrew Dancis), and Isu and porin (gifts from Dr. Roland Lill). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (Promega) were visualized with ECL Prime chemiluminescence detecting reagents.
RESULTS

Growth of crd1Δ at elevated temperature is restored by supplementation with GSH or with glutamate and cysteine:

The data presented in the previous chapter showed that the loss of CL leads to decreased activities of Fe-S enzymes aconitase and sulfite reductase (Patil et al., 2012). Because these enzymes are essential for the synthesis of glutamate and the sulfur-containing amino acids (methionine or cysteine), respectively, I hypothesized that the growth defect in crd1Δ at elevated temperature may be due to depletion of both glutamate and cysteine. Consistent with this prediction, the growth of crd1Δ at elevated temperature is restored by supplementation of glutamate and cysteine (Fig. 3.2). Supplementation of cysteine alone partially rescued the growth defect in crd1Δ, but glutamate alone did not (Fig. 3.3). This suggests that cysteine, and to a lesser extent, glutamate levels are decreased in the crd1Δ mutant.

Both glutamate and cysteine are essential precursors for the synthesis of GSH. Because crd1Δ cells are auxotrophic for glutamate and cysteine, I further predicted that the growth defect in crd1Δ at elevated temperature is due to GSH deficiency. Consistent with this prediction, the growth defect of crd1Δ was rescued by GSH supplementation (Fig. 3.4). These results indicate that GSH deficiency in the CL mutant is due to the depletion of both glutamate and cysteine.
Figure 3.2. Supplementation of glutamate and cysteine restored the growth of \textit{crd1\Delta} at elevated temperature. Cells were precultured in SD galactose overnight, serially diluted, and spotted on SD galactose plates containing the indicated amounts of glutamate and cysteine, and incubated at 37°C for 4 days.
Figure 3.3. Supplementation of cysteine partially rescued the growth of \textit{crd1}\(\Delta\) at elevated temperature. Cells were precultured in SD galactose overnight, serially diluted, and spotted on SD galactose plates containing the indicated amounts of (A) glutamate or (B) cysteine, and incubated at 37°C for 4 days.
Figure 3.4. GSH supplementation restored the growth of *crd1Δ* at elevated temperature. 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.
Overexpression of YAP1 did not rescue growth of crd1Δ at elevated temperature:

GSH is synthesized from glutamate, cysteine, and glycine, through sequential reactions catalyzed by Gsh1 and Gsh2 (Inoue et al., 1998; Ohtake and Yabuuchi, 1991). Expression of both GSH1 and GSH2 is regulated by the transcription factor Yap1 (Sugiyama et al., 2000). Consistent with this, overexpression of YAP1 activates the transcription of GSH1 and GSH2, thereby increasing GSH levels (Sugiyama et al., 2000). To address the possibility that GSH deficiency results from decreased GSH biosynthetic enzymes, YAP1 was overexpressed in crd1Δ cells. In contrast to GSH supplementation, overexpression of YAP1 did not rescue the growth of crd1Δ at elevated temperature (Fig. 3.5). This suggests that GSH deficiency in crd1Δ does not result from deficient enzymes for the synthesis of GSH.

GSH supplementation alleviates growth sensitivity of crd1Δ to oxidants:

Previous studies have shown that the loss of CL leads to increased oxidative stress, and that the crd1Δ mutant is sensitive to growth in the presence of iron and H₂O₂ (Chen et al., 2008a; Patil et al., 2012). In yeast cells, GSH is the most abundant antioxidant molecule that is essential to maintain resistance to oxidants (Jamieson, 1998). Consistent with this, GSH deficient mutants are hypersensitive to H₂O₂ (Jamieson, 1998). To address the possibility that GSH deficiency in crd1Δ may account for growth sensitivity of the mutant to iron and H₂O₂, crd1Δ cells were grown in the presence of 7 mM FeSO₄ or 350 mM H₂O₂,
Figure 3.5. Overexpression of YAP1 did not alleviate the growth defect of *crd1Δ* at elevated temperature. Cells were precultured in SD galactose overnight, serially diluted, spotted on SD galactose plates lacking leucine, and incubated for 4-5 days.
in the presence or absence of 1 mM GSH. GSH supplementation significantly improved the growth of \textit{crd1Δ} in the presence of iron or H$_2$O$_2$ (Fig. 3.6). This suggests that the growth sensitivity of \textit{crd1Δ} to oxidants is due to GSH deficiency.

**Genetic interaction between CRD1 and GSH metabolic pathway genes:**

If the loss of CL leads to GSH deficiency, which accounts for the increased ROS, then further perturbation of GSH synthesis could be expected to exacerbate the growth defect of \textit{crd1Δ}. Consistent with this prediction, deletion of \textit{GSH1} in \textit{crd1Δ} cells leads to synthetic lethality (Fig. 3.7). The genetic interaction between \textit{CRD1} and \textit{GSH1} is consistent with increased oxidative stress in \textit{crd1Δ}, which is further exacerbated by the deletion of \textit{GSH1}.

To preserve a reducing environment, GSH must be maintained in the reduced form, by converting GSSG to GSH. For redox cycling of GSSG to GSH, both glutathione reductase (Glr1) and glucose 6-phosphate dehydrogenase (Zwf1), which provides NADPH for the reaction are required (Grant, 2001; Izawa et al., 1998; Nogae and Johnston, 1990; Outten and Culotta, 2004; Outten et al., 2005). Deletion of either \textit{GLR1} or \textit{ZWF1} is expected to disrupt regeneration of GSH and exacerbate the growth of \textit{crd1Δ}. Consistent with this prediction, deletion of \textit{GLR1} or \textit{ZWF1} in \textit{crd1Δ} cells leads to synthetic sick interactions in galactose-containing media at 39°C. (Fig. 3.8). The synthetic interaction of \textit{crd1Δ} with \textit{zwf1Δ} is stronger than with \textit{glr1Δ}, which suggests that, in addition to alterations in GSH, cytosolic NADPH levels may be decreased in \textit{crd1Δ}. The
Figure 3.6. GSH supplementation alleviated growth sensitivity of crd1Δ to H₂O₂ and FeSO₄. Cells were precultured in YPD overnight, 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.
Figure 3.7. Genetic interaction between \textit{crd1}\Delta and \textit{gsh1}\Delta. Cells were precultured in YPD overnight, serially diluted, spotted on YPD and SD glucose, and incubated at 30°C.
Figure 3.8. Genetic interaction between \textit{crd1Δ}, \textit{zwf1Δ}, and \textit{glr1Δ}. Cells were precultured in YPD overnight, serially diluted, spotted on SD galactose plates, and incubated at 30°C and 39°C.
synthetic genetic interactions of $gsh1\Delta$, $zwf1\Delta$, and $glr1\Delta$ with $crd1\Delta$ indicate that perturbation of GSH metabolism in the CL mutant further contributes to the oxidative stress, which exacerbates the growth defect.

**Loss of CL leads to decreased Yfh1 levels:**

The most likely explanation for GSH deficiency in $crd1\Delta$ cells is decreased maturation of Fe-S proteins aconitase and sulfite reductase, which are essential for the synthesis of glutamate and cysteine (Gerber et al., 2004; Kispal et al., 1999; Lange et al., 2000; Muhlenhoff et al., 2002). To test the possibility that the protein(s) required for mitochondrial Fe-S cluster assembly are decreased in $crd1\Delta$, I carried out a Western blot analysis of the steady-state levels of three core components involved in mitochondrial Fe-S assembly, Yfh1, Nfs1, and Isu. As seen in Fig. 3.9, Yfh1, the putative iron donor for mitochondrial Fe-S assembly, is decreased in $crd1\Delta$. However, Nfs1 and Isu levels are unaltered in the mutant. This suggests that the expression, import or stability of Yfh1 is decreased in $crd1\Delta$, which may account for defective mitochondrial Fe-S biogenesis and GSH deficiency.
Figure 3.9. Decreased Yfh1 in crd1Δ. Western blot analysis of Nfs1, Yfh1, and Isu proteins from mitochondrial extracts of cells grown in SD galactose at 35°C. Por1 was used as a loading-control.
Figure 3.10. Proposed model for GSH deficiency in \textit{crd1}\textgreek{A}. Loss of CL leads decreased Yfh1 protein, causing perturbation of mitochondrial Fe-S biogenesis. Defective Fe-S biogenesis leads to elevated mitochondrial iron levels and decreased maturation of aconitase (Aco1) and sulfite reductase (SiR), which results in reduced synthesis of glutamate and cysteine, precursors required for GSH synthesis. Decreased GSH levels and elevated mitochondrial iron contribute to an increase in ROS, which inhibits the growth of \textit{crd1}\textgreek{A}. 
In this study, we provide evidence that perturbation of CL synthesis leads to GSH deficiency: 1) growth of \textit{crd1Δ} at elevated temperature is restored by supplementation of GSH and GSH precursors; 2) GSH supplementation restored the growth of \textit{crd1Δ} in the presence of oxidants; 3) deletion of genes for GSH synthesis are synthetically lethal with \textit{crd1Δ}; and 4) Yfh1 levels are decreased, suggesting that mitochondrial Fe-S cluster assembly is reduced in \textit{crd1Δ}. Collectively, these findings indicate that GSH deficiency in \textit{crd1Δ} is due to the depletion of precursors for GSH synthesis, which is caused by defective Fe-S biogenesis.

The finding that GSH supplementation alleviated the growth defect of \textit{crd1Δ} at elevated temperature suggests that GSH levels in \textit{crd1Δ} are decreased. To maintain high intracellular GSH concentrations, yeast cells can take up GSH from the growth media or synthesize it through the \textit{de novo} pathway. In synthetic dropout media, which is devoid of specific amino acids and GSH, yeast cells depend entirely on intracellular GSH synthesis from glutamate, cysteine, and glycine. Auxotrophies for glutamate and cysteine suggest that synthesis of both amino acids are decreased in \textit{crd1Δ} (Fig. 3.2), which is further supported by the finding that overexpression of \textit{YAP1} did not restore the growth of \textit{crd1Δ} at elevated temperature (Fig. 3.5) or improve resistance of \textit{crd1Δ} to iron and H$_2$O$_2$ (Patil et al., 2012).
GSH supplementation restored the growth of crd1Δ in presence of both iron and H₂O₂ (Fig. 3.6), which suggests that, compared to WT cells, the CL mutant is unable to increase GSH synthesis to efficiently neutralize the ROS. This is supported by findings that mutations in genes for GSH synthesis and redox cycling lead to synthetic interactions with crd1Δ, which indicates that oxidative stress in the crd1Δ mutant is further exacerbated by perturbations in GSH synthesis (Figs. 3.7 and 3.8).

A decrease in Yfh1 protein in crd1Δ suggests that the transport of iron to the Isu scaffold may be defective, which is consistent with decreased activity of Fe-S enzymes and perturbation of mitochondrial Fe-S biogenesis (Patil et al., 2012). Several possibilities may account for decreased Yfh1 protein in crd1Δ. Previous studies have shown that perturbation of CL synthesis leads to decreased mitochondrial protein import (Gebert et al., 2009; Jiang et al., 2000). Consistent with this, the import of Yfh1 in CL-deficient mitochondria may be decreased. Alternatively, the stability of the protein may be decreased, leading to increased protein turnover. Yfh1 is localized in close proximity to the CL-enriched mitochondrial inner membrane (Campuzano et al., 1997). Interaction of Yfh1 with CL and/or inner membrane protein subunits of succinate dehydrogenase may be required for Yfh1 function (Gonzalez-Cabo et al., 2005).

Based on the data obtained, I propose the following model to depict the role of CL in regulating GSH homeostasis (Fig. 3.10). Perturbation of CL synthesis leads to decreased Yfh1 protein, which disrupts mitochondrial Fe-S biogenesis. Defective Fe-S biogenesis leads to elevated mitochondrial iron levels
and decreased maturation of aconitase and sulfite reductase, which results in reduced synthesis of glutamate and cysteine, precursors required for GSH synthesis. Decreased GSH synthesis and elevated mitochondrial iron contribute to the ROS generated, which inhibits the growth of \textit{crd1Δ}.

The data presented here suggest that CL is a critical regulator of amino acid synthesis and GSH homeostasis. These findings could be potentially used to develop treatments for Barth syndrome (BTHS) patients, as early reports indicate that BTHS patients exhibit metabolic deficiencies similar to those identified in CL-deficient yeast cells (Dr. Richard Kelley, Kennedy Krieger Institute, John Hopkins). Mass spectrometric analyses of the plasma from BTHS patients indicate that the TCA cycle intermediate \textit{cis}-aconitate is elevated. \textit{Cis}-aconitate is an intermediate formed during the conversion of citrate to isocitrate (Krebs and Holzach, 1952). Elevated \textit{cis}-aconitate levels suggest that the enzymatic activity of aconitase may be decreased. BTHS patients also exhibit significant health improvement when orally supplemented with methionine, cysteine, or GSH (personal communication, Dr. Richard Kelley), which would be consistent with depletion of sulfur-amino acids in BTHS patients. In summary, the findings reported in this study and the preliminary data obtained from the studies of BTHS patients indicate that GSH could be an important physiological modifier of CL deficiency in BTHS.
CHAPTER 4

SUPPRESSORS OF *CRD1Δ* GROWTH DEFICIENCY AT ELEVATED TEMPERATURE

INTRODUCTION

The primary hurdle for the effective treatment of BTHS is that the mechanisms linking the pathology and CL deficiency are not known. In addition, the clinical phenotypes of BTHS vary widely, even among patients with the identical mutation, suggesting that genetic and physiological factors exacerbate the consequences of perturbation of CL synthesis. Therefore, there is an imminent need to determine cellular functions of CL.

To obtain an understanding of CL functions that might explain the cellular defects in BTHS, a screen was carried out to identify genes that, when overexpressed, compensate for the loss of CL. A previous screen for suppressors of growth defects of the *pgs1Δ* mutant led to the identification of an unexpected role of PG and/or CL in regulating cell wall biogenesis (Zhong and Greenberg, 2005). A loss-of-function mutation in the gene *KRE5* restored the growth of *pgs1Δ* by strengthening the cell wall, demonstrating that PG and/or CL mediate cross-talk between the mitochondria and the cell wall. The current study was carried out to identify suppressors of *crd1Δ*. The *crd1Δ* mutant is unable to form colonies at elevated temperature from single cells on media containing the
fermentable carbon source, glucose (Jiang et al., 1999; Jiang et al., 2000; Zhong et al., 2004). Interestingly, studies described in chapters two and three indicate that CL-deficient cells exhibit perturbation of mitochondrial Fe-S biogenesis, iron homeostasis, GSH deficiency and growth sensitivity to iron and H₂O₂ in galactose-containing media. Although galactose, like glucose, is a fermentable carbon source, ATP synthesis by fermentation alone is not sufficient in galactose-containing media. First, galactose has to be converted to glucose 6-phosphate by the Leloir pathway enzymes, which comprise ~5% of total cellular proteins (Bhat, 2008). Second, 2 ATP/galactose molecule consumed is inadequate to synthesize galactolytic enzymes and support growth at the same time. Therefore, pyruvate generated by fermentation is simultaneously oxidized via the TCA cycle and respiration. In contrast, in glucose-containing media, yeast cells almost entirely use fermentation to generate ATP, and switch to the respiratory mode after glucose is converted to ethanol (Lagunas, 1986). The difference in utilization of these two carbon sources is evident from the doubling time of rho° cells (cells lacking mitochondrial DNA), which is ~70 minutes in glucose and ~140 minutes in galactose (Bhat, 2008). Therefore, to increase stringency of the screen, I identified suppressors of the growth defect on galactose.

The suppressor screen utilizing a high copy genomic DNA library led to the identification of fifty putative suppressors of crd1Δ. Five plasmids were intially sequenced, and interestingly, each plasmid contained the same region of chromosome III, spaning four genes. One of the genes was LEU2, which is
involved in leucine biosynthesis, a process that is perturbed in the $crd1\Delta$ mutant (chapter two). This supports the finding that perturbation of leucine synthesis likely contributes to the growth defect of $crd1\Delta$. 
MATERIALS AND METHODS

Strains and growth media

The yeast strains used in this work are listed in Table 1. The ingredients of synthetic defined (SD) growth media are as described in the previous chapter. SD glucose and SD galactose contain synthetic defined growth media lacking leucine, and either glucose (2%) or galactose (2%).

*Escherichia coli* (*E. coli*) cells were grown in Luria Bertani (LB) media, which is composed of Bacto-tryptone (10 gm/l), yeast extract (5 gm/l), and NaCl (10 gm/l). *E. coli* transformants were selected in LB media containing ampicillin (100 µg/ml).

Transformation and isolation of plasmids

Transformation of plasmids in *E. coli* and yeast were done by electroporation (Delorme, 1989; Woodall, 2003). Plasmids from *E. coli* were isolated using *Wizard® Plus SV Minipreps* (Promega), whereas plasmids from yeast cells were extracted using the *Zymoprep™ Yeast Plasmid Miniprep II kit* (Zymo Research).
Table 4.1. Yeast and *E. coli* strains, and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGY3</td>
<td><em>MATa, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1</em></td>
<td>(Jiang et al., 1997)</td>
</tr>
<tr>
<td>FGY2</td>
<td><em>MATα, ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1, crd1Δ::URA3</em></td>
<td>(Jiang et al., 1997)</td>
</tr>
<tr>
<td>YEpl51</td>
<td>High-copy number plasmid, <em>LEU2</em> nutritional marker</td>
<td>(Hill et al., 1986)</td>
</tr>
<tr>
<td>YEpl51-genomic DNA library</td>
<td>Derivative of YEpl51, 7-10 kb fragments of yeast genomic DNA, expression driven by the native promoter</td>
<td>(Wemmie et al., 1994)</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>fhuA2, lacUΔ169, phoA, glnV44, Φ80', lacZΔM15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17</em></td>
<td>(Taylor et al., 1993)</td>
</tr>
</tbody>
</table>

Table 4.2. Primers used for sequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 F24</td>
<td>Forward</td>
<td>CGCCAGGGTTTTCCCAGTCACGAC</td>
</tr>
<tr>
<td>M13 R22</td>
<td>Reverse</td>
<td>TCACACAGGAACAGCTATGAC</td>
</tr>
</tbody>
</table>
RESULTS

Transformation of the cDNA library and suppressor screening:

The \( crd1\Delta \) mutant exhibits growth deficiency in galactose-containing media, but not in glucose, at elevated temperature (37° - 38°C) (Fig. 4.1). To identify genes that alleviate the growth deficiency of \( crd1\Delta \) at elevated temperature, \( crd1\Delta \) cells were transformed with a yeast genomic DNA library (Delorme, 1989). This library consists of 7-10 kb partially digested genomic DNA fragments inserted into \( Sall \) digested YEp351 (5.644 kb) plasmid. Transformants were selected on SD glucose Leu\(^-\) plates and replicated on SD galactose Leu\(^-\) plates. Plates were incubated at 30° and 38°C for 3-4 days. The colonies that grew at 38°C compared to colonies transformed with empty vector were selected. To eliminate false-positives, suppressors were streaked on fresh SD galactose Leu\(^-\) plates and incubated at 38°C for 3 days (Fig. 4.2). The colonies that grew at 38°C were re-streaked on fresh SD galactose Leu\(^-\) plates and incubated at 38°C for the final selection (Fig. 4.3). The growth of suppressors at each step was compared with the growth of \( crd1\Delta \) cells containing the empty plasmid (negative control) and WT cells containing the empty plasmid (positive control). In this manner, 15,000 transformants were screened and 50 putative suppressors of \( crd1\Delta \) growth deficiency were obtained.

Identification of suppressors of \( crd1\Delta \) growth deficiency:

To isolate the plasmids, the suppressors were streaked on fresh SD glucose Leu\(^-\) plates, and cells from a single colony were inoculated into SD
Figure 4.1. Growth deficiency of crd1Δ in galactose at elevated temperature. Cells were precultured in YPD overnight, serially diluted, spotted on SD plates containing glucose or galactose, and incubated at 30°C or 37°C.
Figure 4.2. Suppressors of crd1Δ growth deficiency. Suppressors were restreaked on SD galactose plates lacking leucine and incubated at 38°C for 3 days. Arrows indicate WT and crd1Δ cells containing the empty plasmid, while suppressors are circled.
Figure 4.3. Confirmation of suppressors of *crd1Δ* growth deficiency. To eliminate false-positives, suppressors (shown in Fig. 4.2) were re-streaked on fresh SD galactose plates lacking leucine and incubated at 38°C for 3 days.
glucose Leu\textsuperscript{+} liquid media and incubated overnight. Because the plasmid yield from yeast was too low for sequencing analysis, plasmids from yeast were transformed into \textit{E. coli} cells, and transformants were selected on LB plates containing ampicillin. As a pilot experiment, five plasmids were submitted to the AGTC sequencing facility (WSU Medical School) along with the sequencing primers that were specifically designed to amplify the inserted genomic DNA fragment (Table 2).

The obtained sequence information was submitted to the Saccharomyces Genome Database (SGD) BLAST (Basic Local Alignment Search Tool) (Fig. 4.4). Unfortunately, the sequence information obtained from the forward primer (M13 F24) did not match any region in the yeast genomic DNA. Therefore, Table 3 sequence data are based only on the M13 region amplified by the reverse primer (M13 R22). Interestingly, each of the five plasmids contain the same region of chromosome III, which comprise the suppressor genes listed in Table 3.

A surprising finding is that \textit{LEU2} may be a suppressor. The genetic background of the \textit{crd1\Delta} strain in the current study includes the \textit{leu2-\Delta1} mutation; thus, the \textit{LEU2} gene on the plasmid was used as the selectable marker for growth at 30°C. However, the plasmid alone did not rescue the growth of the mutant at elevated temperature. Therefore, overexpression of \textit{LEU2} may compensate for the decrease in leucine biosynthesis that characterizes the \textit{crd1\Delta} mutant, as shown in chapter two.
Figure 4.4. Genomic DNA region identified by the reverse sequencing primers. The dotted vertical lines show the region amplified by the sequencing primer, while the green arrow shows the direction of amplification. The genes present on the ‘Watson’ strand are indicated in red, while the genes present on the ‘Crick’ strand are indicated in blue.
Table 4.3. Putative suppressor genes of *crd1Δ* growth deficiency.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strand</th>
<th>Location on Chromosome III</th>
<th>Gene function*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEU2</td>
<td>Watson</td>
<td>91324 → 92418</td>
<td>β-isopropylmalate dehydrogenase, catalyzes the third step of the leucine biosynthetic pathway</td>
</tr>
<tr>
<td>YCL019W</td>
<td>Watson</td>
<td>85102 → 90415</td>
<td>Retrotransposon TYA Gag and TYB Pol genes</td>
</tr>
<tr>
<td>YCL020W</td>
<td>Watson</td>
<td>85102 → 86418</td>
<td>Retrotransposon TYA Gag gene co-transcribed with TYB Pol.</td>
</tr>
<tr>
<td>YCL021W-A</td>
<td>Watson</td>
<td>83620 → 83997</td>
<td>Putative protein of unknown function</td>
</tr>
</tbody>
</table>

*Source*: Saccharomyces Genome Database (www.yeastgenome.org)
DISCUSSION

This study was carried out to identify genes that suppress the growth defect of crd1Δ in galactose-containing media at elevated temperature. In the five sequenced suppressors, sequences containing LEU2 restored growth of crd1Δ at elevated temperature.

LEU2 encodes the enzyme β-isopropylmalate dehydrogenase, which catalyzes the third step of leucine biosynthesis (Andreadis et al., 1984; Kohlhaw, 1988). The data suggest that overexpression of LEU2 rescues the growth defect of crd1Δ, although the control plasmid, which contains the LEU2 gene as a marker, does not. There may be several possible explanations for this finding. Overexpression of LEU2 may cause a two-fold increase in leucine over the control plasmid, which contains one copy of LEU2. To confirm this finding, LEU2 should be overexpressed in a plasmid containing a different nutritional marker. Alternatively, other genes adjacent to LEU2 may suppress the growth defect in crd1Δ (Table 3). These genes are YCL021W-A, an uncharacterized open reading frame (ORF), and YCL019W and YCL020W, which encode the retrotransposon TYA Gag and TYB Pol genes, respectively. TYA Gag and TYB Pol genes are transcribed and translated as one polyprotein, which is processed to generate nucleocapsid-like protein (Gag), reverse transcriptase (RT), protease (PR), and integrase (IN), analogous to retroviral genes. This process may increase chances of insertion of Ty elements (transposon-yeast) into the genome disrupting a gene, which encodes a protein that is deleterious to crd1Δ.
Interestingly, \textit{LEU2} was isolated as a suppressor that restored the growth of \textit{YFH1} point mutants (\textit{yfh1}^{-}) in respiratory conditions (Bedekovics et al., 2011). The data in this study showed that disruption of leucine synthesis by deletion of \textit{LEU1} or \textit{LEU2} downregulated the transcription of \textit{ACO1}, which exacerbated the phenotype of the \textit{yfh1}^{-} strain in respiratory conditions. The strains used in the studies described in chapters two and three are \textit{leu2Δ}. Therefore, an alternative explanation for \textit{LEU2} as a suppressor of \textit{crd1Δ} could be that \textit{leu2Δ} may exacerbate the phenotype of \textit{crd1Δ} at elevated temperature, whereas overexpression of \textit{LEU2} may restore the growth of \textit{crd1Δ} by increasing \textit{ACO1} transcription.

Finally, leucine may be catabolized to replenish acetyl-CoA levels (Gibson et al., 1993; Vorapreeda et al., 2012). Recent findings in the lab suggest that generation of acetyl Co-A through the β-oxidation pathway in the peroxisomes may be defective in \textit{crd1Δ}. Therefore, the growth deficiency of \textit{crd1Δ} at elevated temperature may be due to the depletion of acetyl-CoA. A two-fold increase in leucine by overexpressing two-copies of \textit{LEU2} may augment acetyl-CoA levels in \textit{crd1Δ}, thereby restoring growth at elevated temperature. In addition to these possibilities, sequencing the remaining suppressors may potentially provide insight into other cellular functions that are defective in \textit{crd1Δ}.
CHAPTER 5

FUTURE DIRECTIONS

The data presented in chapters two and three indicate that the loss of CL leads to perturbation of mitochondrial Fe-S cluster biogenesis, which most likely causes GSH deficiency in the CL mutant. However, the underlying mechanism for defective Fe-S cluster biogenesis and GSH deficiency in crd1Δ cells is not understood. In the process of Fe-S biogenesis in mitochondria, iron (Fe) and sulfide (S) atoms are covalently linked to form Fe-S clusters, which are transferred onto recipient proteins. Key details of this process are shown in Fig. 5.1. An exciting question for future studies is how CL plays a role in this process.

1. What is the role of CL in mitochondrial Fe-S cluster biogenesis?

The data presented in chapter two show that the loss of CL leads to defective mitochondrial Fe-S cluster biogenesis, which results in perturbation of mitochondrial and cytosolic iron homeostasis. As an indication of defective Fe-S cluster biogenesis, the activities of mitochondrial and cytosolic Fe-S enzymes are decreased in crd1Δ (Table 2.3). However, it is unclear how CL in the inner membrane is involved in mitochondrial Fe-S cluster biogenesis, which is largely carried out in the matrix. Most importantly, which step(s) of Fe-S cluster synthesis are perturbed in the absence of CL? Is the assembly of Fe-S clusters on the Isu-scaffold or the transfer of Fe-S clusters from the Isu-scaffold to apoproteins decreased in crd1Δ? Perturbation of either of the two steps in crd1Δ
Fig. 5.1. Mitochondrial Fe-S cluster biogenesis in yeast. Step 1: The assembly of Fe-S clusters in mitochondria begins on the Isu1 scaffold. Cysteine desulfurase complex (Nfs1-Isd11) catalyzes the removal of sulfur (S) from cysteine for delivery to Isu1. Iron (Fe) is delivered by the putative iron chaperone protein, frataxin (Yfh1). The source of electrons for the Fe-S assembly is most likely NADH, which is transferred by ferredoxin reductase (Arh1) and ferredoxin (Yah1). Step 2: The 2Fe-2S clusters assembled on Isu1 are transferred to the recipient apoproteins by interaction between Isu1 and Hsp70 chaperone Ssq1, a process that is assisted by Jac1, Mge1, and Grx5 proteins.
may suggest a direct role of CL in Fe-S cluster biogenesis.

2. Why are Yfh1 levels decreased in crd1Δ?

The experiment depicted in Fig. 3.9 indicates that steady-state levels of Yfh1 are decreased in crd1Δ, although Nfs1 and Isu proteins are not altered. What is the cause of decreased Yfh1 in crd1Δ? At least three possibilities may explain this decrease. First, CL is required for optimal protein import, as CL deficient cells exhibit decreased import of precursor proteins into mitochondria (Jiang et al., 2000; Kutik et al., 2008). Therefore, mitochondrial import of Yfh1 may be decreased in crd1Δ. Protein import assays would address this possibility. Alternatively, the mRNA levels of YFH1 in crd1Δ may be decreased. Finally, the stability of Yfh1 in CL-deficient mitochondria should be examined. Any of these mechanisms may explain decreased Yfh1 in crd1Δ cells.

3. CL and glutathione deficiency:

The data presented in chapter three show that the growth defect of crd1Δ at elevated temperature and in the presence of oxidants (iron and hydrogen peroxide) is rescued by GSH supplementation. This suggests that crd1Δ cells are deficient in GSH. I suggest the following experiments to gain insight into the mechanism of GSH deficiency in crd1Δ cells:

I. Amino acid deficiencies in crd1Δ. The data presented in chapter two suggest that glutamate and cysteine are depleted in crd1Δ cells. I have shown that crd1Δ exhibits decreased aconitase activity (Table 2.3), which is
expected to cause depletion of α-ketoglutarate, a precursor required for the synthesis of glutamate. Similarly, decreased activity of sulfite reductase (Table 2.3) is expected to cause depletion of cysteine. These findings are supported by the observation that crd1Δ cells are auxotrophic for both glutamate and cysteine (Fig. 3.2). Because glutamate and cysteine are required for GSH synthesis, I hypothesize that GSH deficiency in crd1Δ is due to depletion of both amino acids. Amino acids analysis to quantify glutamate and cysteine levels in cell extracts from crd1Δ should be performed.

II. How does GSH supplementation restore the growth of crd1Δ at elevated temperature? I suggest two possibilities. First, the synthesis of GSH may be decreased, as mentioned above. Alternatively, ROS produced in crd1Δ cells may exceed the cellular capacity to respond by synthesizing and regenerating GSH. This would be consistent with previous studies showing that crd1Δ exhibits elevated mitochondrial iron levels and increased ROS production (Chen et al., 2008a; Patil et al., 2012). Therefore, quantification of mitochondrial and cytosolic GSH should be carried out to determine if GSH levels or the GSH:GSSG ratio is decreased in crd1Δ. A decrease in GSH levels would suggest that GSH synthesis is perturbed, whereas a decrease in the GSH:GSSG ratio would be consistent with increased oxidative stress due to elevated ROS production.

III. Are activities of Fe-S enzymes restored by GSH supplementation? Previous reports have shown that Fe-S clusters in proteins such as aconitase and isopropylmalate isomerase are particularly sensitive to
degradation by ROS (Kispal et al., 1997; Kispal et al., 1999; Moreno-Cermeno et al., 2010; Vasquez-Vivar et al., 2000). Therefore, if ROS accounts for decreased activities of Fe-S enzymes, then GSH supplementation may restore the activities of aconitase and isopropylmalate isomerase to WT levels.

4. **Suppressors of crd1Δ growth deficiency at elevated temperature:**

In the suppressor screen described in chapter four, fifty putative suppressors of crd1Δ were obtained. From five sequenced suppressor plasmids, **LEU2**, which encodes the β-isopropylmalate dehydrogenase enzyme essential for the synthesis of leucine, was identified. An intriguing question is, how does overexpression of **LEU2** alleviate the growth defect of crd1Δ when the control plasmid (YEp351), which contains the **LEU2** marker, does not? The plasmid used in this study contains a 2-micron origin of replication, which maintains 50-100 copies of the plasmid per cell (Zakian et al., 1979). The **LEU2** insert in suppressors is expected to cause a two-fold increase in leucine content over the control plasmid. This would suggest that supplementation of excess leucine in the growth media may alleviate the growth defect of crd1Δ at elevated temperature.

Interestingly, **LEU2** was obtained as a suppressor that restored the growth of YFH1 point mutants (yfh1Δ) in respiratory conditions (Bedekovics et al., 2011). This study showed that leu2Δ abolished respiratory growth of yfh1, whereas overexpression of **LEU2** restored growth of the mutant by activating expression of ACO1 (Bedekovics et al., 2011). The data presented in chapters two and three show that CL is required for mitochondrial Fe-S biogenesis and CL deficiency
leads to decreased Yfh1 protein, which suggests that in addition to the Fe-S defect, $leu2\Delta$ may exacerbate the phenotype of $crd1\Delta$, causing the growth defect at elevated temperature. Therefore, overexpression of $LEU2$ may restore decreased activity of Aco1 by increasing $ACO1$ transcription. To determine if expression of $ACO1$ and the activity of the enzyme in $crd1\Delta$ are restored to WT levels, $LEU2$ should be overexpressed in a plasmid containing a different nutritional marker (e.g. $URA3$, $TRP1$, or $HIS3$). In addition to elucidating the role of $LEU2$ in $crd1\Delta$, the 45 suppressor plasmids should be sequenced to identify the genes present.

In summary, the findings presented in chapters two and three show for the first time that CL is required for maintaining Fe-S biogenesis and GSH homeostasis. However the mechanism linking CL to these two processes is not known. I invite my hard-working colleagues in the lab to identify the mechanism based on the questions proposed here.
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Cardiolipin (CL) is the signature phospholipid of mitochondrial membranes, where it is synthesized locally and plays a critical role in mitochondrial bioenergetic functions. Inside the mitochondria, CL is a critical target of mitochondrial generated reactive oxygen species (ROS) and regulates signaling events related to apoptosis and aging. CL deficiency causes perturbation of signaling pathways outside the mitochondria, including the PKC-Slt2 cell integrity pathway and the high osmolarity glycerol (HOG) pathway, and is a key player in the cross-talk between the mitochondria and the vacuole. The importance of CL in human health is underscored by the observation that perturbation of CL biosynthesis causes the severe genetic disorder Barth syndrome.

In order to fully understand the cellular response to the loss of CL, genome-wide expression profiling was carried out in the yeast CL mutant crd1Δ. The 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. The crd1Δ mutant exhibits decreased 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, which encode proteins involved in the export of mitochondrial generated Fe-S co-factors to the cytosol and a transcription factor that regulates several antioxidant genes, respectively, 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.

Consistent with the role of CL in mitochondrial Fe-S biogenesis, perturbation of CL synthesis leads to decreased Yfh1 protein, a putative iron donor for mitochondrial Fe-S cluster assembly, and reduced activities of Fe-S enzymes aconitase and sulfite reductase, which are required for the synthesis of glutamate and sulfur-containing amino acids (methionine and cysteine). The data presented in this study show that the synthesis of glutamate and cysteine are decreased in crd1Δ. Interestingly, both amino acids are required for the synthesis of an essential antioxidant, glutathione (GSH), a tripeptide of glutamate, cysteine, and glycine. The growth defect of crd1Δ at elevated temperature and in the
presence of oxidants is rescued by GSH supplementation, which is consistent with decreased synthesis of GSH. Collectively, these findings indicate that GSH deficiency in crd1Δ is due to the depletion of precursors for GSH synthesis, which is caused by defective Fe-S biogenesis.

To obtain an understanding of CL functions that might explain the cellular defects in BTHS, a screen was carried out to identify genes that, when overexpressed, suppress the growth deficiency of crd1Δ in galactose-containing media at elevated temperature. The suppressor screen utilizing a high copy genomic DNA library led to the identification of fifty putative suppressors of crd1Δ. Five plasmids were initially sequenced, and interestingly, each plasmid contained the same region of chromosome III, spanning four genes. One of the genes was LEU2, which is involved in leucine biosynthesis, a process that is perturbed in the crd1Δ mutant. This supports the finding that perturbation of leucine synthesis likely contributes to the growth defect of crd1Δ.
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