1-1-2012

Identification of cellular functions of cardiolipin as physiological modifiers of barth syndrome

Amit Shridhar Joshi
Wayne State University,

IDENTIFICATION OF CELLULAR FUNCTIONS OF CARDIOLIPIN AS PHYSIOLOGICAL MODIFIERS OF BARTH SYNDROME

by

AMIT S. JOSHI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2012

MAJOR: BIOLOGICAL SCIENCES

Approved by:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

To my parents, my sister, and my fiancée, with all my love.

ACKNOWLEDGEMENTS

I owe my deepest gratitude to my mentor, Dr. Miriam L. Greenberg for her continuous support and encouragement during my PhD. I am grateful to her for always being the source of inspiration, confidence, and passion. Her patience, guidance, and motivation have helped me achieve my professional goals. I would also like to extend my sincere gratitude to my committee members, Dr. Maik Hüttemann, Dr. Karen Beningo, and Dr. Victoria Meller for their continuous support and valuable suggestions during my graduate study. I would like to thank Dr. Nikolaus Pfanner for his collaboration and support.

It is my pleasure to thank all the past and current members of the Greenberg laboratory for their support, assistance and above all their friendship. All of you have given me wonderful memories that I will treasure my whole life. Special thanks go to Dr. Guiling Li and Gnanada Kulkarni for guiding me during my rotation in the Greenberg laboratory. I would also like to thank all the rotation, masters and the undergraduate students who worked with me, especially Janani Ganesh and Naomi Fei.

I am grateful to all my friends for their support and encouragement. Cheers to our friendship and togetherness!

Lastly, I would like to thank my parents, Shridhar Joshi and Archana Joshi, my dearest sister Prajakta Joshi-Tanksale for their unconditional love, sacrifices and support. I thank my fiancée, Manasi Apte for her love, support and friendship. Thank you all!!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Parts of this chapter have been published in *Biochemica et Biophysica Acta* **1793, 212-218, 2009.**

Eukaryotic cells require mitochondria to play a vital role in producing cellular energy and in the metabolism of amino acids, lipids, heme and iron. Mitochondria are surrounded by outer and inner membranes, which are made up of a phospholipid bilayer of unique composition and proteins anchored into the membrane. Besides acting as glue for the membrane proteins, the phospholipids play an important role in providing shape to the mitochondria. Cardiolipin (CL) is the signature phospholipid of mitochondria, which is synthesized and is predominantly present in the inner membrane. The work in this thesis identifies key roles of CL in mitochondrial protein import, mitochondrial fusion and metabolic pathways including the TCA cycle, glyoxylate cycle and β-oxidation.

1. Structural and physiological role of cardiolipin

Cardiolipin (CL) (1,3 diphosphatidyl-*sn*-glycerol) is a unique and ubiquitous anionic phospholipid that, in eukaryotes, is localized primarily in the mitochondrial inner membrane. CL was first isolated from beef heart, hence its name (Pangborn, 1948). While it is most abundant in the heart, CL is found in all mammalian tissues. Unlike the other membrane phospholipids, it has a

dimeric structure in which two phosphatidyl moieties are linked by a glycerol (Figure 1.1) (Lecocq and Ballou, 1964). As a result, CL is hydrophobic by virtue of four fatty acyl groups and acidic due to two phosphates. CL interacts with a wide variety of mitochondrial proteins by both hydrophobic and electrostatic interactions (Hoch, 1992; Schlame et al., 2000), and stabilizes proteins in the mitochondrial respiratory chain (Fry and Green, 1981). CL molecules can form lamellar or inverted hexagonal structures. The hexagonal phase is favored in the presence of divalent cations (Vasilenko et al., 1982). Although the biological relevance of these structures is not known, it is plausible that CL is involved in the formation of local non-bilayer structures within biological membranes. Such structures are believed to be involved in membrane fusion and in trans-bilayer movement of solutes (de Kruijff et al., 1985). The finding that mitochondrial biosynthesis of the non-bilayer forming phospholipid phosphatidylethanolamine is essential for the viability of yeast mutants lacking CL suggests a critical role of CL in the formation of these structures (Gohil et al., 2005), although it should be noted that these structures have not been convincingly demonstrated in vivo.

The fatty acid composition of CL plays an important role in the function of the lipid, as aberrant CL remodeling (replacing one fatty acid with another) underlies the genetic disorder Barth syndrome (BTHS) (Schlame et al., 2005). However, no single species of fatty acid is required for function, as the acyl species of CL from different organisms vary considerably. Bacterial CL contains saturated and mono-unsaturated fatty acyl chains 14-19 carbons in length (Kito et al., 1972). Mitochondrial CL is mainly composed of

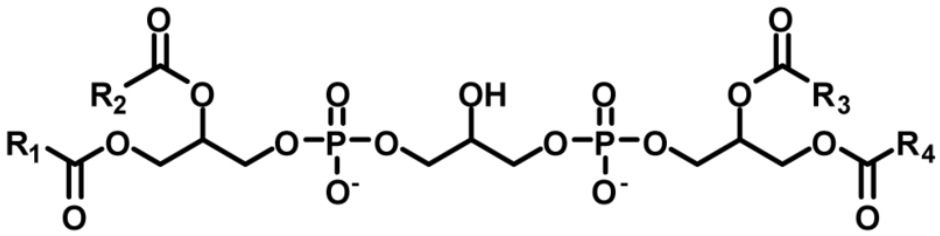


Figure 1.1: Structure of cardiolipin (CL): R1, R2, R3, R4 represent fatty acyl chains.

monounsaturated and diunsaturated fatty acyl chains of 16-18 carbons in length, resulting in a much higher unsaturation index than that of bacterial CL. In mammals and higher plants, the predominant species is linoleic acid, whereas oleic acid and palmitoleic acid species exist in yeast (Schlame et al., 1993). In humans, CL acyl species vary with tissue type, although the predominant species in heart is tetralinoleoyl CL. This species is absent from BTHS cells (Barth et al., 1999b). While specific CL acyl species vary among eukaryotic cardiolipin, the feature shared by different organisms is that the dominant species of CL contains only one or two types of fatty acid. This leads to symmetry and structural uniformity among CL species (Schlame et al., 2005).

The most exciting finding pertaining to CL in the last few years is that this phospholipid plays an important role not only in mitochondrial bioenergetics, which is not unexpected given the interaction of CL with mitochondrial proteins, but also in essential cellular functions not generally associated with respiratory function. Some of these include mitochondrial protein import, mitochondrial fusion, vacuolar functions, cell wall biogenesis, translational regulation of electron transport chain (ETC) components, aging, and apoptosis, and it is likely that this list will be longer in a few years. The importance of CL in these and other processes is underscored by the finding that mutations in tafazzin, the CL remodeling enzyme, lead to BTHS. This chapter focuses on CL biosynthesis and remodeling, cellular functions of CL and its relevance to BTHS.

2. CL biosynthesis and remodeling

All of the enzymes for de novo synthesis of CL are present in the mitochondria. As seen in Figure 1.2, phosphatidylglycerolphosphate (PGP) synthase (Pgs1p) catalyzes the committed step, forming PGP from CDP-DG and glycerol-3-phosphate (G-3-P) (Chang et al., 1998a). PGP is then dephosphorylated to PG by PGP phosphatase Gep4p (PTPMT1 in mammals) (Osman et al., 2010; Zhang et al., 2011). CL synthase (Crd1p) catalyzes an irreversible condensation reaction in which CDP-DG is linked to PG via cleavage of a high-energy anhydride bond to form CL (Chang et al., 1998b; Hostetler et al., 1971; Hostetler et al., 1972; Jiang et al., 1997; Schlame et al., 1993; Tamai and Greenberg, 1990; Tuller et al., 1998). CL then undergoes remodeling in which deacylation by the CL specific deacylase Cld1p leads to the formation of monolysocardiolipin (MLCL) (Beranek et al., 2009). In rat liver, mitochondria associated phospholipase A_2 was shown to catalyze the deacylation step to form MLCL (Hauff and Hatch, 2006). MLCL is then reacylated with another fatty acid (Schlame and Rustow, 1990), which is catalyzed by tafazzin (Taz1p) (Gu et al., 2004). Schlame et al. demonstrated that tafazzin is a CoA-independent transacylase that transfers acyl chains preferentially from phosphatidylcholine (PC) to CL (Schlame and Ren, 2006).

In a study comparing CL species from a wide variety of organisms, Schlame and co-workers showed that the most abundant species of CL contained only one or two types of fatty acids, which results in a high degree of structural uniformity and molecular symmetry in cardiolipin (Schlame et al., 2005). In contrast, tafazzin-deficient cells were characterized by multiple species of CL.

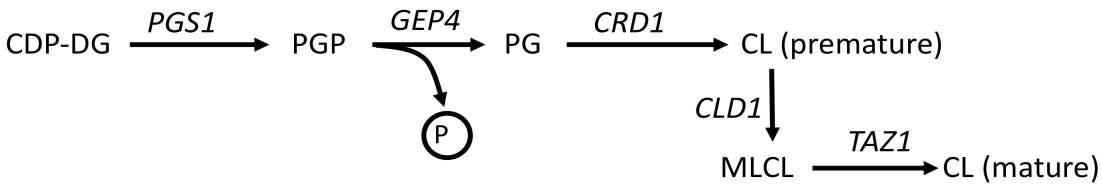


Figure 1.2: Biosynthesis and remodeling of CL: *PGS1* encodes phosphatidylglycerol phosphate (PGP) synthase, which converts glycerol-3-phosphate (G-3-P) and CDPdiacylglycerol (CDP-DAG) to PGP. PGP is dephosphorylated to phosphatidylglycerol (PG) by *GEP4*, which encodes a PGP phosphatase. *CRD1* encodes CL synthase, which converts CDP-DAG and phosphatidylglycerol (PG) to CL. In the remodeling part of the pathway, CL is deacylated to monolysocardiolipin (MLCL) by the CL specific phospholipase Cld1p. MLCL is then reacylated to mature CL by the *TAZ1*-encoded tafazzin.

The accumulation of MLCL in yeast and human tafazzin-deficient cells (Claypool et al., 2006; Gu et al., 2004; Valianpour et al., 2005; Vaz et al., 2003) is consistent with the two-step pathway of remodeling shown in Figure 1.2. BTHS patients display reduced CL, accumulation of MLCL, and aberrant CL species (Hauff and Hatch, 2006). The importance of remodeling is underscored by its role in BTHS, a rare X-linked disorder caused by mutations in tafazzin.

3. CL and BTHS

The CL pathway is crucial for mitochondrial bioenergetics and for essential cellular functions not generally associated with respiration. It is, therefore, not surprising that perturbation of this pathway in humans leads to deleterious consequences. As discussed above, BTHS is a disorder resulting from loss of the CL remodeling enzyme tafazzin. About ten years after the first description of BTHS, the locus was mapped to Xq28 (Bolhuis et al., 1991) and mutations were identified in G4.5, the tafazzin gene (Bione et al., 1996). The link between CL and BTHS was first reported by Peter Vreken and colleagues, who demonstrated that fibroblast cultures from BTHS patients contain less CL than control cultures (Vreken et al., 2000). Furthermore, BTHS cells were defective in acylation of CL and PG with unsaturated fatty acids. Subsequent analysis showed that BTHS cells contained a decrease in total CL content (Vreken et al., 2000) and an accumulation of MLCL (Valianpour et al., 2005). The predominant acyl species, tetralinoleoyl-CL is absent from BTHS cells (Barth et al., 1999b).

Lymphoblast mitochondria from patients with BTHS exhibit hyperproliferation, impaired coupling, and abnormalities in energy metabolism (Xu et al., 2005) consistent with studies in tafazzin deficient yeast (Ma et al., 2004). Due to the adhesion of opposing membranes, the intracrista space in BTHS mitochondria appears to be deformed (Acehan et al., 2007), which may explain the decrease in mitochondrial membrane potential in BTHS (Xu et al., 2005) and in yeast *taz1*Δ (Ma et al., 2004).

Approximately 28 different mutations resulting in single amino acid changes in tafazzin have been identified in BTHS patients (Schlame and Ren, 2006). The mutations result in a complete loss of tafazzin or in expression of a severely truncated protein (Claypool et al., 2006). Interestingly, the clinical presentation of BTHS varies a great deal, from those who have severe incapacitating disease to those who are nearly asymptomatic, even among patients with identical mutations. This variation indicates that physiological modifiers play a significant role in the BTHS phenotype. Thus, while it is clear that tafazzin is a CL transacylase, the cellular consequences of defective tafazzin and the molecular basis underlying the pathologies observed in BTHS patients are not understood. Homologues of human tafazzin are present throughout eukaryotic species from yeast to mammals, and yeast (Claypool et al., 2006; Gu et al., 2004; Vaz et al., 2003), Drosophila (Xu et al., 2006a), zebrafish (Khuchua et al., 2006) and mouse (Acehan et al., 2011) models of BTHS have been characterized. Genetic studies in these model systems will help to elucidate the mechanisms linking tafazzin to the cellular

defects in BTHS, and to identify the physiological modifiers of the BTHS phenotype.

4. Yeast as a model system to elucidate the cellular role of CL

The power of the yeast system in elucidating the function of CL derives from the characterization of the yeast genes that encode Pgs1p, Crd1p, and Taz1p and the availability of null mutants of these genes (Chang et al., 1998a; Chang et al., 1998b; Claypool et al., 2006; Gu et al., 2004; Jiang et al., 1997; Tuller et al., 1998; Vaz et al., 2003). These mutants are powerful molecular tools to elucidate the role of CL in vivo. The yeast *taz1Δ* null mutant exhibits biochemical defects similar to those observed in BTHS (Gu et al., 2004; Li et al., 2007; Schlame et al., 2002; Valianpour et al., 2003; Vreken et al., 2000). These defects are complemented by expression of the human tafazzin gene in the *taz1Δ* mutant (Ma et al., 2004). Many studies with the yeast mutants have shown that the CL pathway is required for optimal mitochondrial function, as discussed below (section 5). The mutants exhibit growth defects with varying degrees of severity on non-fermentable carbon sources. The *taz1Δ* mutant grows poorly on ethanol at elevated temperature (Gu et al., 2004; Jiang et al., 2000), *crd1*Δ exhibits growth defects at elevated temperature on several carbon sources (Jiang et al., 1999; Jiang et al., 2000; Zhong et al., 2004), and *pgs1*Δ cannot grow at all on non-fermentable carbon sources (Chang et al., 1998a; Dzugasova et al., 1998). Interestingly, *pgs1Δ* and *crd1Δ* exhibit growth defects even on glucose, suggesting that the CL pathway is required for essential cellular processes not directly associated

with respiration. The *crd1Δ* mutant exhibits a strain dependent inability to form colonies at elevated temperature on glucose medium, and *pgs1Δ* cannot grow at all at 37^{o}C on glucose unless supplemented with sorbitol (Jiang et al., 1999; Zhong et al., 2004). Moreover, *pgs1Δ* also loses mitochondrial DNA, which may account to some degree for the inability of the mutant to grow on non-fermentable carbon sources. The studies summarized in section 5 describe cellular functions that are perturbed when CL synthesis is blocked.

5. Cellular functions of CL

As discussed above, the loss of CL in yeast leads to growth defects not only in non-fermentable but also in fermentable media, indicating that CL is required for non-mitochondrial functions. Both mitochondrial and non-mitochondrial functions of CL in yeast (Figure 1.3) are discussed in this section.

5.1 CL and mitochondrial bioenergetics: CL is highly enriched in membranes designed to generate an electrochemical gradient for ATP synthesis, such as the bacterial plasma membrane (Dowhan, 1997) and the inner mitochondrial membrane (Daum, 1985). This ubiquitous and intimate association between CL and energy transducing membranes suggests an important role for CL in bioenergetic reactions. CL modulates the catalytic activities of interacting proteins, such as the ADP–ATP carrier (Beyer and Klingenberg, 1985) and/or provides stability, as reported for complex III (Fry and Green, 1980) and complex IV (Sedlak and Robinson, 1999). In vivo

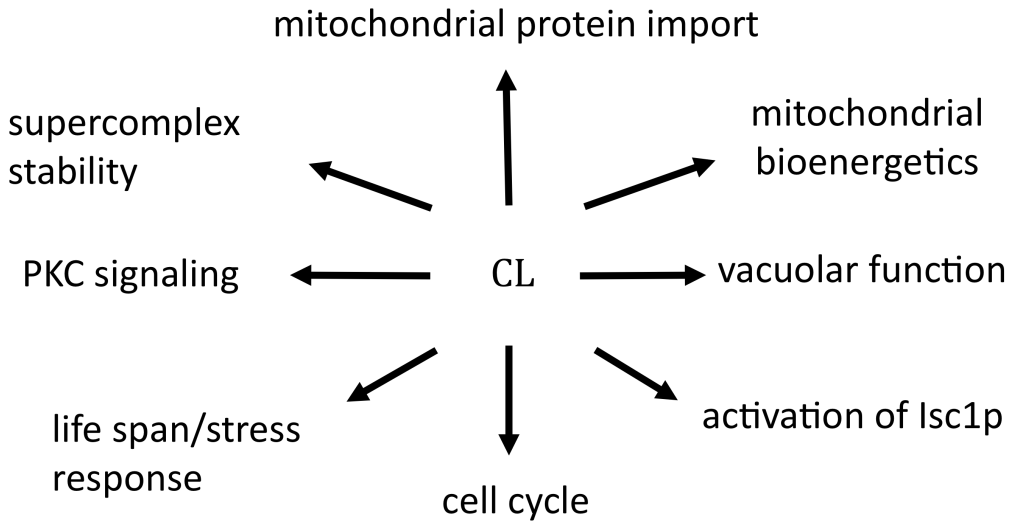


Figure 1.3: Cellular functions of CL in yeast.

studies have shown that loss (*crd1*Δ) and/or decreased CL (*taz1*Δ) results in unstable respiratory chain supercomplexes (McKenzie et al., 2006; Pfeiffer et al., 2003; Xu et al., 2010; Zhang et al., 2005). Unstable supercomplex leads to leakage of electrons that react with oxygen to form reactive oxygen species (ROS). Thus, it is possible that an unstable supercomplex increases oxidative stress that might contribute to the pathogenesis of BTHS. Indeed, during growth on ethanol as sole source of carbon, both *crd1*Δ and *taz1*Δ exhibit an increase in protein carbonylation, an indicator for increased ROS (Chen et al., 2008a).

5.2 CL and mitochondrial protein import: More than 98% of mitochondrial proteins are encoded in the nucleus and synthesized as precursors in the cytosol. These preproteins are imported into the mitochondria via translocases present in the outer and inner mitochondrial membrane (Pfanner et al., 1994). The preproteins are targeted to receptor proteins on the outer mitochondrial membrane and to general import proteins of the TOM complex (Lill and Neupert, 1996). Transport across the inner membrane is mediated by the TIM complex (Lill and Neupert, 1996).

A role for CL in protein import was first suggested by the finding that protein import was blocked when yeast cells were treated with the CL-binding compound doxorubicin (Eilers et al., 1989). Subsequent studies with the *crd1Δ* mutant indicated that the absence of CL leads to a reduced membrane potential and decreased protein import (Jiang et al., 2000). More recently, a reconstitution study indicated that the membrane integration of mitochondrial

preproteins is most efficient when a presequence translocase is reconstituted in CL-containing membranes (van der Laan et al., 2007). The import defect was greater for a preprotein that required a membrane potential.

A link between BTHS and protein import was suggested by the finding that a BTHS-like illness known as dilated cardiomyopathy with ataxia (DCMA) syndrome is caused by mutations in the protein import gene DNAJC19/TIM14 (Davey et al., 2006). Like BTHS, DCMA syndrome is characterized by cardiomyopathy, neutropenia and elevated 3-methylglutaconic acid. The DNAJC19 protein shares sequence similarity with Tim14p, a protein that is associated with the inner mitochondrial membrane motor complex of Tim23p. Because the clinical presentation of DCMA is very similar to that of BTHS, it is interesting to speculate that the defect in BTHS may be caused or exacerbated by defective mitochondrial protein import. Studies discussed in Chapter 2 provide evidence that CL is required for assembly of outer membrane protein translocases (TOM) (Gebert et al., 2009).

5.3 CL is associated with apoptosis and aging: CL binds specifically and irreversibly to cytochrome c (Rytomaa and Kinnunen, 1994), limiting the soluble pool of the protein. Therefore, it may play an important regulatory role in cytochrome c release, which triggers the downstream events in apoptosis (Kagan et al., 2005). A role for CL has been implicated in the inter-related processes of aging and apoptosis (Iverson and Orrenius, 2004; McMillin and Dowhan, 2002; Pollack and Leeuwenburgh, 2001). Loss of CL in yeast leads to decreased replicative life span. These defects were rescued by down

regulating the high osmolarity glycerol stress response pathway or by promoting cell integrity, suggesting that stress response is perturbed in cells lacking CL (Zhou et al., 2009).

5.4 Mitochondrial anionic phospholipids and cell wall biogenesis: Experiments to isolate suppressors of the *pgs1Δ* temperature sensitivity phenotype led to the identification of a loss of function mutant of *KRE5*, a gene involved in cell wall biogenesis (Zhong et al., 2005). Suppression of *pgs1Δ* temperature sensitivity by *kre5Δ* suggested a connection between the CL pathway and cell wall biogenesis (Zhong et al., 2005), consistent with the finding of Lussier et al. that disruption of the *PGS1* promoter leads to hypersensitivity to cell wall perturbing agents such as zymolyase, calcofluor white, papulacandin and caffeine (Lussier et al., 1997). Biochemical analysis of the cell wall of *pgs1Δ* indicated that the mutant has reduced levels of β-1,3-glucan (Zhong et al., 2005). Consistent with this, cytological studies revealed that *pgs1Δ* cells exhibited the enlarged cell phenotype characteristic of cell wall mutants. Levels of β-1, 3-glucan were increased in the *kre5* suppressor mutant, and the phenotypes of temperature sensitivity and enlarged spherical morphology were suppressed by *kre5*. What are the mechanisms linking mitochondrial anionic phospholipids to the cell wall, from which these lipids are absent? A clue to this question comes from the finding that cell wall defects in the *pgs1Δ* mutant are associated with perturbation of the cell integrity pathway (Zhong et al., 2007). In this pathway, activation of Pkc1p in response to cell wall stress results in activation of a cascade of proteins in the

Mpk1/ Slt2 mitogen activated protein kinase (MAPK) pathway, culminating in the activation/dual phosphorylation of Slt2. The dual phosphorylated Slt2p activates transcription factors that up-regulate genes involved in cell wall synthesis (Jung and Levin, 1999; Terashima et al., 2000). Interestingly, the *pgs1Δ* mutant exhibits defective Slt2p activation, which is restored by the *kre5* suppressor (Zhong et al., 2007). The mechanism linking PG/CL to the Slt2 pathway is not known.

5.5 CL, inositol sphingolipid phospholipase C (Isc1p), and translational regulation of electron transport chain (ETC) components: Dowhan et al. made the surprising observation that *pgs1Δ* exhibits defective translational regulation of several mitochondria-encoded ETC components and of Cox4p, a nuclear-encoded component of the ETC (Ostrander et al., 2001). The translational defect resulted from the lack of PG/CL in the mitochondrial membrane, as re-introduction of *PGS1* on a high copy plasmid restored expression of Cox4p. RNA levels were not affected, and in vitro studies indicated that the defect was not due to decreased protein import but rather to a failure of translation (Su and Dowhan, 2006). Deletion analysis of the upstream non-coding region of *COX4* suggested that a cis-acting sequence with two stem-loops in the 5′ UTR appeared to be responsible for inhibition of *COX4* translation. Trans-acting factors that bind to this region have not been identified; however, binding of a protein factor(s) to this sequence was observed with cytoplasm from *pgs1Δ* but not wild type *PGS1* cells, and loss of function mutants that allowed expression of reporter constructs under control

of the *COX4* promoter were isolated. These findings identify a novel cross talk pathway between mitochondria and the nucleus, in which translation of nuclear-encoded proteins destined for the mitochondrial membrane respond to a deficiency of mitochondrial anionic lipids PG and/or CL (Su and Dowhan, 2006).

Defective translation of Cox4p was also seen with the loss of inositol phosphosphingolipid phospholipase C (Isc1p), a member of the family of neutral sphingomyelinases that regulate ceramide synthesis. Isc1p is localized to the mitochondrial outer membrane. Its activity is impaired in the *pgs1Δ* mutant, suggesting that PG/CL is required for activation of this enzyme (Vaena de Avalos et al., 2005). Interestingly, the phenotypic defects of *isc1Δ* and *pgs1Δ* are similar, which suggests that these genes may have overlapping functions. These findings led to the speculation that PG regulates translation of the ETC proteins indirectly by activation of Isc1p.

5.6 CL and the vacuole: Recent studies provide interesting evidence that the loss of CL affects vacuolar function. The loss of CL leads to vacuolar defects, including swollen vacuolar morphology and loss of vacuolar acidification at the non-permissive temperature (Chen et al., 2008b). Consistent with this, the *crd1*Δ mutant showed decreased vacuolar H (+)-ATPase activity and proton pumping. Deletion of *NHX1*, which is required for Na^+ and K^+ ion exchange, and for vacuolar fusion, alleviates the temperature sensitive (ts) phenotype of *crd1*Δ, suggesting that vacuolar function is essential for survival of *crd1*Δ. Interestingly, deletion of *RTG2*, a sensor for mitochondrial dysfunction, also

rescues the ts phenotype and defects in vacuolar morphology of *crd1*Δ mutant cells (Chen et al., 2008b). The role of CL in the cross talk between vacuole and mitochondria is yet unknown.

5.7 CL and the cell cycle: The loss of mitochondrial DNA in cells lacking CL leads to elevated expression of the morphogenesis checkpoint protein Swe1p (Chen et al., 2010b). The elevated expression of Swe1 is most likely due to activation of the retrograde pathway, as deletion of *RTG2* and *RTG3* rescues the elevated expression of Swe1. In addition, deletion of Swe1 in rho^0 cells of the *crd1*Δ mutant rescues the cell cycle defect (Chen et al., 2010b). The mechanistic link between CL and the cell cycle has not been elucidated.

6. Project outline

The objective of the studies described in this thesis is to understand the cellular functions of CL that may identify physiological modifiers of BTHS. Utilizing power of yeast genetics, I showed that CL is required for mitochondrial protein import (Chapter 2), mitochondrial fusion (Chapter 3), and the mitochondrial retrograde pathway (Chapter 4).

As discussed above, BTHS is caused by mutations in tafazzin. BTHS patients exhibit a wide range of clinical symptoms, indicating that physiological modifiers influence the BTHS phenotype. Based on these observations, I hypothesized that the identification of synthetic lethal interactions with CL mutants will identify potential physiological modifiers. Using yeast as my model system, a targeted synthetic lethality screen was

performed with the CL mutants to identify genes that share common pathway/functions with CL. These studies showed that CL mutants genetically interacted with several mutants of mitochondrial protein import and mitochondrial morphology. Studies in Chapter 2 describe the role of CL in protein import and in the maintenance of mitochondrial morphology.

Previous findings have reported that cells lacking both CL and mitochondrial PE are inviable, suggesting an overlapping role of these phospholipids. Because these lipids affect membrane curvature, I hypothesized that they have overlapping roles in mitochondrial fusion. The studies in Chapter 3 show that the loss of both CL and mitochondrial PE leads to highly fragmented mitochondria caused by defective mitochondrial fusion. These results strongly suggest that CL and mitochondrial PE have overlapping functions in mitochondrial fusion and are required to maintain tubular mitochondrial morphology. Thus, Chapter 3 identifies a specific role of CL in mitochondrial fusion, which could be a modifier of tafazzin deficiency in BTHS.

Chapter 4 identifies a role of CL in essential metabolic pathways, including the TCA cycle, β-oxidation pathway and the glyoxylate cycle. Recent findings from the Greenberg lab showed that the loss of CL leads to decreased activities of TCA cycle enzymes aconitase and succinate dehydrogenase (Vinay Patil, unpublished). A block in the TCA cycle leads to activation of the mitochondrial retrograde (RTG) pathway in WT cells. The studies in Chapter 4 test my hypothesis that *crd1*Δ exhibits defective activation of the RTG pathway at elevated temperature. Consistent with this

prediction, I report that the loss of CL leads to metabolic defects that are not alleviated due to defect in activation of the RTG pathway.

While the studies described here identify fascinating cellular functions of CL, many questions remain unanswered. Future studies to elucidate the mechanisms whereby CL affects these functions are suggested in Chapter 5.

CHAPTER 2

GENETIC SCREEN TO IDENTIFY PHYSIOLOGICAL MODIFIERS THAT EXACERBATE THE LOSS OF CARDIOLIPIN IN *S. CEREVISIAE*

Parts of this chapter have been published in *Current Biology* **19(24): 2133-9, 2009**. I thank Gnanada Kulkarni and Naomi Fei for assistance with tetrad dissection.

INTRODUCTION

Barth Syndrome (BTHS) is a rare X-linked disorder characterized by cardiomyopathy, skeletal myopathy, neutropenia, 3-methylglutaconic aciduria and growth retardation due to abnormal mitochondria and defective oxidative phosphorylation (Barth et al., 1981). Other metabolic abnormalities found in patients include elevated urinary excretion of 3-methylglutaconic acid (Cardonick et al., 1997) and hypocholesterolemia (Mazzocco et al., 2007). About ten years after the first description of BTHS, mutations were identified in G4.5, commonly referred to as tafazzin (*TAZ1*) (Bione et al., 1996). Mutations result in the complete loss of Taz1p or in expression of a severely truncated Taz1p (Bione et al., 1996). The characteristic symptoms of BTHS vary widely, even among patients with identical mutation, and there is considerable variation in the age of onset and in disease progression (Barth et al., 2004). Cardiomyopathy is a characteristic feature and primary cause of

death in BTHS patients (Barth et al., 1999a). Cardiomyopathy is a structural disease of the heart muscle that is marked by rigidity and loss of flexibility of the heart walls, leading to weakness and fatigue (Barth et al., 1983). Skeletal myopathy and cardiomyopathy lead to delayed motor development in some BTHS patients, and to changes in cardiac mitochondrial appearance (Xu et al., 2006a). Skeletal myopathy and cardiomyopathy are due to muscle weakness, which can be related to deficient oxidative phosphorylation (Barth et al., 1999b). Sudden neutropenia, an abnormally low number of neutrophils, is a major cause of death in BTHS patients due to bacterial infections (Barth et al., 2004). Neutropenia in BTHS is cyclic, and thus not always detectable, but it is a major cause of concern as it often leads to chronic bacterial infections. 3-methylglutaconic aciduria (3-MGA) in the urine of BTHS patients is caused by deficiency of the enzyme 3-methylglutaconyl-CoA hydratase, involved in the mitochondria localized leucine degradation pathway (Schmidt et al., 2004).

As mentioned, the clinical symptoms of BTHS vary among patients even those with identical mutations (Gonzalez, 2005). These range from severe incapacitating disease to those who are nearly asymptomatic (Gonzalez, 2005). The cellular function of Taz1p and the molecular basis underlying the pathologies observed in BTHS patients are not well understood. Peter Vreken and colleagues demonstrated that fibroblast cultures from BTHS patients contain less CL than control cultures (Vreken et al., 2000). Furthermore, BTHS cells were defective in acylation of CL and PG with unsaturated fatty acids. Subsequent analysis showed that BTHS cells

contained a decrease in total CL content (Vreken et al., 2000) and an accumulation of MLCL (Valianpour et al., 2005). The predominant acyl species, tetralinoleoyl-CL, is absent from BTHS cells (Schlame et al., 2003).

The role of tafazzin is highly conserved from yeast to humans. To elucidate the role of tafazzin in BTHS, the Greenberg lab has developed a yeast model for the disorder (Gu et al., 2004). The *taz1*Δ mutant, a null mutant of tafazzin, has decreased CL, increased MLCL, and aberrant acyl species, similar to the biochemical profile in BTHS. These defects are complemented by expression of the human *TAZ1* cDNA (Ma et al., 2004). Therefore, the yeast model for BTHS is a powerful tool for *in vivo* studies of tafazzin function. To elucidate the function of tafazzin and identify the physiological factors that exacerbate its loss, we used the powerful genetic tool of synthetic lethality. Synthetic lethal analysis exploits the possibility that two non-lethal mutations in genes that provide an essential function lead to an inviable cell when present together (Hartman et al., 2001). An example of this is demonstrated by previous studies in the lab, which have identified a synthetic lethal interaction between the genes for mitochondrial phosphatidylethanolamine (PE) synthesis and CL synthase (Gohil et al., 2005). This study indicated that mitochondrial biosynthesis of PE is essential for yeast *crd1*Δ cells, which lack CL.

In this chapter, I report a screen for synthetic lethality with *crd1*Δ and *taz1*Δ, screening target genes that represent pathways relevant to the clinical presentation in BTHS patients. Several mutants from functional categories such as mitochondrial protein import and mitochondrial morphology exhibited

synthetic lethal or synthetic sick phenotypes with the CL mutants, suggesting a possible overlapping role of CL in these mitochondrial functions. Consistent with the prediction, we show that CL is required for outer membrane protein biogenesis (Gebert et al., 2009). In addition, the following chapter (Chapter 3) shows that CL is also required for mitochondrial fusion (Joshi et al., 2012).

Materials and methods

Yeast strains and growth media:

The yeast strains used in this study are isogenic to BY4741 and BY4742. Complex medium (YP) contained 1% yeast extract (US Biological), 2% peptone (Fischer Scientific) and 2% glucose (Fischer Scientific) in YPD and 3% glycerol (EMD) in YPG. Complete synthetic medium (CSM) contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), and uracil (20 mg/liter), vitamins, salts (essentially components of Difco Vitamin Free Yeast Base without amino acids), and glucose (2%). Synthetic drop out medium contained all of the above ingredients except the amino acid used as a selectable marker. Sporulation medium contained potassium acetate (1%), glucose (0.05%), and the essential amino acids. Solid medium was prepared by adding 2% agar.

Construction of double mutants by tetrad dissection:

The CL mutants *crd1*Δ*::URA3* and *taz1*Δ*::URA3 MATα*/*MATa* were crossed with mutants of opposite mating type obtained from the yeast deletion collection. The heterozygous diploids were selected on dropout media lacking methionine and lysine, sporulated, and tetrads were dissected. The synthetic interaction between CL and deletion mutants was determined by examining the growth of the double mutant compared to isogenic parent strains and wild type on YPD.

Extraction, separation and analysis of yeast total phospholipids:

Yeast cells were grown in the presence of $^{32}P_i$ (10 µCi/ml) in the indicated growth conditions. Total phospholipids were extracted and analyzed by TLC as described (Vaden et al., 2005). The developed chromatograms were analyzed by phosphorimaging and the phospholipids were quantified using Image Quant software.

Fluorescence microscopy:

Fluorescence microscopy was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3 digitally charge-coupled device camera operated by QCapture2 software. All pictures were taken at 1,000 X. To stain mitochondrial DNA, yeast cells were cultured to the mid-log phase, fixed in 70% ethanol at room temperature for 30 min, washed two times with distilled water, and stained with 1 µg/ml DAPI (Sigma) for 5 min.

Results

Acyltransferase and sterol metabolism mutants are not synthetically lethal with CL mutants

The first group tested for synthetic lethality with *taz1*Δ was the acyltransferases. Tafazzin is a transacylase that remodels CL (Xu et al., 2006b). We hypothesized that other acyltransferases or their products could compensate for the loss of *TAZ1.* Loss of these compensatory enzymes or products might thus be lethal in a *TAZ1* deficient cell. The second group tested for synthetic lethality with CL mutants was sterol metabolism. The sterol metabolism pathway is relevant because hypocholesterolemia is a prevalent clinical abnormality in BTHS (Spencer et al., 2006). Sterols are a vital component of mitochondrial membranes. In addition to this, sterol synthesis is highly conserved in yeast and mammals (Guo et al., 2001; Hampton et al., 1996; Lees et al., 1999). Therefore, we hypothesized that loss of sterol might be lethal in *taz1*Δ, as the loss might exacerbate defective mitochondrial function. To test these hypotheses, we crossed nine acyltransferase mutants (Table 2.1) and three sterol metabolism mutants (Table 2.2) obtained from the deletion collection with the *taz1*Δ mutant, and performed tetrad analysis. In all twelve crosses, the double mutants (CL mutant and sterol or acyltransferase mutant) were viable, indicating that the *taz1*Δ mutant is not synthetically lethal with acyltransferase or sterol metabolism mutants (Tables 2.1 and 2.2).

Table 2.1: Genetic interaction of acyltransferase mutants with *taz1*Δ. The acyltransferase mutants in the *MATa* genetic background were crossed with the *taz1*Δ mutant (*MATα*) to obtain double mutants. Genetic interaction was determined based on the viability of the double mutant. Inviable double mutants would suggest a synthetic lethal interaction; viable double mutants with growth defects would indicate a synthetic sick interaction, and double mutants with no growth defect would indicate no genetic interaction. No genetic interactions were found in these crosses.

Acyltransferase mutants	Number of tetrads dissected	Genetic interaction
*slc1*Δ	18	No
*are1*Δ	20	No
*lro1*Δ	25	No
*dga1*Δ	16	No
*sct1*Δ	15	No
*gpt2*Δ	16	No
*mum3*Δ	20	No
*YBR042C*Δ	20	No
*YDR018C*Δ	18	No

Table 2.2: Genetic interaction of sterol metabolism mutants with *taz1*Δ. The sterol metabolism mutants were crossed with the *taz1*Δ mutant to obtain double mutants. Genetic interaction was determined as in Table 2.1. No genetic interaction was found.

Sterol metabolism mutants	Number of tetrads dissected	Genetic interaction
*atf1*Δ	18	No
*osh6*Δ	17	No
*izh4*Δ	18	No

CL is required for assembly of the outer mitochondrial membrane protein complex

Previous studies in the Greenberg lab suggested that CL is required for mitochondrial protein import at elevated temperature (Jiang et al., 2000). Interestingly, a BTHS-like illness known as dilated cardiomyopathy with ataxia (DCMA) syndrome is caused by mutations in the protein import gene DNAJC19/TIM14. These findings strongly suggest that the defect in BTHS may be caused or exacerbated by defective mitochondrial protein import. To test this possibility, we screened mutants of the outer and inner mitochondrial membrane complexes for genetic interactions with *crd1*Δ and *taz1*Δ. The outer mitochondrial membrane consists of TOM, SAM and MDM complexes (Figure 2.1). The TOM machinery is made up of at least seven subunits: the channel forming unit Tom40p, the receptor components Tom22p, Tom20p, and Tom70p, and small Tom proteins Tom5p, Tom6p, and Tom7p (Bolender et al., 2008). The TOM complex is involved in the import of some outer membrane precursors, but whether it is required for import and assembly of all outer membrane proteins is unclear (Rapaport, 2003). The SAM complex is required for the insertion of ß-barrel proteins in the outer membrane (Kozjak et al., 2003). As seen in Figure 2.2, the double mutants exhibited a range of phenotypes. The *crd1*Δ*tom5*Δ and *crd1*Δ*mdm12*Δ mutants were synthetically lethal. The *tom5*Δ mutant was also lethal with *taz1*Δ. The mutants *tom70*Δ, *tom7*Δ, *mdm10*Δ, *mdm12*Δ, *mmm1*Δ, *sam37*Δ were synthetically sick with *crd1*Δ and *taz1*Δ, as double mutants grew at 30^0C but not at elevated temperatures. Double mutants *tom6*Δ*crd1*Δ and *tom6*Δ*taz1*Δ grew similar to

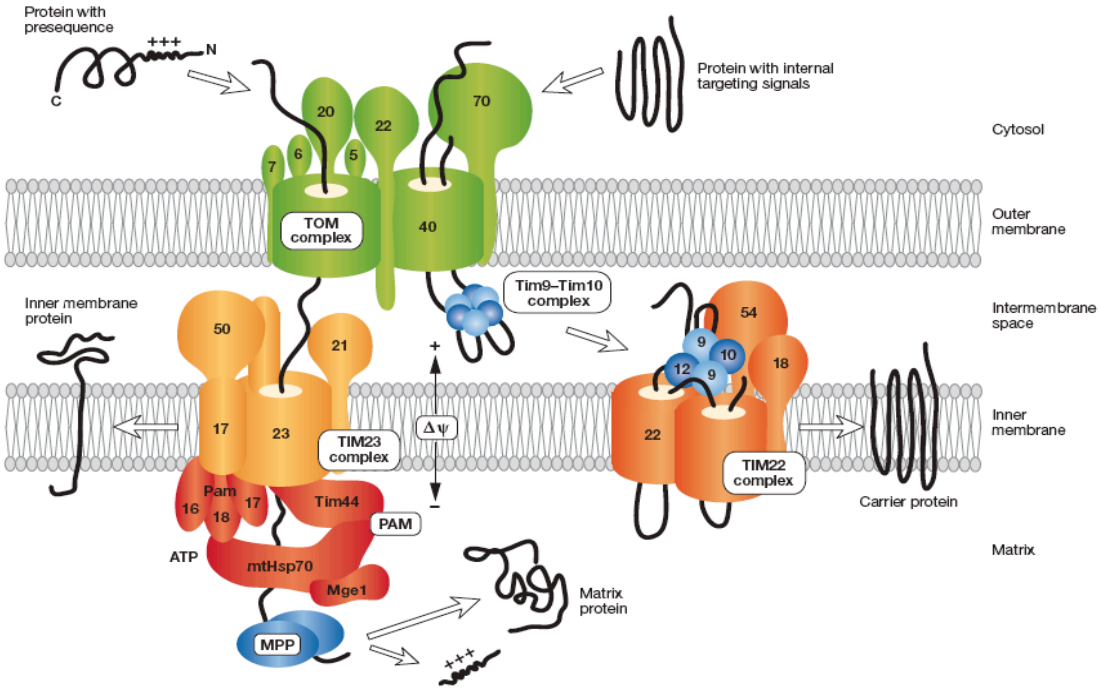


Figure 2.1: Mitochondrial protein import machinery. Nuclear encoded proteins are imported into the mitochondria via the translocase of the outer membrane (TOM) and two translocases of the inner membrane (TIM22 and TIM23). The presequence directs preproteins to the TOM complex at the outer membrane, then to the TIM23 and PAM complexes at the inner membrane. Carrier (transporter) proteins with internal target sequences are targeted to Tom70, and then inserted into the inner membrane by the TIM22 complex (Bolender et al., 2008).

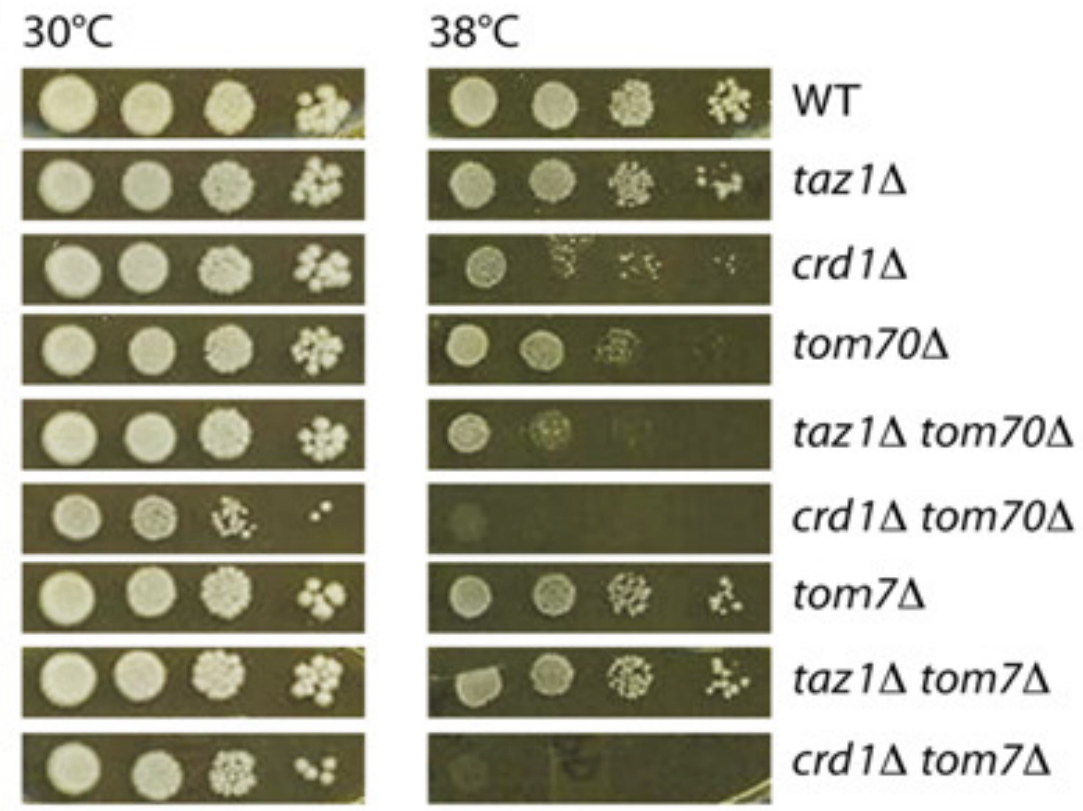


Figure 2.2: Genetic interactions and synthetic growth defects. Cells were grown at 30^0C in liquid YPD to the early stationary phase, serially diluted, spotted on YPD plates, and incubated at the indicated temperatures for 3 to 5 days.

WT. The growth defects in import mutants were more severe with *crd1*Δ than *taz1*Δ, supporting the hypothesis that the defects were due to CL. The genetic interactions of TOM, SAM and MDM complex mutants with the *taz1*Δ mutant suggest that aberrant CL species (lacking unsaturated fatty acids), increased MLCL or decreased CL in the *taz1*Δ mutant was insufficient to fully support the function of CL in mitochondrial protein import. The genetic interactions of the CL mutants were observed not only with the TOM complex (Table 2.3) but also with the SAM and MDM (Table 2.4) complexes, which are involved in the assembly of outer membrane β-barrel proteins, suggesting that CL is required for the assembly of these proteins. In collaboration with Dr. Nikolaus Pfanner (University of Freiburg), we revealed a new role for CL in protein sorting at the outer mitochondrial membrane (Gebert et al., 2009).

CRD1 genetically interacts with TIM and PAM complexes

Pre-proteins translocated from the TOM complex are directed to the inner membrane translocase, the Tim23p complex (Bauer et al., 1996). An alternate pathway for insertion of inner membrane proteins (transporter proteins) is the carrier pathway mediated by the Tim22p complex. To identify the function of CL in inner mitochondrial protein import, we crossed the available deletion mutants from the Tim23 and Tim22 complexes, mitochondrial intermembrane space import and assembly machinery (MIA) complex, presequence translocase-associated motor (PAM) complex that is associated with Tim23, and chaperones of intermembrane space (small TIM proteins) complexes with the CL mutants (Figure 2.1).

Table 2.3: Genetic interaction of CL mutants with the TOM complex. The TOM complex mutants were crossed with the CL mutants to obtain double mutants. The genetic interaction was determined based on the viability of the double mutants. Key: (-): no growth; (+/-): poor growth; (+): good growth.

Mitochondrial protein import mutants	Growth of double mutants			
	*taz1*Δ		*crd1*Δ	
	30^0C	38^0C	30^0C	38^0C
*tom5*Δ	-	-	-	-
*tom6*Δ	+	+	+	+
*tom7*Δ	+	+	+/-	+/-
*tom70*Δ	+	+/-	+/-	+/-

Table 2.4: Genetic interaction of CL mutants with the SAM and MDM complexes. The SAM and MDM complex mutants were crossed with the CL mutants to obtain double mutants. The genetic interaction was determined based on the viability of the double mutants. Key: (-): no growth; (+/-): poor growth; (+): good growth.

Mitochondrial protein import mutants	Growth of double mutants			
	*taz1*Δ		*crd1*Δ	
	30^0C	37^0C	30^0C	37^0C
*mdm10*Δ	+	+/-	+/-	-
	30^0C	36^0C	30^0C	36^0C
*sam37*Δ	+	+/-	+	+/-
	30^0C	38^0C	30^0C	38^0C
*mdm12*Δ	+/-	-	-	-
*mmm1*Δ	+	+/-	+	+/-

TIM23 and PAM complexes: The Tim23p complex mediates a membrane potential and ATP driven import of proteins across the mitochondrial inner membrane (Truscott et al., 2001). This complex, along with mtHsp70 and the PAM complex, drive the import of the preprotein into the matrix in an ATP dependent manner (Krayl et al., 2007). An alternative route for import of proteins by the TIM23 complex includes Tim17p and Tim21p (Figure 2.1). This pathway is independent of function of mtHsp70 and ATP (Chacinska et al., 2005). Even though the CL mutants did not genetically interact with the *tim21*Δ mutant from the Tim23 complex, the *crd1*Δ mutant was synthetic sick with *pam17*Δ (Table 2.5). Pam17p modulates the function of the core Tim23 complex by affecting its conformation (van der Laan et al., 2005). The genetic interaction studies indicate that CL might affect the assembly of TIM23 by affecting the function of the PAM complex. Our studies indicate that CL is specifically required for the ATP and membrane potential dependent functions of the TIM23 complex.

TIM22 complex: The TIM22 complex is required for insertion of hydrophobic proteins, usually carrier proteins such as the ADP/ATP carrier (transporters) (Wiedemann et al., 2001). The import pathway that involves the TIM22 complex is, thus, termed the carrier pathway. The TIM22 complex core consists of Tim22p, Tim54p, Tim18p, and Sdh3p (Figure 2.1). The core of the TIM22 complex is associated with small TIM proteins (Tim9p, Tim10p, Tim12p) from the intermembrane space (Figure 2.1). The small TIM proteins facilitate the transit of proteins during import from the outer membrane complex (TOM) to the inner membrane complex (TIM22) (Curran et al., 2002).

Table 2.5: Genetic interaction of CL mutants with mitochondrial inner membrane protein import complexes. The TIM and PAM complex mutants were crossed with the CL mutants to obtain double mutants. The genetic interaction was determined based on the viability of the double mutants. Key: (-): no growth; (+/-): poor growth; (+): good growth.

Mitochondrial protein import mutants	Growth of double mutants			
	*taz1*Δ		*crd1*Δ	
	30^0C	37^0C	30^0C	37^0C
*hot13*Δ	+	+	+	+
*tim13*Δ	+	+	+	+
*tim18*Δ	+	+	-	-
*pam17*Δ	+	+	+/-	+/-
*tim21*Δ	+	+	+	+

Interestingly, the *tim18*Δ mutant was synthetically lethal with *crd1*Δ but not with *taz1*Δ (Table 2.5). These results indicate that the complete loss of CL, as in *crd1*Δ, affects the function of the TIM22 complex while the presence of decreased levels of CL, aberrant CL species or increased MLCL, as in the *taz1*Δ mutant, are sufficient to support Tim22 function. Tim18p is a subunit of Tim22 complex that assembles with Sdh3p to function in protein biogenesis (Gebert et al., 2011). In addition to the synthetic lethal interaction between *crd1*Δ and *tim18*Δ, recent studies have shown that the loss of CL leads to decreased activity of the SDH complex (Vinay Patil, unpublished), indicating that CL is required for the function of the subunits of the TIM22 complex.

MIA complex and small TIM proteins: The MIA complex proteins Mia40p and Erv1p, along with Hot13p, stabilize proteins imported from the TOM complex and relay them to the small TIM proteins or to the TIM22 complex (Curran et al., 2002; Rissler et al., 2005). The *crd1*Δ and the *taz1*Δ mutants did not genetically interact with the available deletion mutants, including *hot13*Δ, from the intermembrane space MIA complex, and the small TIM protein mutant *tim13*Δ (Table 2.5). These findings strongly suggest that the function of CL is limited to the membrane proteins required for mitochondrial protein import.

Deletion of mitochondrial morphology genes exacerbates the growth defect in *crd1*Δ

CL is involved in mitochondrial membrane structure and stability due to its unique physical properties. This structurally unique lipid assembles in the

presence of divalent cations as inverted hexagonal structures (Vasilenko et al., 1982). Recent studies have demonstrated that CL is required for maintaining mitochondrial morphology (Kuroda et al., 2011b; Osman et al., 2009b; Tamura et al., 2009). We recently demonstrated a specific function of CL in mitochondrial fusion (Chapter 3) (Joshi et al., 2012). Mitochondria from cells with mutated tafazzin exhibit abnormal mitochondrial morphology, further supporting a role for CL in mitochondrial morphology (Acehan et al., 2011; Acehan et al., 2007; Xu et al., 2005). The lack of CL could indirectly affect morphology due to reduced mitochondrial functions such as defective mitochondrial protein import and loss of mitochondrial DNA. Therefore, we hypothesized that deletion of genes with functions related to maintenance of mitochondrial morphology would exacerbate the growth defects of cells lacking CL. To characterize the function of CL in mitochondrial morphology, we performed a targeted synthetic lethality screen assessing genetic interactions between mutants lacking CL and mutants in mitochondrial morphology. The genetic screen in the current study identified both lethal and sick interactions. The genes that affect mitochondrial morphology are grouped according to their individual functions in Table 2.6.

UPS proteins: Ups1p was first shown to mediate processing of Mgm1p, an inner mitochondrial membrane protein required for mitochondrial fusion, to small and long isoforms (Sesaki et al., 2006). Recent findings have shown that Ups1 and Ups2 proteins regulate the levels of mitochondrial PE and CL

Table 2.6: Genetic interaction of CL mutants with mitochondrial morphology mutants. Mitochondrial morphology mutants were crossed with

*crd1*Δ to obtain double mutants. Key: (-): No growth; (+/-): poor growth; (+): good growth.

Mitochondrial morphology mutants		*crd1*Δ	
		30^0C	37^0C
UPS protein	*ups1*Δ	-	-
	*ups2*Δ	-	-
	*ups3*Δ	+	+
GET complex	*get1*Δ	+	+/-
	*get2*Δ	+/-	+/-
	*get3*Δ	+	+/-
Mitochondrial inheritance	*mdm31*Δ	+	+
	*mdm32*Δ	+	+
ERMES complex	*gem1*Δ	+/-	+/-
F-box protein association	*mfb1*Δ	+	+
Imported protein cleavage	*cym1*Δ	+	+/-
Fatty acid synthesis	*htd2*Δ	+	+/-
Similarity to hemolysins	*mam3*Δ	+	+/-
Mitochondrial dynamics	*dnm1*Δ	+/-	-
	*fis1*Δ	+	+
	*mdv1*Δ	+	+
	*mdm36*Δ	+	+/-
	*ugo1*Δ	-	-
	*fzo1*Δ	-	-
	*mgm1*Δ	+	+

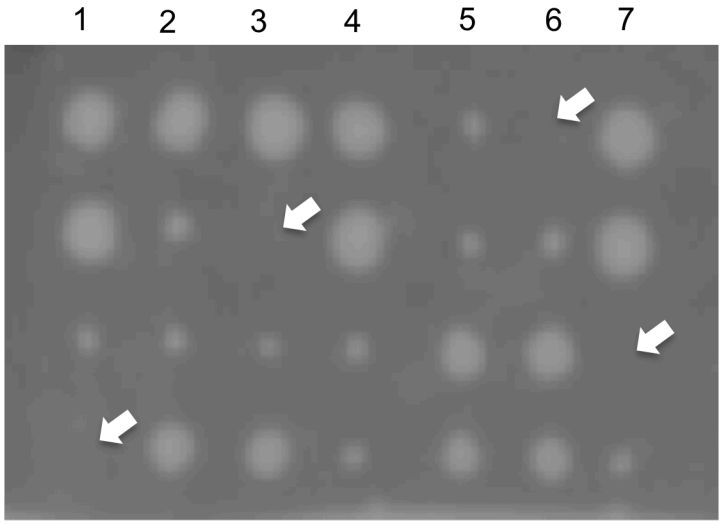


Figure 2.3: *crd1*Δ is synthetically lethal with *ups1*Δ. A diploid strain heterozygous for deletion of *CRD1* and *UPS1* was sporulated, and tetrads were dissected and incubated on YPD for 3-4 days at 30°C. Tetrads 1,3,6 and 7 are tetratype asci, and 2,4 and 5 are parental ditypes. Arrows indicate inviable double mutant haploid spores.

(Tamura et al., 2009). Thus, to determine if UPS proteins and CL have overlapping cellular functions, we tested if UPS mutants genetically interacted with *crd1*Δ. As seen in Figure 2.3, *CRD1* is synthetically lethal with *UPS1*, as *crd1*Δ*ups1*Δ is not viable. Earlier studies showed that the *ups1*Δ mutant has seven-fold decreased levels of CL (Osman et al., 2009a). We demonstrate here that CL is essential for viability of the *ups1*Δ mutant. Consistent with earlier studies (Osman et al., 2009a), we observed synthetic lethality between *crd1*Δ and *ups2*Δ. The synthetic lethality is very likely due to the decreased PE levels in *ups2*Δ (Osman et al., 2009a; Tamura et al., 2009), as our previous studies showed that synthesis of mitochondrial PE is essential in cells lacking CL (Gohil et al., 2005). *UPS1* and *UPS2* antagonistically regulate CL levels, affect the assembly of mitochondrial protein import complexes such as TIM23, which is present in the inner membrane, and regulate mitochondrial morphology (Tamura et al., 2009). The mechanism by which *UPS1* and *UPS2* regulate CL levels remains unknown.

GET complex: The second group of mutants that interact with *CRD1* is the GET complex. Recent studies have suggested that the GET complex is required for targeting newly synthesized tail-anchored (TA) proteins to the ER membrane. This process involves formation of the Get3p-TA complex in the cytosol, which is then recruited by the Get1/2p receptor proteins present in the ER membrane to insert the TA proteins in the lipid bilayer (Mariappan et al., 2011; Schuldiner et al., 2008; Stefer et al., 2011; Wang et al., 2011). Both *crd1*Δ*get1*Δ and *crd1*Δ*get3*Δ exhibited growth defects compared to the parent strains (Figure 2.4). In contrast, genetic interaction was not observed between

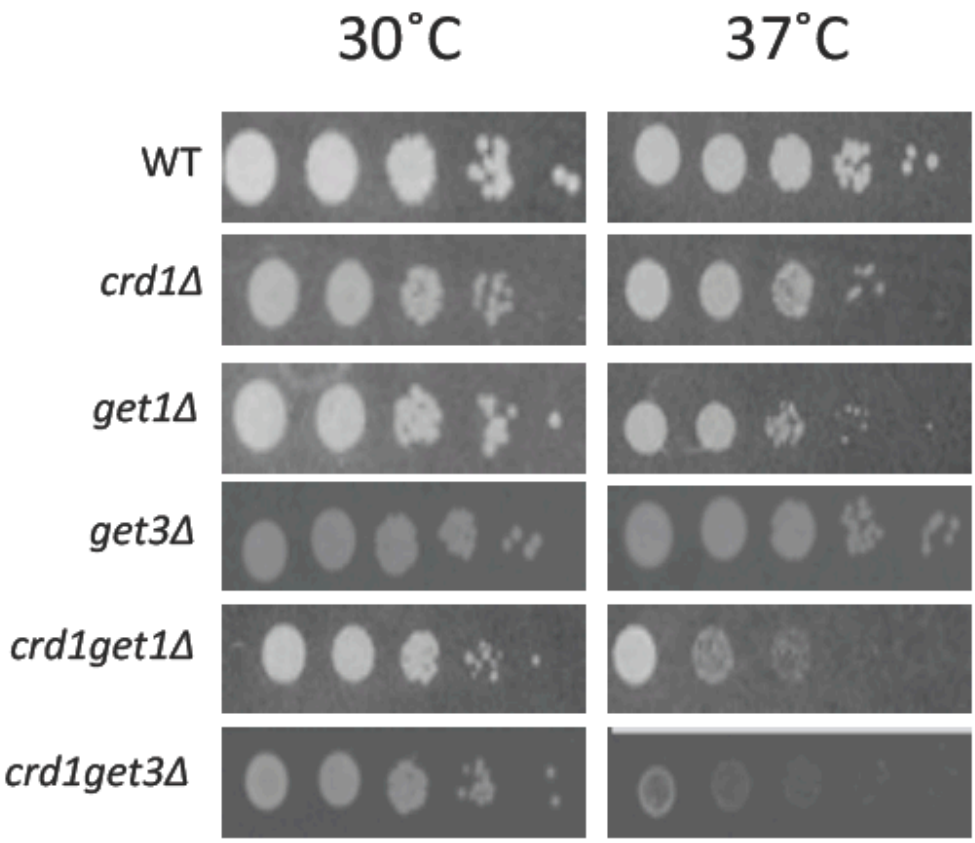


Figure 2.4: *crd1*Δ is synthetically sick with *get1*Δ and *get*Δ*3*. Yeast cells were pre-cultured overnight in YPD at 30°C, serially diluted and spotted on YPD plates. The plates were incubated at 30°C and 37°C for 3-4 days.

*crd1*Δ and *get2*Δ. These findings suggests that at elevated temperature, CL is essential for the growth of *get1*Δ and *get3*Δ but not *get2*Δ, or that the function of Get1p or Get3p, but not Get2p, is essential for growth of *crd1*Δ. These findings may indicate that CL shares a novel essential function with Get1p and Get3p but not with Get2p.

Surprisingly, the GET complex mutants *get1*Δ and *get2*Δ, but not *get3*Δ, exhibited a decrease in CL levels (~25% and ~55%, respectively) compared to WT (Figure 2.5, Table 2.7). This is the first demonstration that ER membrane receptor proteins Get1p and Get2p regulate the levels of CL. While the loss of mitochondrial DNA leads to a decrease in CL (Chen et al., 2010b), this cannot explain the reduced CL levels in *get1*Δ and *get2*Δ, as DAPI staining indicated the presence of mitochondrial DNA (mtDNA) in the GET mutants (Figure 2.6). Although the GET mutants were not deficient in mtDNA, they exhibited a severe growth defect in respiratory medium, indicating reduced mitochondrial function (Figure 2.7). These results indicate that import of precursors of CL or enzymes required for CL biosynthesis in the mitochondria might be affected in the *get1*Δ and *get2*Δ mutants. Our results clearly identify a new role for the GET complex in regulating the levels of CL and mitochondrial function.

MDM proteins and GEM1: An independent study demonstrated synthetic interactions between *crd1*Δ and MDM complex mutants *mdm10*Δ, *mmm1*Δ, *mdm12*Δ and *mdm34*Δ, consistent with our finding (Table 2.4) (Gebert et al., 2009), which led to identification of the ER-mitochondria tethering complex

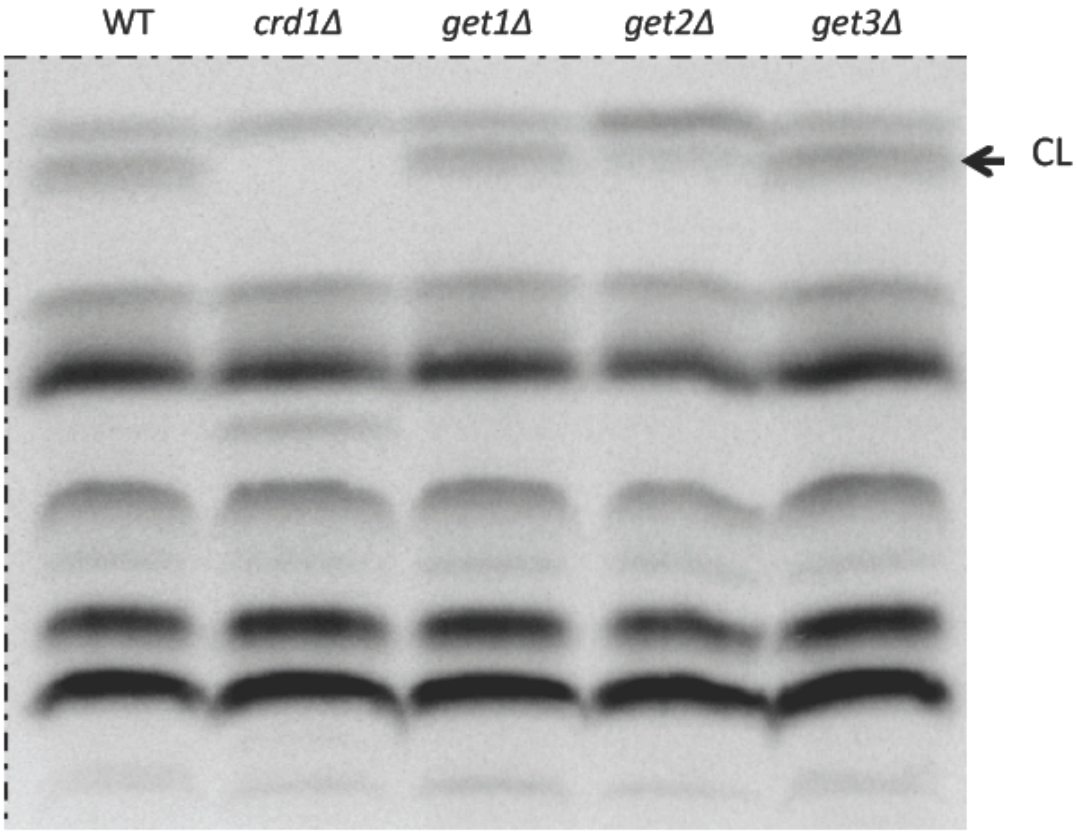


Figure 2.5: Separation of phospholipids from *get1*Δ, *get2*Δ and *get3*Δ mutants. Yeast cells were grown in YPD containing $^{32}P_i$ at 30°C to the early stationary phase. Total phospholipids were extracted and analyzed by one-dimensional TLC as described (Vaden et al., 2005).

Table 2.7: Quantitation of CL in the GET and the mitochondrial fusion complex mutants. Yeast cells were grown in YPD with $^{32}P_i$ at 30°C until early stationary phase. Total phospholipids were extracted and analyzed by 1D-TLC as described (Vaden et al., 2005). Values indicated represent mean ± S.D (n=2) for GET mutants and ± S.E (n=3) for fusion mutants.

Strains	% CL (Mean ± S.D)	Strains	% CL (Mean ± S.E)
WT	100	WT rho zero	100
*get1*Δ	77.7 ± 4.7	*mgm1*Δ	89.6 ± 12.7
*get2*Δ	45.3 ± 0.7	*ugo1*Δ	32.0 ± 15.5
*get3*Δ	90.7 ± 9.3	*fzo1*Δ	134.4 ± 18.11

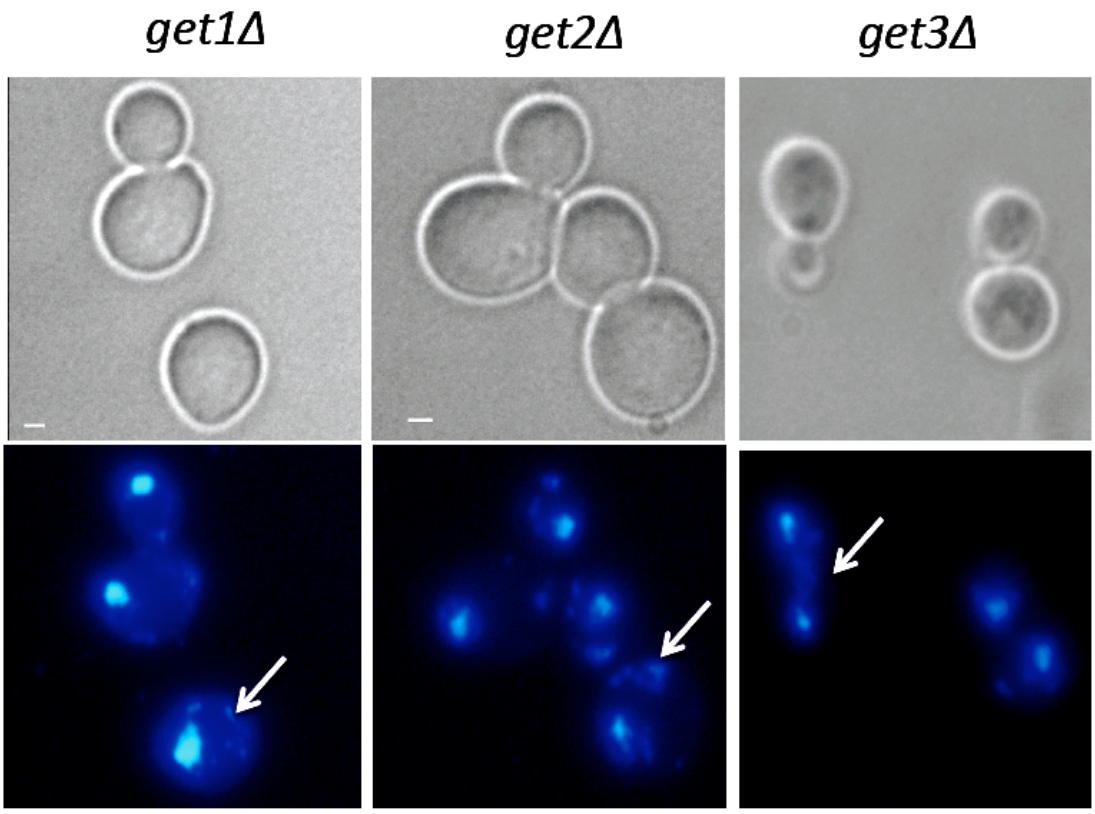


Figure 2.6: The *get1*Δ, *get2*Δ and *get3*Δ mutants do not exhibit loss of mitochondrial DNA. Yeast cells were cultured to the mid-log phase, fixed in 70% ethanol at room temperature for 30 min, washed two times with distilled water, and stained with 1 µg/ml DAPI (Sigma) for 5 min. Arrows indicate mtDNA.

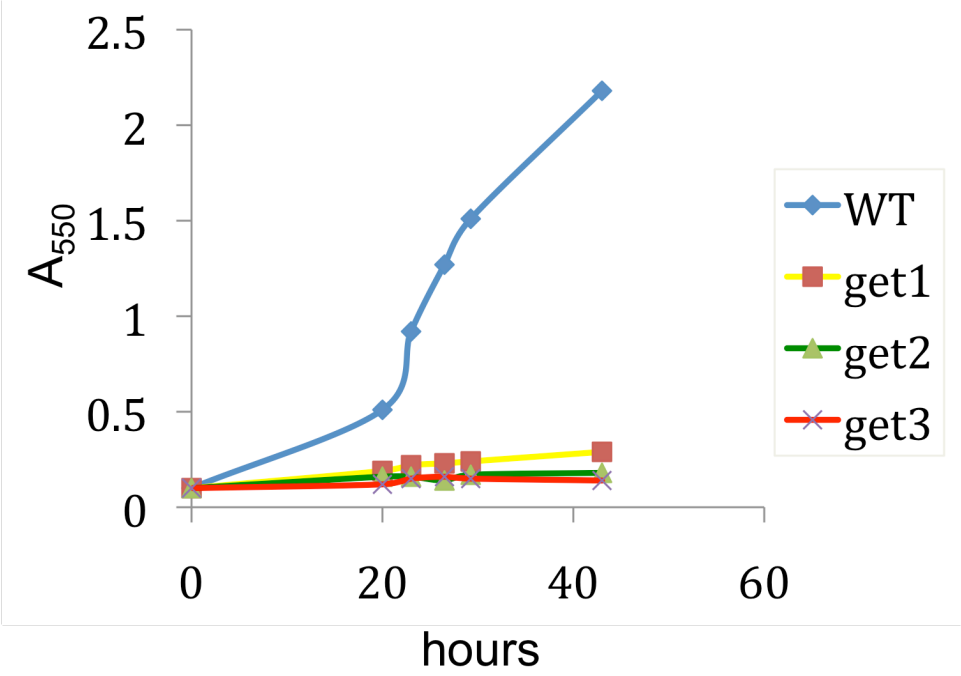


Figure 2.7: GET complex mutants exhibit decreased mitochondrial function. Yeast cells grown in liquid YPD for 1 day were inoculated into liquid YPG to an A_{550} of 0.1 and grown at 30^0C with shaking at 230 rpm. Aliquots of each culture were taken and cell density was measured at A_{550}.

(ERMES). The ERMES complex was shown to be involved in phospholipid exchange, mitochondrial protein import, maintenance of mitochondrial DNA and morphology (Kornmann et al., 2009). In a recent finding, Kornmann et al. suggested that Gem1p is an integral component of the ERMES complex. It was shown to regulate the number and size of ERMES complexes and mediate phospholipid exchange. Consistent with this study, we showed that *crd1*Δ genetically interacts with *gem1*Δ (Table 2.6), supporting the function of the ERMES complex in phospholipid exchange (Kornmann et al., 2011).

Mitochondrial dynamics: *CRD1* genetically interacted with genes involved in mitochondrial fusion and fission. *CRD1* is synthetically lethal with mitochondrial fusion genes *UGO1* and *FZO1* (Table 2.6). The *fzo1*Δ mutant also exhibited synthetic interaction with *fmp30*Δ, a mitochondrial inner membrane protein required for maintaining mitochondrial morphology and regulating CL levels (Kuroda et al., 2011b). To determine if fusion genes regulate CL levels, we examined CL levels in the fusion mutants. Because loss of mitochondrial DNA leads to decreased CL (Chen et al., 2010b) and fusion mutants lose mitochondrial DNA (Guan et al., 1993; Hermann et al., 1998; Nunnari et al., 1997), we compared CL levels of *fzo1*Δ, *ugo1*Δ and *mgm1*Δ with WT rho^0 cells. Except for *ugo1*Δ, fusion mutants did not exhibit a significant decrease in CL levels (Table 2.7). *CRD1* also specifically interacted with the *DNM1* and *MDM36* genes in the fission pathway, as the *dnm1*Δ and *mdm36*Δ mutants exhibited synthetic interaction with *crd1*Δ (Table 2.6). *DNM1* is involved in mitochondrial fission, participates in endosomal trafficking, and regulates peroxisome abundance (Gammie et al., 1995; Kuravi et al., 2006;

Otsuga et al., 1998), while *MDM36* is suggested to promote mitochondrial fission (Hammermeister et al., 2010). Genetic interaction of the mitochondrial dynamics mutants with *crd1*Δ strongly suggests that CL is required for maintaining mitochondrial morphology.

Other mitochondrial morphology genes: *CRD1* also genetically interacted with *CYM1*, *HTD2*, and *MAM3*, suggesting that these genes have overlapping functions with *CRD1* in common essential cellular pathways (Table 2.6). The genetic screen has provided evidence that point towards specific players required for regulation of CL levels and mitochondrial function in yeast.

Discussion

In this study, a targeted genetic screen was performed in *S. cerevisiae* to identify the cellular functions of CL that might be potential physiological modifiers of BTHS. We identified genetic interactions between CL mutants and mutants from mitochondrial protein import complexes (TOM, SAM, MDM, TIM and PAM) and mutants with defective mitochondrial morphology (the UPS, and GET complexes mutants, and mitochondrial fusion and fission mutants), suggesting that CL is required for mitochondrial protein import and maintenance of morphology.

How does the loss of CL affect mitochondrial protein import? A previous report from the Greenberg lab indicated that the loss of CL leads to decreased protein import and membrane potential at non-permissive temperature (Jiang et al., 2000). In the current study, we observed that several genes from the TIM complex genetically interact with cells lacking CL (Table 2.5). Loss of CL affects interaction between the TIM and PAM complexes (Tamura et al., 2009). In addition to this, it affects the electrophoretic mobility of the TIM22 complex, required for insertion of metabolic carrier proteins in the inner mitochondrial membrane (Tamura et al., 2009). It appears from these studies that mitochondrial protein import in *crd1*Δ mutant cells might not only be affected by a decrease in membrane potential but also by altered activity and assembly of these protein complexes. Interestingly, we also showed that several genes from the TOM complex genetically interact with *crd1*Δ and *taz1*Δ mutants, strongly indicating that CL

shares functions with the outer membrane proteins required for protein import. Consistent with this, we provide evidence that a detectable amount of CL is present in the outer membrane and is required for assembly of the β barrel protein in yeast as well as in mitochondria from lymphoblasts isolated from a Barth patient (Gebert et al., 2009). Thus, CL is required for outer membrane biogenesis. Taken together, the genetic interaction data suggest that CL is required for the biogenesis of translocases of the inner and outer membrane.

What is the function of CL in the maintenance of mitochondrial morphology? Mitochondrial morphological defect in cells lacking CL could be due to decreased membrane potential or decreased mitochondrial import (Stojanovski et al., 2006). Recent studies have shown that CL could have a direct effect on mitochondrial morphology (Chapter 3) (DeVay et al., 2009; Joshi et al., 2012; Rujiviphat et al., 2009). In the genetic screen with mitochondrial morphology mutants, we have shown that the GET and UPS complexes genetically interacted with the *crd1*Δ mutant, indicating that these modifiers share cellular functions with CL. Our findings indicate that the GET complex might be required for the regulation of CL levels, as the loss of GET proteins such as Get1p and Get2p leads to decreased CL levels. In addition to this, the *crd1*Δ mutant interacted with several other mitochondrial morphology mutants, indicating that CL is required for the maintenance of mitochondrial morphology.

The goal of the synthetic lethality screen performed in this study was to identify functions of CL that might be physiological modifiers of BTHS. In the current study, we showed that CL is required for mitochondrial protein import

and maintenance of mitochondrial morphology. How could mitochondrial protein import and mitochondrial morphology act as physiological modifiers of BTHS? Import of proteins into the mitochondria is an intricate process that requires the presence of target sequences in the proteins, targeting of protein to the TOM complex present in the outer membrane, and the translocation of proteins into the mitochondria. Errors in these events may lead to disease in humans due to decreased mitochondrial proteins. As discussed previously, DCMA syndrome is an illness caused by mutation in a protein (Tim14p) required for import. Pyruvate dehydrogenase deficiency is a disease caused by mutation in the targeting signal of one subunit of pyruvate dehydrogenase enzyme (PDH). As a result, the mitochondrial import of PDH is defective. PDH deficiency is the most common cause of lactic acidosis in infants and children. Interestingly, BTHS patients exhibit lactic acidosis upon exercise (Sweeney et al., 2008). Thus, it is possible that import of PDH is decreased in cells with defective CL. Mutation in Hsp60, a mitochondrial matrix chaperone, causes atypical mitochondrial disease involving, perturbation of oxidative phosphorylation, β-oxidation, and pyruvate metabolism (Agsteribbe et al., 1993; Briones et al., 1997). Consistent with this, we observed that cells lacking CL appear to be defective in these metabolic pathways, as described in Chapter 4. Thus, mitochondrial protein import is potentially a vital physiological modifier that may lead to metabolic deficiencies in BTHS.

The functional state of mitochondria affects its morphology. Defects in mitochondrial protein import, decreased ATP levels or increased ROS lead to defective morphology (Stojanovski et al., 2006). The genetic screen discussed

in the current study identified several players that might act as physiological modifiers of CL in BTHS, including the UPS and GET complexes. GET complex mutants, which have defective mitochondrial morphology, regulate CL levels. In addition, subsequent studies following up on the synthetic lethal data with fusion mutants (discussed further in chapter 3) indicate that CL is required for mitochondrial fusion (Joshi et al., 2012). Therefore, mitochondrial fusion could be a physiological modifier of BTHS. The role of mitochondrial fusion as a physiological modifier of BTHS is discussed in Chapter 3. Thus, genes that interact with *CRD1* may identify functions that affect the BTHS phenotype. An increased understanding of the role of CL and its regulation could identify potential avenues for new treatments of BTHS.

CHAPTER 3

CARDIOLIPIN AND MITOCHONDRIAL PHOSPHATIDYLETHANOLAMINE HAVE OVERLAPPING FUNCTIONS IN MITOCHONDRIAL FUSION IN *SACCHAROMYCES CEREVISIAE*

The work in this chapter is published in the *Journal of Biological Chemistry* 2012 May 18;287(21):17589-97. I thank Morgan N. Thompson for constructing the *crd1*Δ*psd1*Δ conditional mutant, Naomi Fei for assistance with tetrad dissection and microscopy, and Dr. J. Michael McCaffery, Integrated Imaging Centre (Johns Hopkins University), for providing EM images.

INTRODUCTION

Mitochondria exist as dynamic, double membrane-bound organelles. Mitochondrial membranes are enriched in phospholipids and proteins that are required for mitochondrial biogenesis and for maintenance of mitochondrial morphology and the tubular network (Gohil and Greenberg, 2009). CL and PE are non-bilayer forming phospholipids in the mitochondrial membranes (Ardail et al., 1990; Gonzalvez and Gottlieb, 2007) that play an essential role in mitochondrial function. Although cells lacking CL or mitochondrial PE are viable, the loss of both phospholipids is lethal, suggesting that these lipids have overlapping functions that are essential (Gohil et al., 2005). Several recent studies have implicated the involvement of CL and mitochondrial PE in the maintenance of mitochondrial morphology (Kuroda et al., 2011a; Osman

et al., 2009a; Tamura et al., 2009). CL and PE are fusogenic phospholipids that form hexagonal phases in the presence of divalent cations, which confer negative curvature to the mitochondrial membrane (Rand and Sengupta, 1972; van den Brink-van der Laan et al., 2004). In the current study, we investigated the role of CL and PE in mitochondrial fusion. Highly conserved protein machinery strictly regulates the process of mitochondrial fusion, and recent studies suggest that phospholipids also play a vital role in this process. Mitochondrial fusion in the yeast *Saccharomyces cerevisiae* primarily requires three proteins. These include the outer membrane GTPase, Fzo1p (Mfn1 and Mfn2 in mammals) (Hermann et al., 1998; Rapaport et al., 1998), the inner membrane GTPase, Mgm1p (Opa1 in mammals) (Wong et al., 2000; Wong et al., 2003), and the outer membrane protein Ugo1p, which links the two GTPases to form a functional complex (Hoppins et al., 2009; Sesaki and Jensen, 2001; Sesaki and Jensen, 2004). In *S. cerevisiae*, Mgm1p exists as long (l-Mgm1p) and short isoforms (s-Mgm1p), both of which are required for mitochondrial fusion (Herlan et al., 2003; Zick et al., 2009). *In vitro* studies demonstrated that CL stimulates the GTPase activity of the s-Mgm1p (DeVay et al., 2009; Rujiviphat et al., 2009). Moreover, it was shown *in vitro* that s-Mgm1p and l-Mgm1p assemble in a CL dependent manner (DeVay et al., 2009). We hypothesized that the mitochondrial phospholipids CL and PE have overlapping functions in mitochondrial fusion *in vivo*. Consistent with this hypothesis, we demonstrate that cells lacking both CL and mitochondrial PE have reduced levels of both Mgm1p isoforms and exhibit excessive fragmentation of mitochondria and defects in mitochondrial fusion.

MATERIAL AND METHODS

Yeast strains, plasmids and growth media:

The *S. cerevisiae* strains used in this study, listed in Table 3.1, are isogenic to BY4741 and BY4742. The single mutants were obtained from the *MATa* yeast deletion collection obtained from Dr. John Lopes. Double and triple mutants used in this study were obtained by tetrad dissection. Synthetic complete media contained standard concentration of amino acids, all the essential components of DIFCO vitamin-free yeast nitrogen base, 0.2% ammonium sulphate and glucose (2%). Synthetic dropout media contained all of the aforementioned ingredients except the amino acid used as a selectable marker. Complex media contained yeast extract (1%), peptone (2%), with glucose (2%) (YPD) or galactose (2%) (YP-galactose) as carbon source. All the plasmids were amplified and extracted using standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol (Chen et al., 1992). The v5 epitope-tagged *CRD1* gene was cloned into the pCM189 plasmid (ATCC), in which, the TET_{OFF} promoter regulates the expression of cloned gene, using the BamHI and NotI restriction sites. The existing *URA3* marker of the plasmid was replaced by *HIS3* using EcoRV and ClaI restriction sites. Bacterial transformations were performed using dam^- *E.coli* to avoid Dam methylase sensitivity to the ClaI restriction enzyme.

Fluorescence microscopy:

Fluorescence microscopy was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3 digitally charge-coupled device camera operated by QCapture2 software. All pictures were taken at 1,000 X. To stain mitochondrial DNA, yeast cells were cultured to the mid-log phase, fixed in 70% ethanol at room temperature for 30 min, washed two times with distilled water, and stained with 1 µg/ml DAPI (Sigma) for 5 min. Mitochondria were visualized by transforming the cells with either plasmid pYX142 or pYX122 expressing GFP fused to the mitochondrial presequence, pre Su9 (Westermann and Neupert, 2000) (provided by Dr. Benedikt Westermann) or pYX142-mtRFP expressing mitochondria targeted RFP (provided by Dr. Janet Shaw). Cells were harvested in the appropriate medium and viewed under fluorescence microscopy.

Electron microscopy:

Cells were grown in 100 ml YPD to an A_{550} of 0.5. After harvesting, cells were prepared for EM using the osmium thiocarbohydrazide osmium fixation method (Willingham and Rutherford, 1984).

In vivo fusion assay:

The mitochondrial *in vivo* fusion assay was performed as described (Nunnari et al., 1997; Wong et al., 2003). *MAT*α cells of WT, *crd1*Δ, and *psd1*Δ, were transformed with pYX122-mtGFP and *MATa* cells were transformed with pYX142-mtRFP. *MATa* cells of the conditional mutant *crd1*Δ*psd1*Δ were

transformed with pYX142-mtRFP, and *MAT*α cells with pYX142-mtGFP. *MATa* cells of the conditional mutant *crd1*Δ*psd1*Δ*fis1*Δ were transformed with pYX142-mtGFP, and *MAT*α with pYX142-mtRFP. *MAT*α cells of *crd1psd1dnm1*Δ were transformed with pYX142-mtGFP and *MATa* with pYX142-mtRFP. Cells were grown in 5 ml selective media to an A_{550} of 0.5. After centrifugation, cells of opposite mating type were mixed and spotted on an YPD plate. After 3.5 hours of incubation at 30°C, cells were observed for mitochondrial fusion. The images were merged and analyzed using Image J software.

Extraction, separation and analysis of yeast total phospholipids:

Yeast cells were grown in the presence of $^{32}P_i$ (10 µCi/ml) in the indicated growth conditions. Total phospholipids were extracted and analyzed by TLC as described (Vaden et al., 2005). The developed chromatograms were analyzed by phosphorimaging and the phospholipids were quantified using Image Quant software.

Flow cytometry:

Mitochondrial membrane potential was measured using whole cells as described (Ludovico et al., 2001). Cells were grown in YP-galactose media to the mid-logarithmic phase. Actively growing cells ($5x10^4$ cells) were incubated at 30°C with the dye tetramethyl rhodamine methyl ester (TMRM) (50nM) for 30 mins. To induce a decrease in membrane potential, control cells were treated with sodium azide (20 mM). Fluorescence was measured using a flow

cytometer. The results were analyzed using WinMDI2.9 software.

SDS-PAGE and Western blot analysis:

Proteins were extracted from cells grown to an A_{550} of 0.5, separated by 8% SDS-PAGE, transferred to PVDF membrane and analyzed using primary antibodies to Fzo1p (1: 1000), Ugo1p (1:1000), Mgm1p (1:500) (provided by Dr. Jodi Nunnari) and α-tubulin (1:1000) (Santa Cruz Biotechnology). Proteins were visualized using appropriate secondary antibody conjugated with horseradish peroxidase (1:3000) followed by detection using the ECL chemiluminescence system (GE Healthcare).

Table 3.1: Strains used in this study.

Strains	Genotype	Reference
BY4741	*MATa, his 301, leu 200, met 1500, ura 300*	**Invitrogen**
BY4742	*MATα , his 301, leu 200, lys 200, ura 300*	**Invitrogen**
VGY1	*MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3*	**Gohil et al., 2005**
crd1Δ	*MATa, his 301, leu 200, met 1500, ura 300, crd1Δ::KanMX4*	**Invitrogen**
psd1Δ	*MATa, his 301, leu 200, met 1500, ura 300, psd1Δ::KanMX4*	**Invitrogen**
psd1Δ	*MATα, his 301, leu 200, lys 200, ura 300, psd1Δ::KanMX4*	**This study**
dnm1Δ	*MATa, his 301, leu 200, met 1500, ura 300, dnm1Δ::KanMX4*	**Invitrogen**
fis1Δ	*MATa, his 301, leu 200, met 1500, ura 300, fis1Δ::KanMX4*	**Invitrogen**
crd1Δpsd1Δ	*MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, pCM189-CRD1*	**This study**
crd1Δpsd1Δ	*MATa, his 301, leu 200, lys 200, met 1500, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, pCM189-CRD1*	**This study**
crd1Δpsd1Δ fis1Δ	*MATa, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, fis1Δ::KanMX4, pCM189-CRD1*	**This study**
crd1Δpsd1Δ fis1Δ	*MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, fis1Δ::KanMX4, pCM189-CRD1*	**This study**
crd1Δpsd1Δ dnm1Δ	*MATa, his 301, leu 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, dnm1Δ::KanMX4, pCM189-CRD1*	**This study**
crd1Δpsd1Δ dnm1Δ	*MATα, his 301, leu 200, lys 200, met 1500, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, dnm1Δ::KanMX4, pCM189-CRD1*	**This study**

RESULTS

Maintenance of a mitochondrial network and mitochondrial fusion is defective in the absence of CL and mitochondrial PE

Previous studies have shown that loss of CL (*crd1Δ*) is lethal in combination with loss of mitochondrial PE (*psd1Δ*), but not cytosolic PE (*psd2*Δ) (Gohil et al., 2005). To gain insight into the overlapping roles of these mitochondrial lipids, we constructed a conditional mutant, *crd1*Δ*psd1*Δ, in which *CRD1* is expressed from a plasmid under the control of the TET_{OFF} promoter. This mutant lacks mitochondrial PE and CL in the presence of tetracycline, but contains CL in the absence of tetracycline. We used this conditional mutant as a tool to identify functions of these phospholipids in mitochondrial morphology and mitochondrial fusion. The conditional double mutant grew normally on YPD. The addition of tetracycline (200 μg/ml), which shut off *CRD1* expression, inhibited growth of the double mutant but did not affect growth of WT, *crd1*Δ, or *psd1*Δ cells (Figure 3.1A). To determine if tetracycline did indeed regulate *CRD1* expression, we measured the levels of CL in *crd1*Δ*psd1*Δ cells. In *psd1*Δ, CL was synthesized, although levels were reduced compared to those of WT, consistent with previous studies (Gohil et al., 2005). In *crd1*Δ*psd1*Δ grown in the absence of tetracycline, CL levels were 40% of those of *psd1*Δ, indicating that CL levels from plasmid *CRD1* are less than CL levels obtained from genomic *CRD1*. In the presence of tetracycline, CL was greatly diminished to only 14% of the levels in *psd1*Δ, indicating that expression from the TET_{OFF} promoter was greatly (but not completely)

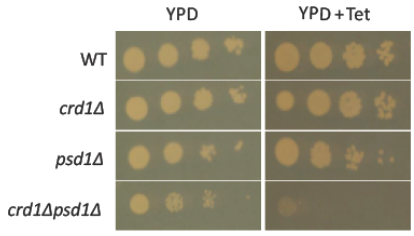
repressed. Tetracycline itself did not affect CL levels in cells lacking the plasmid, which were similar in *psd1*Δ cells grown in the presence and absence of the drug (Figure 3.1B).

To determine if CL and mitochondrial PE play a role in the maintenance of mitochondrial morphology, we compared the mitochondrial network in WT, *crd1*Δ*, psd1*Δ, and *crd1*Δ*psd1*Δ cells transformed with plasmids expressing mitochondria targeted GFP (mtGFP) (Westermann and Neupert, 2000) (Figure 3.2A). At least 500 cells of each strain were observed for each biological replicate (Figure 3.2B). Cells exhibited a normal tubular mitochondrial network in *crd1*Δ*,* consistent with earlier findings (Chen et al., 2010b), indicating that the lack of CL by itself does not affect the mitochondrial network. The lack of mitochondrial PE had a small but significant effect on the mitochondrial network, as ~23% of *psd1*Δ cells exhibited fragmented mitochondria. Unlike the WT-like tubular mitochondrial network, the majority of *psd1Δ* cells had short tubular mitochondria consistent with a mitochondrial morphology defect in these cells. The morphology of *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline was similar to that of *psd1*Δ cells. However, the addition of tetracycline severely affected the mitochondrial network, leading to excessive mitochondrial fragmentation similar to that observed in fusion mutants (Figure 3.2A, 3.2B). These findings suggested that loss of both CL and mitochondrial PE leads to a defect in mitochondrial fusion. Tetracycline by itself did not affect the mitochondrial network in WT, *crd1*Δ and *psd1*Δ cells (data not shown). To determine if the increase in mitochondrial fragmentation correlated with the loss of CL, a time

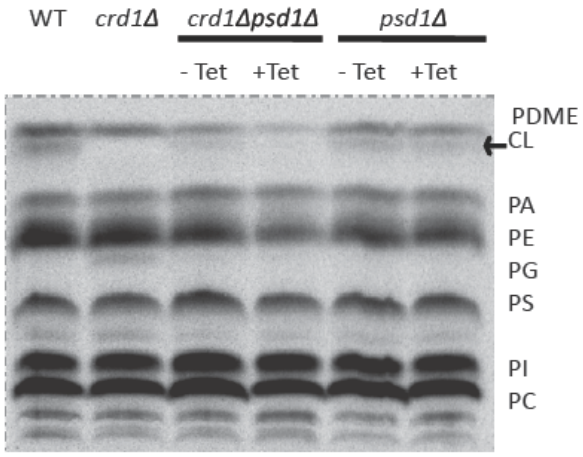
course experiment was performed in which *crd1*Δ*psd1*Δ cells were grown in YPD containing $^{32}P_i$, in the presence or absence of tetracycline. Total phospholipids and mitochondrial morphology were examined at 5, 8 and 11 hrs. Total CL decreased by ~11%, ~31%, and ~55% while the percentage of mitochondrial fragmentation increased during this time to ~20%, ~45% and ~96% at 5, 8 and 11 hrs, respectively (Figure 3.2C, 3.2D). These findings indicate that the increase in mitochondrial fragmentation corresponded with a decrease in CL in the *crd1*Δ*psd1*Δ cells.

Electron microscopic examination of the mutants revealed that *crd1*Δ mitochondria were somewhat smaller than those of WT, but relatively unremarkable (data not shown). Mitochondria in *psd1*Δ cells and in *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline also appeared smaller than WT. This phenotype was even more striking in *crd1*Δ*psd1*Δ cells grown in the presence of tetracycline. Thus, the loss of both CL and mitochondrial PE led to highly fragmented mitochondria, consistent with defective fusion (Figure 3.2F). To determine the role of CL and mitochondrial PE in mitochondrial fusion, we performed an *in vivo* fusion assay (Nunnari et al., 1997; Wong et al., 2003) as described in “Materials and methods.” In this assay, we examined the mitochondrial fusion events in zygotes acquired by mating haploids of opposite mating types of WT, *crd1*Δ, *psd1*Δ, and *crd1*Δ*psd1*Δ cells transformed with either mtGFP or mitochondria tagged RFP (mtRFP). As expected, *crd1*Δ cells exhibited complete mixing of mitochondrial content, indicating that the lack of CL alone does not affect mitochondrial fusion (Figure 3.3A). Fusion occurred but was decreased in *psd1*Δ cells,

A

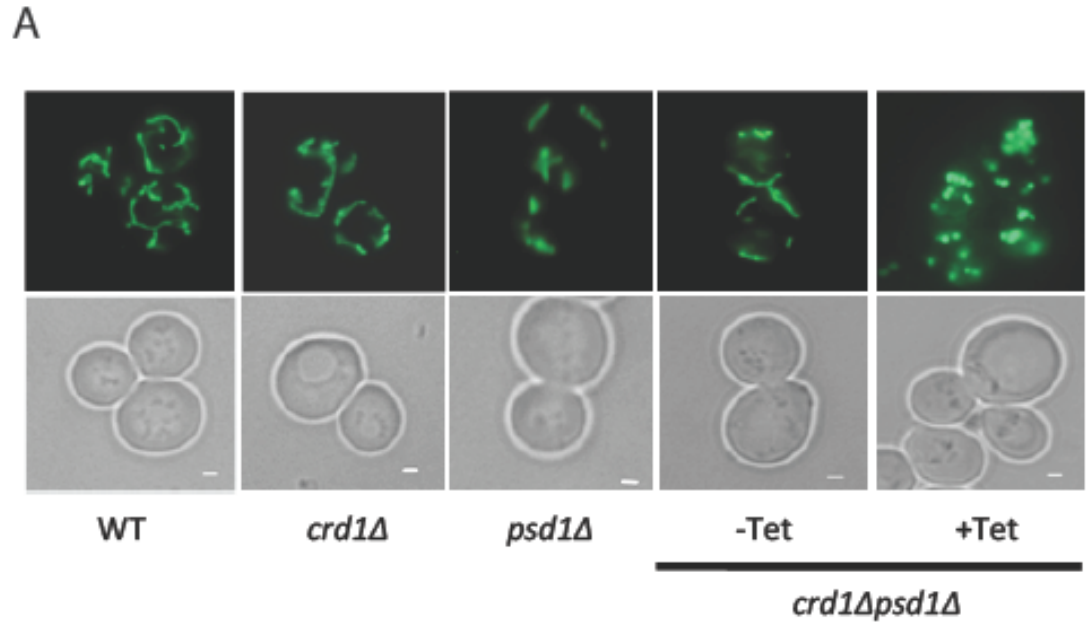


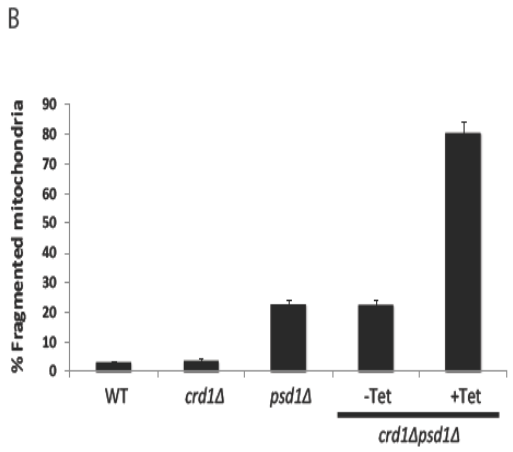
B

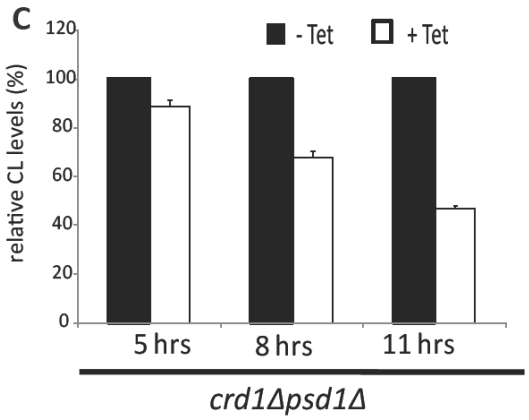


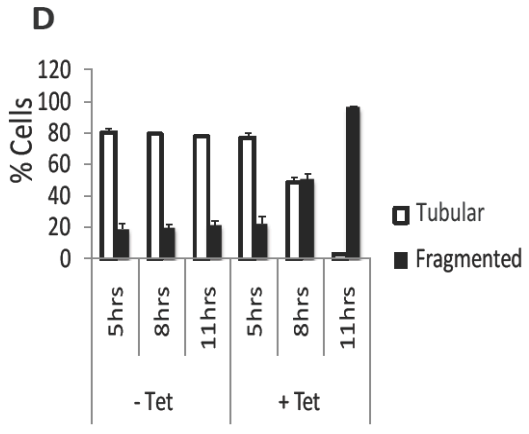
Strains	% CL
BY4742 (WT)	100
crd1Δ	Not detectable
crd1Δpsd1Δ (- Tet)	18.5 ± 1.62
crd1Δpsd1Δ (+ Tet)	7.1 ± 0.14
psd1Δ (- Tet)	51.2 ± 1.13
psd1Δ (+ Tet)	46.6 ± 4.80

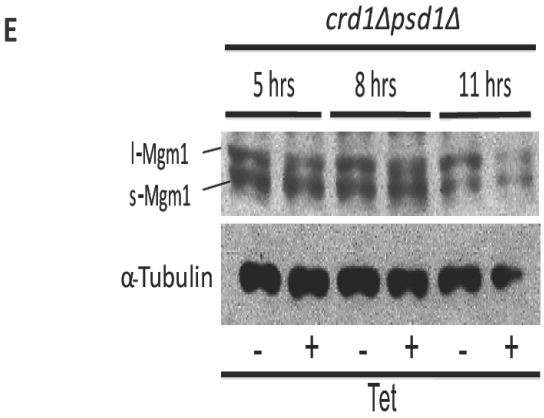
Figure 3.1: Tetracycline-dependent growth of the conditional mutant *crd1*Δ*psd1*Δ. A) 10-fold serial dilutions of cell suspensions were spotted on YPD plates supplemented with 200 µg/ml tetracycline (Tet) where indicated and incubated at 30°C. B) Cells were grown in YPD for 12 h in the presence or absence of tetracycline. Steady state labeling, phospholipid extraction, one dimensional TLC, phosphorimaging, and quantification were carried out as described under “Materials and methods.” CL levels are quantified as percent of total phospholipids. Mean values ± S.D. of two independent experiments are shown. *PC,* phosphatidylcholine; *PI,* phosphatidylinositol; *PS,* phosphatidylserine; *PG,* phosphatidylglycerol; *PA,* phosphatidic acid.


A
WT
crd1Δ
psd1Δ
-Tet
+Tet
crd1Δpsd1Δ


B
% Fragmented mitochondria
WT
crd1Δ
psd1Δ
-Tet
+Tet
crd1Δpsd1Δ


C
- Tet
+ Tet
relative CL levels (%)
5 hrs
8 hrs
11 hrs
crd1Δpsd1Δ


D
% Cells
Tubular
Fragmented
5hrs
8hrs
11hrs
- Tet
+ Tet


E
crd1Δpsd1Δ
5 hrs
8 hrs
11 hrs
l-Mgm1
s-Mgm1
α-Tubulin
Tet

F

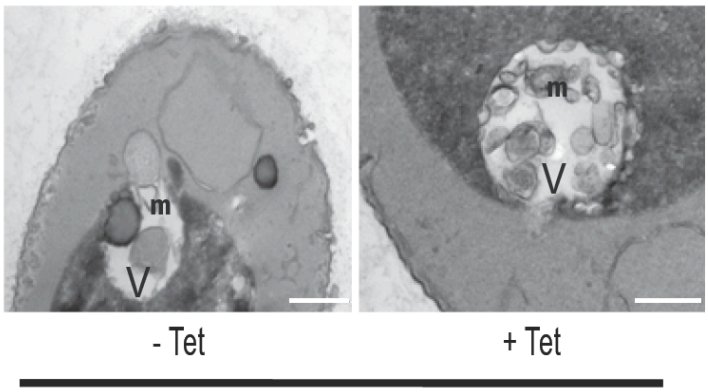


Figure 3.2: Mitochondrial fragmentation observed in *crd1Δpsd1ΔCRD1* cells. A) Mitochondria were visualized using mtGFP. Cells were grown at 30°C to log phase in synthetic leucine deficient medium with or without 200 µg/ml tetracycline (Tet) and examined by fluorescence microscopy. Bars,1 µm. B) Quantitation of cells containing fragmented mitochondria. Values are mean ± S.E. (n=3). At least 500 cells were visualized in each experiment. C–F, the *crd1Δpsd1ΔCRD1* mutant cells were grown at 30° C in the presence or absence of 200 µg/ml tetracycline and harvested at the indicated times. C) CL levels were analyzed by one-dimensional TLC as described under “Materials and methods” and relative levels of CL are indicated. Values are mean ± S.E. (n=3). D) Cells containing fragmented and tubular mitochondrial morphology were quantified. Values are mean ± S.E. (n =3). E) Total cell proteins were extracted and analyzed by SDS-PAGE followed by Western blot. F) Aliquots of crd1Δpsd1Δcells were fixed as described under “Materials and methods” and thin sections were examined by electron microscopy. Labels m and V indicate mitochondria and vacuole (white area), respectively. Bars, 500 nm.

suggesting that the lack of PE causes somewhat reduced fusion even when CL is present. As expected, the fusion phenotype of *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline was similar to that of *psd1*Δ cells. However, in the presence of tetracycline, a complete block of mitochondrial fusion was observed in all the *crd1*Δ*psd1*Δ zygotes examined, consistent with the defective mitochondrial network observed in the absence of both CL and mitochondrial PE (Figure 3.3A). These results indicate that when both CL and mitochondrial PE are deficient, mitochondrial fusion does not occur.

Loss of mitochondrial DNA and reduced mitochondrial membrane potential in cells lacking CL and mitochondrial PE

Several studies have reported that cells defective in mitochondrial fusion lose mitochondrial DNA (mtDNA) (Chen et al., 2010a; Guan et al., 1993; Herlan et al., 2003; Hermann et al., 1998; Nunnari et al., 1997). Therefore, we hypothesized that *crd1*Δ*psd1*Δ cells would exhibit mtDNA loss. To address this possibility, WT, *crd1*Δ, *psd1*Δ, and *crd1*Δ*psd1*Δ cells were grown with or without tetracycline to the mid-logarithmic growth phase at 30°C. Cells were observed under the fluorescence microscope after DAPI staining for the presence of mtDNA (Figure 3.4A). As expected, the majority of *crd1*Δ*psd1*Δ cells (~80%) grown in the absence of tetracycline at the permissive temperature of 30°C retained the mtDNA. This was consistent with our previous study showing that *crd1*Δ cells retained mtDNA at 30°C but exhibited mtDNA loss only at elevated temperatures (Zhong et al., 2004). However, in

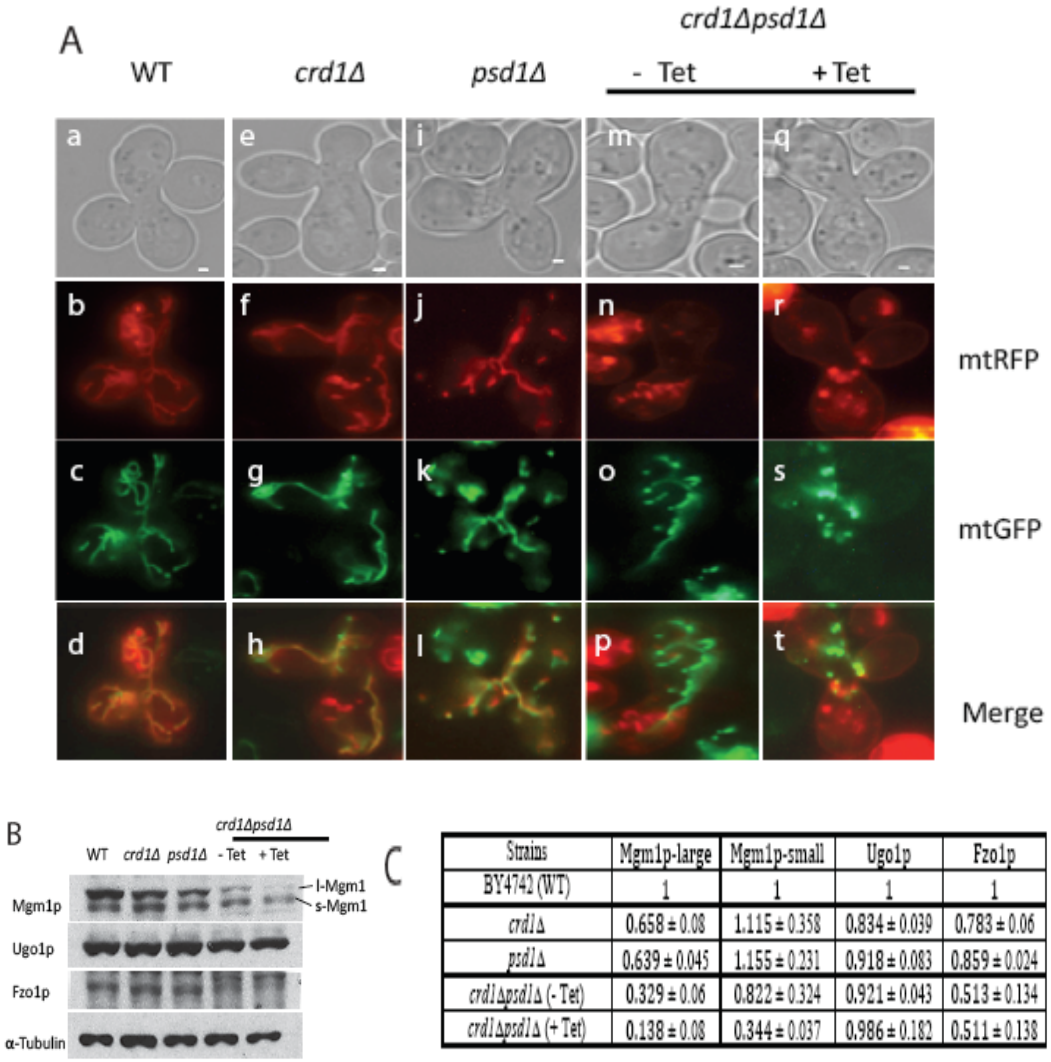
the presence of tetracycline, only ~20% of *crd1*Δ*psd1*Δ cells had mtDNA (Figure 3.4B).

Mitochondrial fusion as determined by *in vitro* assay involves distinct steps of outer and inner membrane fusion (Meeusen et al., 2004). In addition to functional protein complexes, fusion of the outer membrane requires low GTP levels and a proton gradient, while inner membrane fusion requires large amounts of GTP and an inner membrane potential. It is therefore possible that a decreased membrane potential led to the fusion defect in *crd1*Δ*psd1*Δ cells. To test this possibility, we used a flow cytometry assay to measure mitochondrial membrane potential (ΔΨm) in intact WT, *crd1*Δ, *psd1*Δ, and *crd1*Δ*psd1*Δ cells grown with or without tetracycline (Ludovico et al., 2001) in YP-galactose rather than YP-glucose to ensure actively respiring mitochondria. Cells were grown at 30°C to the mid-logarithmic growth phase and then incubated with the voltage-dependent probe tetramethylrhodamine methyl ester (TMRM) (50 nM) for 30 minutes. The accumulation of TMRM in mitochondria is driven by the ΔΨm, which is determined by the difference in yellow fluorescence and forward scatter in the form of fluorescence peaks (Ludovico et al., 2001). Values were calculated relative to the control, i.e., *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline. As seen in Figure 3.4C, *crd1*Δ*psd1*Δ cells in the presence of tetracycline exhibited a decrease in membrane potential similar to that observed in these cells in the presence of sodium azide, a cytochrome *c* oxidase inhibitor that reduces the ΔΨm (Ludovico et al., 2001). These observations were consistent with a reduced membrane potential in cells lacking both CL and mitochondrial PE. It was

recently demonstrated that mitochondrial fusion in mammalian cells requires high ΔΨm levels and is prevented by depolarization (Mitra et al., 2009). Thus, the observed decrease of ΔΨm could be one explanation for the fusion defects in the *crd1*Δ*psd1*Δ mutant cells.

Deletion of *DNM1* in *crd1Δpsd1Δ* cells restores normal mitochondrial tubular network

We wished to determine if the mitochondrial fragmentation observed in *crd1*Δ*psd1*Δ cells could be explained by increased fission rather than decreased fusion. Fusion and fission regulate mitochondrial morphology in an antagonistic manner (Hoppins et al., 2007). Previous studies have shown that three major proteins regulate mitochondrial fission, Dnm1p (Bleazard et al., 1999; Mozdy et al., 2000; Sesaki and Jensen, 1999), Fis1p (Mozdy et al., 2000), and Mdv1p (Tieu and Nunnari, 2000; Tieu et al., 2002). Abolishing mitochondrial fission by deletion of any of these genes leads to net-like mitochondria. In contrast, eliminating fusion by deletion of *MGM1*, *FZO1* or *UGO1* leads to fragmentation, which can be restored to normal tubular morphology by deletion of the fission gene *DNM1* (Sesaki and Jensen, 1999; Wong et al., 2003). If mitochondrial fragmentation in *crd1*Δ*psd1*Δ cells results from a defect in fusion and not increased fission, then disruption of mitochondrial fission would restore mitochondria to the normal tubular morphology. Therefore, we examined if the fragmented mitochondrial morphology of *crd1*Δ*psd1*Δ cells could be rescued to normal tubular mitochondrial morphology by deletion of the fission gene *DNM1*. To do so, we



Strains	Mgm1p-large	Mgm1p-small	Ugo1p	Fzo1p
BY4742 (WT)	1	1	1	1
crd1Δ	0.658 ± 0.08	1.115 ± 0.358	0.834 ± 0.039	0.783 ± 0.06
psd1Δ	0.639 ± 0.045	1.155 ± 0.231	0.918 ± 0.083	0.859 ± 0.024
crd1Δpsd1Δ (- Tet)	0.329 ± 0.06	0.822 ± 0.324	0.921 ± 0.043	0.513 ± 0.134
crd1Δpsd1Δ (+ Tet)	0.138 ± 0.08	0.344 ± 0.037	0.986 ± 0.182	0.511 ± 0.138

Figure 3.3: *crd1*Δ*psd1*Δ cells exhibit defective mitochondrial fusion. (A) Cells of opposing mating type were transformed with either mtGFP or mtRFP. Mitochondrial fusion was examined by observing merged images of mtGFP and mtRFP in WT (a-d), *crd1*Δ (e-h), *psd1*Δ (i-l) and *crd1*Δ*psd1*Δ cells grown without (m-p) or with (q-t) tetracycline. Bars, 1 μm. (B) Total cellular proteins were analyzed by SDS-PAGE followed by Western blot. Steady state levels of Mgm1p, Fzo1p and Ugo1p were measured. α-tubulin was used as a loading control. (C) Quantitation of fusion proteins. Values are mean ± SE (n = 3).

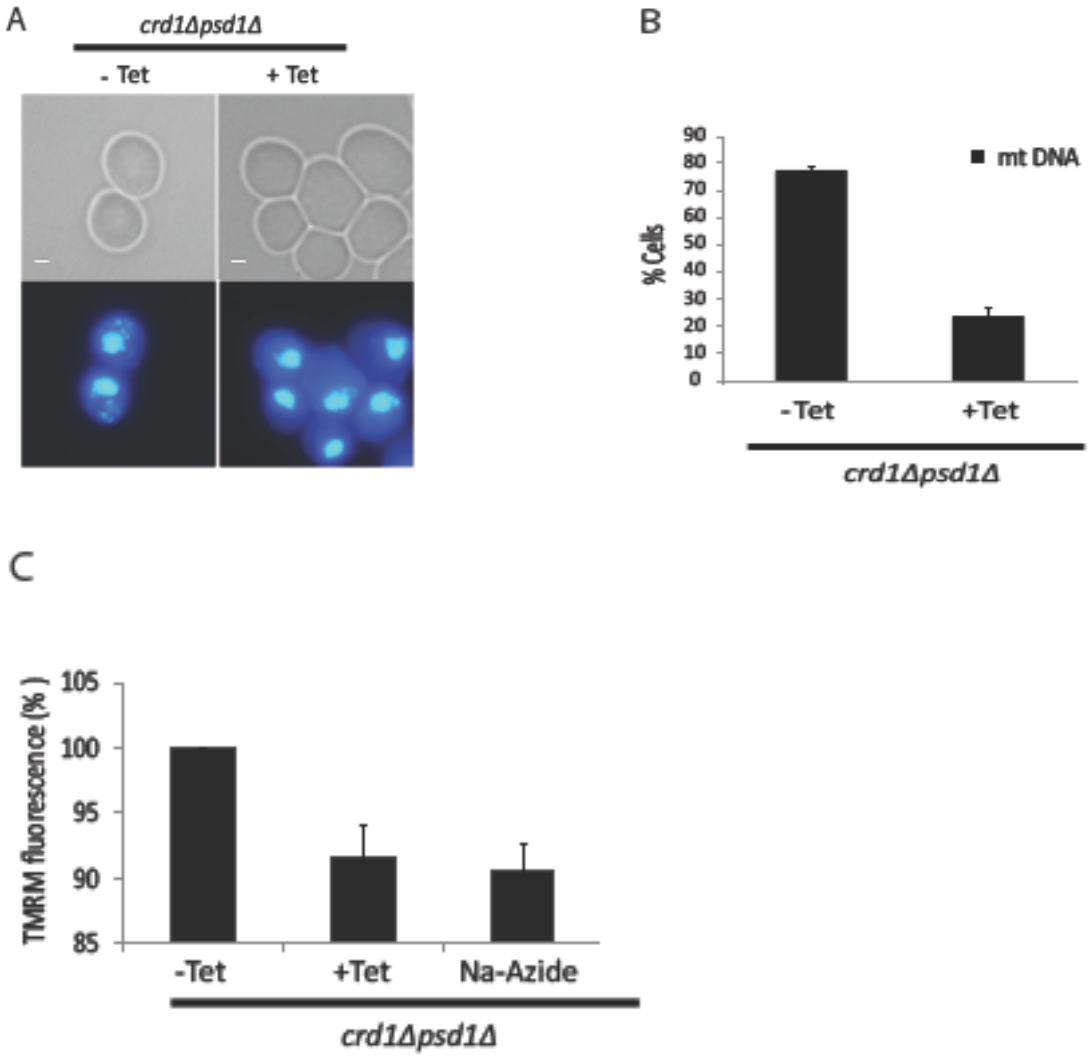


Figure 3.4: *crd1*Δ*psd1*Δ cells exhibit loss of mitochondrial DNA and reduced membrane potential. (A) Cells were grown in YP-gal to log phase at 30°C with or without 200 µg/ml tetracycline (Tet) and stained with DAPI. Bars, 1µm. (B) Quantitation of cells containing mtDNA. Values are mean ± SE (n = 3). At least 500 cells were visualized in each experiment. (C) Dissipation of the mitochondrial membrane potential demonstrated as TMRM fluorescence (%) in *crd1*Δ*psd1*Δ cells grown to log phase in YP-gal with or without tetracycline and stained with TMRM. Cells were also treated with sodium azide as control.

constructed a *crd1*Δ*psd1*Δ*dnm1*Δ conditional mutant containing the plasmid with the TET_{OFF} regulated *CRD1* expression plasmid, as well as a plasmid expressing mtGFP (Figure 3.5A). In the absence of tetracycline, when *CRD1* is expressed, the *crd1*Δ*psd1*Δ*dnm1*Δ cells would be expected to exhibit net-like mitochondria characteristic of a fission defect. However, in the presence of tetracycline, the cells would be predicted to lack both fission and fusion and, hence, would exhibit WT tubular mitochondrial morphology.

As seen in Figure 3.5B, ~76% of *crd1*Δ*psd1*Δ*dnm1*Δ cells grown in the absence of tetracycline exhibited net-like mitochondria, the predicted phenotype. The remaining cells (~18%) exhibited tubular mitochondria, most likely because fusion was decreased in these cells due to the low level of expression of *CRD1* (Figure 1). In the presence of tetracycline, only ~32% of cells exhibited net-like mitochondria while the majority (~45%) exhibited tubular mitochondria, as predicted. These findings suggest that both fission and fusion were defective in these cells and that the fragmented mitochondrial morphology in *crd1*Δ*psd1*Δ cells was rescued by deletion of the fission gene *DNM1* (Figure 3.5A, 3.5B). Tetracycline itself did not affect the mitochondrial morphology in *dnm1*Δ (data not shown). Interestingly, a significant number of *crd1*Δ*psd1*Δ*dnm1*Δ cells (~22%) grown in the presence of tetracycline, had fragmented mitochondria, as the network exhibited the appearance of a string of beads (data not shown). This morphology suggested the presence of a persistent fusion defect in the absence of CL and mitochondrial PE.

To further investigate the block in fusion, we performed an *in vivo* mitochondrial fusion experiment by mating *crd1*Δ*psd1*Δ*dnm1*Δ cells of

opposite mating types, in which one mating type contained mtGFP and the other mating type contained mtRFP. We observed decreased fusion in cells grown without tetracycline, and a complete block in mitochondrial fusion in cells grown with tetracycline (Figure 3.5C). Cells grown without tetracycline that exhibited net-like structures had no defect in mitochondrial fusion (Figure 3.5C). Cells grown in the presence of tetracycline displayed a complete block of mitochondrial fusion. Similar observations were made in the conditional mutant *crd1*Δ*psd1*Δ*fis1*Δ (data not shown). These experiments suggest that *crd1*Δ*psd1*Δ*dnm1*Δ cells exhibited a fusion defect due to loss of *CRD1* and *PSD1*. Taken together, these studies indicate that mitochondrial fragmentation observed in *crd1*Δ*psd1*Δ cells is a result of defective fusion and not due to increased fission.

To determine if deletion of the fission gene *FIS1* or *DNM1* could rescue the lethality of the double mutant, we crossed *crd1*Δ*dnm1*Δ and *crd1*Δ*fis1*Δ with *psd1*Δ and carried out meiotic tetrad analysis to identify viable triple mutants. However, triple mutants were not detected in 72 tetrads of the diploid *crd1Δfis1ΔPSD1/CRD1FIS1psd1*Δ or 75 tetrads of the diploid *crd1Δdnm1ΔPSD1/CRD1DNM1psd1*Δ. Therefore, while CL and mitochondrial PE have overlapping functions in mitochondrial fusion, rescue of the fusion defect could not rescue the synthetic lethality.

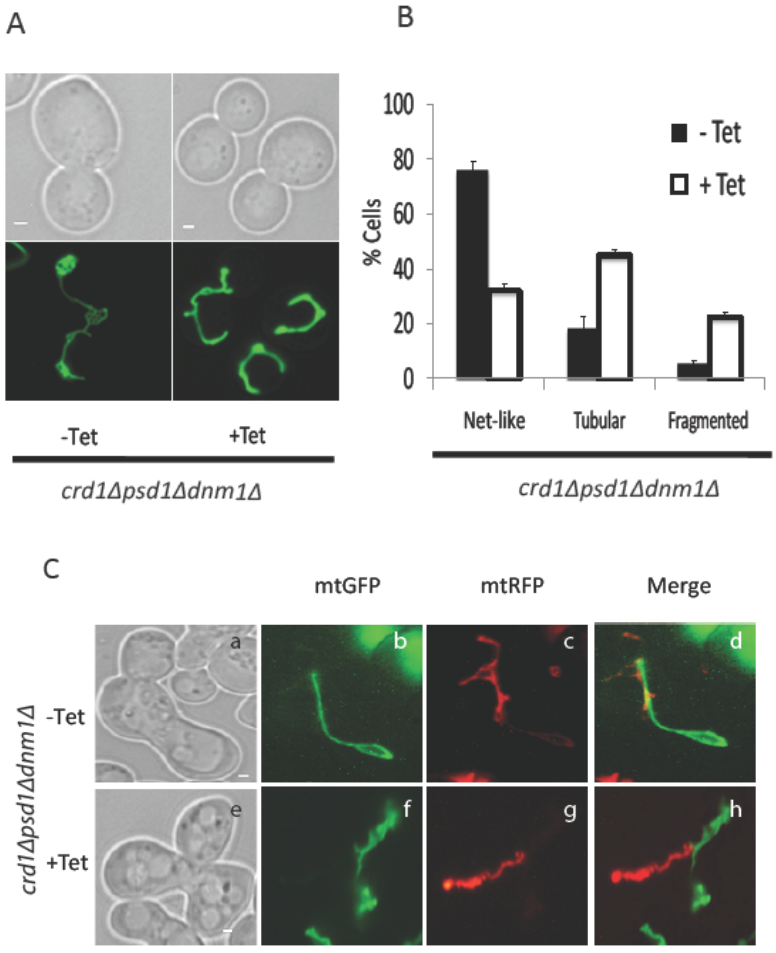


Figure 3.5: *crd1*Δ*psd1*Δ*dnm1*Δ cells are defective in mitochondrial fusion. (A) Mitochondria were visualized in the *crd1*Δ*psd1*Δ*dnm1*Δ mutant using mtGFP. Cells were grown at 30°C to log phase in synthetic deficient glucose medium with 200 µg/ml tetracycline (Tet) where indicated and examined by fluorescence microscopy. Bars, 1µm. (B) Cells containing tubular, fragmented and net-like mitochondria were quantified. Values are mean ± SE (n = 3). At least 100 cells were visualized in each experiment. (C) *crd1*Δ*psd1*Δ*dnm1*Δ cells of opposing mating type were transformed with either mtGFP or mtRFP. Mitochondrial fusion was examined by observing merged images of mtGFP and mtRFP in zygotes of *crd1*Δ*psd1*Δ*dnm1*Δ grown without (a-d) or with (e-h) tetracycline. Bars, 1µm.

Reduced steady state levels of l-Mgm1 and s-Mgm1 isoforms in cells lacking CL and mitochondrial PE

The current study suggests that one common function of CL and PE is mitochondrial fusion. It has been reported that the lack of CL destabilizes the anchoring, assembly, and GTPase activity of fusion protein Mgm1p *in vitro* (Ban et al., 2010; DeVay et al., 2009; Rujiviphat et al., 2009). To test if mitochondrial PE compensates for the loss of CL and stabilizes the fusion proteins *in vivo*, we determined the steady state levels of fusion proteins Fzo1p, Ugo1p, l-Mgm1p and s-Mgm1p in WT, *crd1*Δ, *psd1*Δ, and *crd1*Δ*psd1*Δ cells. The *crd1*Δ*psd1*Δ cells exhibited significantly diminished levels of l-Mgm1p and s-Mgm1p (Figure 3.3B, 3.3C). Fzo1p levels were slightly decreased and Ugo1p was not affected (Figure 3.3B, 3.3C). To determine if the loss of Mgm1p isoforms correlated with the loss CL in *crd1*Δ*psd1*Δ, cells were grown in the presence or absence of tetracycline, proteins were extracted from cells harvested at 5, 8 and 11 hrs, and the levels of Mgm1p isoforms were determined by Western blot (Figure 3.2E). The isoform levels were severely diminished at 11 hrs, which correlated with increased mitochondrial fragmentation as seen in Figure 3.2C. These data indicate that the defect in mitochondrial fusion in *crd1*Δ*psd1*Δ can be attributed at least in part to the reduced levels of s-Mgm1p and the 1-Mgm1p.

DISCUSSION

In this study, we demonstrate that *crd1Δpsd1Δ* cells lacking both CL and mitochondrial PE have fragmented mitochondria due to a defect in mitochondrial fusion. In addition to this defect, we show that *crd1Δpsd1Δ* cells exhibit loss of mtDNA, decreased membrane potential, and reduced steady state levels of short and long isoforms of Mgm1p, a mitochondrial inner membrane protein essential for fusion. The fragmented mitochondrial morphology along with the fusion defect observed in *crd1Δpsd1Δ* cells were rescued by deletion of the fission genes *DNM1* or *FIS1*. These data indicate that CL and mitochondrial PE are required for mitochondrial fusion in vivo.

Our previous studies have shown a synthetic lethal interaction between *crd1Δ* and *psd1Δ* mutant cells, suggesting essential overlapping roles of CL and mitochondrial PE (Gohil et al., 2005). PE synthesized by the non-mitochondrial pathway (Psd2p catalyzed PE synthesis in Golgi/vacuole) (Trotter et al., 1993; Trotter et al., 1995; Trotter and Voelker, 1995) did not rescue this lethality. Externally synthesized PE is inefficiently transported to the inner mitochondrial membrane, as reduced levels of PE were observed in the inner mitochondrial membrane of *psd1Δ* mutant cells (Burgermeister et al., 2004). Taken together, these studies suggested that PE synthesized in the mitochondrial inner membrane has functions that cannot be compensated by externally synthesized PE. In the current study, we demonstrate that the loss of mitochondrial phospholipids CL and PE leads to mitochondrial fragmentation (Fig. 2A, 2B, and 2F) and defective mitochondrial fusion (Fig.

3A). Although mitochondrial fusion is an overlapping function of CL and PE, the lack of mitochondrial fusion is probably not the cause of lethality observed in *crd1Δpsd1Δ* cells, as lethality was not rescued by deletion of the fission gene *FIS1* or *DNM1*. Mitochondria are required not only for cellular bioenergetics, but also for the synthesis of essential metabolites. In addition, our previous studies have shown that CL is required for non-mitochondrial functions, including vacuolar function, the high osmolarity glycerol (Schuller et al., 1994) pathway, and cell wall synthesis (Chen et al., 2008b; Zhong et al., 2007; Zhou et al., 2009). Thus, it is possible that lethality in cells lacking CL and PE could be caused by deficiencies in both mitochondrial and non-mitochondrial functions. The identification of suppressors of *crd1Δpsd1Δ* synthetic lethality will very likely identify the essential cellular functions shared by these phospholipids. These studies are currently in progress.

How do CL and mitochondrial PE affect mitochondrial fusion? Non-bilayer lipids are known to affect the function and stability of many mitochondrial membrane proteins (Schlame and Ren, 2009). Recent studies have proposed that scaffolding proteins such as prohibitin recruit membrane proteins to CL and PE rich regions, forming protein rich lipid domains (Osman et al., 2011). The lack of CL and mitochondrial PE might influence the distribution of these domains, which in turn would affect several mitochondrial processes, including mitochondrial fusion. Although early studies suggested that the non-bilayer forming phospholipids CL and PE play an important role in mitochondrial fusion, very little was known about the mechanism by which this could occur (Cullis and de Kruijff, 1979; Furt and Moreau, 2009; van den

Brink-van der Laan et al., 2004). In this study, we show that the lack of CL and mitochondrial PE leads to reduced steady state levels of both large and small isoforms of Mgm1p (Fig. 2E, 3B), which are required for fusion. Recent studies have shown that l-Mgm1p acts as an anchor in the inner membrane (Zick et al., 2009). Both CL and PE are synthesized and predominantly localized in the inner mitochondrial membrane, and the loss of both CL and mitochondrial PE might affect the stability of this isoform, leading to its degradation. The formation of s-Mgm1p requires functional mitochondrial protein import machinery, membrane potential and adequate ATP levels, all of which are defective in cells lacking CL (Claypool et al., 2008; Duvezin-Caubet et al., 2006; Gebert et al., 2009; Herlan et al., 2004; Jiang et al., 2000). This is a first report describing the overlapping roles of CL and mitochondrial PE in fusion in vivo, and suggests a mechanistic role for these phospholipids in regulating mitochondrial structure and function.

How is the role of CL and PE in mitochondrial fusion relevant to human disease? The role of mitochondrial phospholipids in fusion is relevant to studies that implicate function of mitochondrial fusion in cardiac function (Dorn et al., 2011). Fragmented mitochondria are associated with the loss of Opa1 (the human homolog of Mgm1p) in mitochondrial myopathies involving cardiac and skeletal muscle (Duvezin-Caubet et al., 2006) and in ischemic cardiomyopathy (Chen et al., 2009). Overexpression of the fusion proteins Mfn1/2 (human homolog of Fzo1p) prevents cardiac cell death from ischemia (Ong et al., 2010). Elucidating the role of CL and PE in mitochondrial fusion may also shed light on defects observed in lymphoblast mitochondria from

patients with Barth syndrome (BTHS), a severe genetic disorder characterized by dilated cardiomyopathy and skeletal myopathy (Barth et al., 1996; Bolhuis et al., 1991). BTHS is caused by mutation in the CL remodeling enzyme tafazzin, resulting in decreased CL and altered fatty acid composition of major mitochondrial phospholipids, including CL and PE (Xu et al., 2005). Defects in mitochondrial fusion may account for the observed morphological variation in BTHS mitochondria, including enlarged size, fragmentation, adhesion of opposing membranes and deformed intercristae space observed in BTHS lymphoblasts as well as in cardiac and skeletal muscle mitochondria of the mouse model of BTHS (Acehan et al., 2011; Acehan et al., 2007). Identifying the role of CL and PE in mitochondrial fusion may thus explain, in part, the wide variation in the clinical presentation observed in BTHS.

CHAPTER 4

PERTURBATION OF THE TCA CYCLE IN CELLS LACKING CARDIOLIPIN CANNOT BE ALLEVIATED DUE TO DEFECTIVE MITOCHONDRIAL RETROGRADE RESPONSE

INTRODUCTION

Cardiolipin (CL) is synthesized in the mitochondrial inner membrane and interacts with various inner membrane protein complexes (Houtkooper and Vaz, 2008; Pfeiffer et al., 2003). CL mutants exhibit deficiencies in mitochondrial bioenergetics (Koshkin and Greenberg, 2000; Koshkin and Greenberg, 2002), perturbation of mitochondrial protein import, decreased membrane potential (Jiang et al., 2000), and defective assembly of mitochondrial outer membrane complexes in both yeast and Barth syndrome (BTHS) lymphoblasts (Gebert et al., 2009). Clearly, perturbation of CL synthesis disrupts numerous important mitochondrial functions.

The mitochondrion is an essential organelle that plays a crucial role in cellular bioenergetics, programmed cell death, and metabolism. The tricarboxylic acid cycle (TCA) is the central metabolic pathway that occurs in the mitochondrial matrix of eukaryotic cells. The TCA cycle is an amphibolic pathway that is critical for oxidation of acetyl-CoA and for the production of the reducing agent NADH, which is used by the respiratory complexes to generate ATP. It is also important for biosynthetic processes, including amino

acid and heme biosynthesis. TCA cycle enzymes are more important for cell growth when non-fermentable carbon sources are metabolized. Nevertheless, the first three steps of the TCA cycle leading to the synthesis of α-ketoglutarate are expressed at basal levels even during growth on glucose. α-ketoglutarate is a precursor for synthesis of glutamate, which is utilized for the synthesis of glutamine, proline and arginine.

Yeast cells respond to the loss of mitochondrial DNA or to decreased mitochondrial function resulting from perturbation of the TCA cycle by signaling the nucleus to modulate the expression of genes required to replenish key mitochondrial metabolites (Epstein et al., 2001). In particular, the expression of genes required for glutamate biosynthesis is upregulated by activation of the mitochondrial retrograde (RTG) pathway (Gangloff et al., 1990; Liu and Butow, 1999). Target genes that respond to RTG signaling include *CIT2*, *CIT1*, *ACO1*, *IDH1* and *IDH2*, which are required for glutamate synthesis, and *DLD3*, a cytosolic D-lactate dehydrogenase (Chelstowska and Butow, 1995; Liao and Butow, 1993; Liao et al., 1991; Liu and Butow, 1999). Thus, the central role of the RTG pathway is to replenish TCA cycle intermediates, such as α-ketoglutarate, which is a precursor for glutamate biosynthesis.

The RTG pathway is regulated by several positive (Rtg1p, Rtg2p, Rtg3p, Grr1p) and negative (Mks1p, Bmh1p, Bmh2p, Lst8p) regulators that control expression of the RTG target genes (Figure 4.1) (Liu and Butow, 2006). In response to decreased mitochondrial function, the RTG pathway is

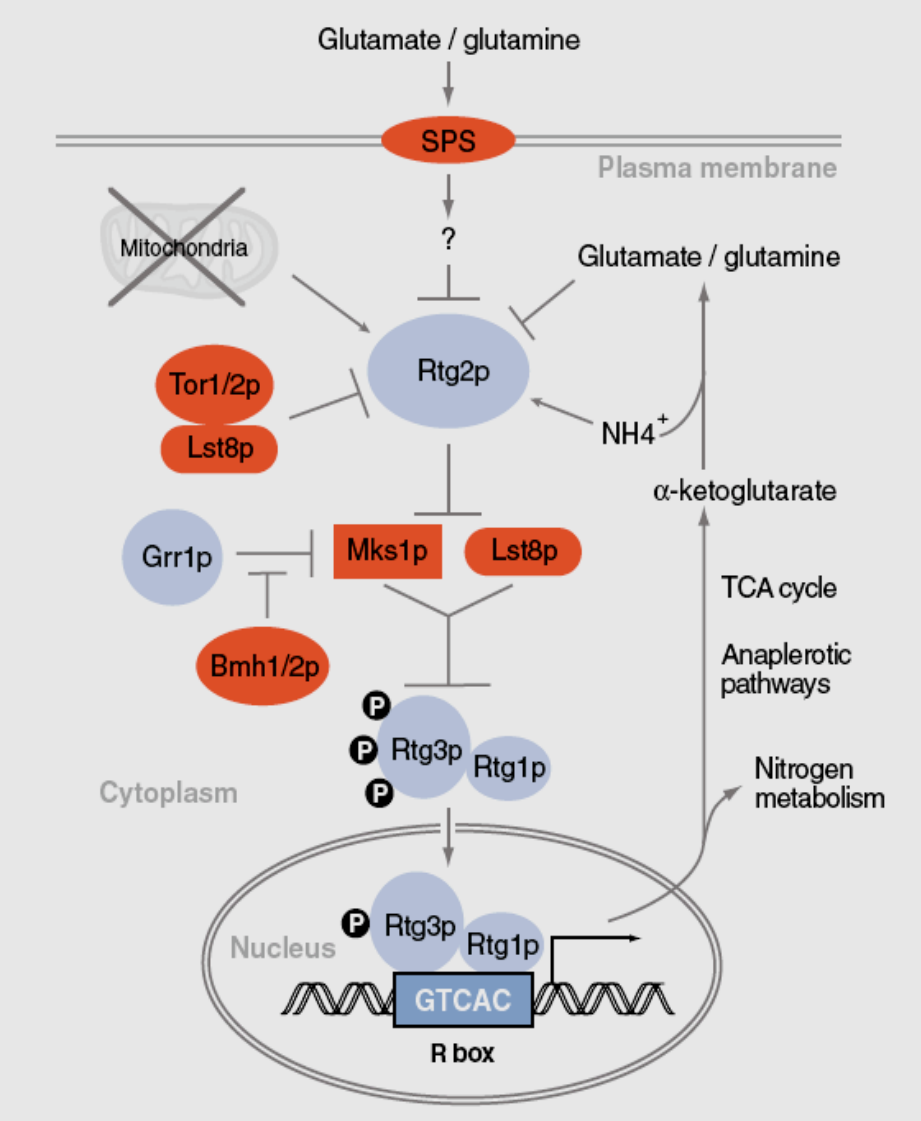


Figure 4.1: The positive (blue) and negative (red) regulators of the RTG pathway. Based on the functional state of mitochondria, and availability of glutamate, the positive and negative regulators of the RTG pathway modulate expression of the target genes (Liu and Butow, 2006).

activated by dephosphorylation and translocation of Rtg3p/Rtg1p into the nucleus (Sekito et al., 2000). Rtg1p and Rtg3p are transcription factors that bind to the promoter region (R box) of target genes such as *CIT2* (Sekito et al., 2000). Mks1p, a negative regulator of the RTG pathway, prevents the translocation of Rtg3p/Rtg1p into the nucleus by promoting phosphorylation of Rtg3p. It acts downstream of Rtg2p and upstream of Rtg1/3p (Dilova et al., 2004). Thus, Rtg2p is required for dephosphorylation of Rtg3p and nuclear translocation of Rtg1/3p (Liu et al., 2003). In addition, Rtg2p binds to the promoter region of *CIT2* and increases its expression under inducible conditions (Pray-Grant et al., 2002). Bmh1p and Bmh2p (14-3-3 proteins) act as negative regulators of the RTG pathway by preventing degradation of Mks1p. These proteins are known to bind different phosphorylated proteins; hence, they have diverse cellular functions, in addition to their role in the RTG pathway (Gelperin et al., 1995; Kakiuchi et al., 2007).

In addition to the RTG pathway, other routes to replenish the TCA cycle metabolites in yeast include β-oxidation and the glyoxylate cycle (Figure 4.2). These two metabolic pathways complement each other and replenish vital metabolites such as acetyl CoA, succinate, and citrate. Acetyl CoA is a central metabolite of carbon assimilation in eukaryotic cells. In addition to synthesis of acetyl CoA from pyruvate by pyruvate dehydrogenase (PDH) in the mitochondria, acetyl-CoA is imported into the mitochondria via two pathways (Elgersma et al., 1995; van Roermund et al., 1999). One requires *CIT2*, a peroxisomal citrate synthase that converts acetyl-CoA into the glyoxylate cycle intermediates succinate and citrate. These metabolites are

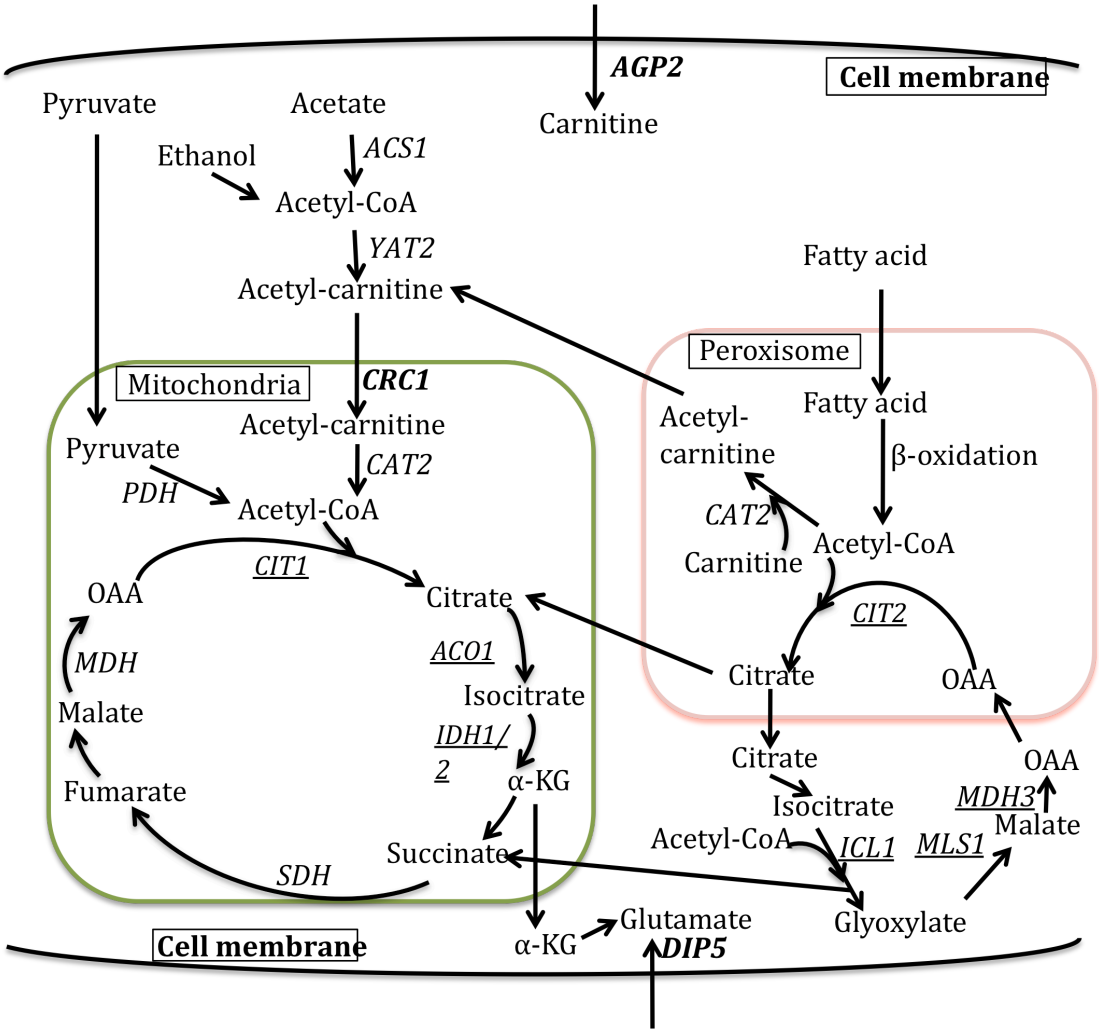


Figure 4.2: Metabolic pathways that replenish the TCA cycle. The glyoxylate cycle and the β-oxidation pathway compensate the TCA cycle by providing metabolic intermediates. The glyoxylate cycle provides citrate and succinate while the β-oxidation pathway provides acetyl-CoA to the TCA cycle. Glyoxylate cycle and glutamate biosynthesis genes that require Rtg1p, Rtg2p, and Rtg3p for expression are underlined. Metabolite transporters are in bold. Modified from Liu and Butow, 2006.

imported into the mitochondria by carrier proteins. The second pathway involves transport of acetyl-carnitine into the mitochondria via the carnitine transporter Crc1p (van Roermund et al., 1999). Interestingly, some BTHS patients have decreased levels of carnitine and accumulation of fat droplets due to decreased transport of acetyl CoA into the mitochondria (Barth et al., 1983). In the mitochondria, carnitine acetyl CoA transferase catalyzes the release of acetyl units, which can be utilized subsequently in the TCA cycle.

Studies from the Greenberg lab have shown that: 1) CL mutants exhibit decreased activity of the TCA cycle enzymes aconitase and succinate dehydrogenase (Vinay Patil, unpublished); 2) CL mutants are synthetically lethal with pyruvate dehydrogenase mutants, suggesting that acetyl CoA synthesis is decreased in the mutants (Vaishnavi Raja, unpublished); and 3) genes that play a role in acetate metabolism, uptake of acetyl CoA into the mitochondria, and transporters of lactate and several amino acid are up-regulated in the *crd1*Δ mutant (Vishal Gohil, unpublished). These findings suggest that CL deficiency leads to perturbation of the TCA cycle. As discussed above, a block in the TCA cycle leads to activation of the mitochondrial RTG pathway by up regulation of *CIT2* gene expression. In this study, I report that *CIT2* is not upregulated in cells lacking CL at elevated temperature despite the mitochondrial dysfunction present in these cells. In addition, I find that overexpression of *RTG2* or supplementation of glutamate rescues the growth defect of *crd1*Δ at elevated temperature. Based on these findings, I hypothesize that CL deficiency leads to perturbation of the TCA

cycle, which is not alleviated due to either defective or insufficient activation of the RTG pathway.

Materials and methods

Yeast strains and growth media:

The yeast strains used in this study are isogenic to BY4741 and BY4742 (Table 4.1). Complex medium (YPD) contained 1% yeast extract (US Biological), 2% peptone (Fischer Scientific) and 2% glucose (Fischer Scientific). Synthetic medium contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), and uracil (20 mg/liter), vitamins, salts (essentially components of Difco Vitamin Free Yeast Base without amino acids), and glucose (2%), acetate (2%) or oleic acid (0.1%) as carbon source. Glutamate was added to a final concentration of 0.05% when indicated. Sporulation medium contained potassium acetate (1%), glucose (0.05%), and the essential amino acids. Solid medium was prepared by adding 2% agar.

Construction of double mutants by tetrad dissection:

The CL mutant strain *crd1Δ::URA3 MATα* was crossed with mutants of the opposite mating type obtained from the yeast deletion collection. Heterozygous diploids were selected on synthetic media lacking methionine and lysine, sporulated, and tetrads were dissected.

Real time PCR:

Yeast cultures (10 ml) were grown to an A_{550} of 1.0 in YPD at 30^0C, cells were harvested, and total RNA was isolated using the acid phenol method (Collart and Oliviero, 2001). The RNA was then purified with an RNeasy Plus Mini Kit (Qiagen). cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche). Real time PCR reactions were performed using a quantitative PCR SYBR green mix (Stratagene). Duplicates for each sample were included for each reaction. RNA levels of the gene of interest were normalized to *ACT1*, which was used as an internal control.

Table 4.1: Strains and plasmids used in this study

Strains	Genotype	Reference
BY4742	*MATα, his 301, leu 200, lys 200, ura 300*	Invitrogen
VGY1	*MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3*	Gohil et al., 2005
BY4742	*rho^0 MATα , his 301, leu 200, lys 200, ura 300*	Shuliang Chen
VGY1	*rho^0 MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3*	Shuliang Chen
rtg1Δ	*MATa, his 301, leu 200, met 1500, ura 300, rtg1Δ::KanMX4*	Invitrogen
rtg2Δ	*MATa, his 301, leu 200, met 1500, ura 300, rtg2Δ::KanMX4*	Invitrogen
rtg3Δ	*MATa, his 301, leu 200, met 1500, ura 300, rtg3Δ::KanMX4*	Invitrogen
mks1Δ	*MATa, his 301, leu 200, met 1500, ura 300, mks1Δ::KanMX4*	Invitrogen
bmh1Δ	*MATa, his 301, leu 200, met 1500, ura 300, bmh1Δ::KanMX4*	Invitrogen
bmh2Δ	*MATa, his 301, leu 200, met 1500, ura 300, bmh2Δ::KanMX4*	Invitrogen
crd1Δrtg1Δ	*MATα*, *his 301*, *leu 200*, *lys 200*, *ura 300*, *crd1Δ::URA3*, *rtg1Δ::KanMX4*	This study
crd1Δrtg2Δ	*MATa*, *his 301*, *leu 200*, *lys 200*, *met 1500*, *ura 300*, *crd1Δ::URA3*, *rtg2Δ::KanMX4*	This study
crd1Δrtg3Δ	*MATa*, *his 301*, *leu 200*, *lys 200*, *ura 300*, *crd1Δ::URA3*, *rtg3Δ::KanMX4*	This study
crd1Δmks1Δ	*MATα*, *his 301*, *leu 200*, *lys 200*, *ura 300*, *crd1Δ::URA3*, *mks1Δ::KanMX4*	This study
crd1Δbmh1Δ	*MATa*, *his 301*, *leu 200*, *ura 300*, *crd1Δ::URA3*, *bmh1Δ::KanMX4*	This study
crd1Δbmh2Δ	*MATα*, *his 301*, *leu 200*, *lys 200*, *met 1500*, *ura 300*, *crd1Δ::URA3*, *bmh2Δ::KanMX4*	This study
pYPGK18	2 µm, *LEU2*	Vaz et al., 2003
pYPGK18-RTG2	Derivative of pYPGK18, expresses *RTG2* from PGK1 promoter	Chen et al., 2008

Results

Overexpression of *RTG2* alleviates the temperature sensitivity of cells lacking CL

I utilized the genetic approach of suppressor analysis to identify functions of CL that are deficient in CL mutants. To this end, I performed a suppressor screen to isolate suppressors of the ts phenotype of *taz1*Δ*tom70*Δ (Figure 2.2). The rationale for using the *taz1*Δ*tom70*Δ mutant is that it exhibited a more severe growth defect than *crd1*Δ, thus enabling more stringent conditions for isolating suppressors. We obtained 45 putative suppressors after screening 3119 transformants. *RTG1* was a putative suppressor identified in this screen. As the goal of the study was to identify cellular functions of CL, I checked if *RTG1* rescued the ts phenotype of *crd1*Δ. Overexpression of *RTG1* or *RTG3* alone did not rescue the ts phenotype. In contrast, I observed that overexpression of *RTG2* rescued the ts phenotype of the *crd1*Δ mutant (Figure 4.3). This suggested that the function of the RTG pathway was deficient in *crd1*Δ at elevated temperature. Consistent with this, deletion of *RTG1*, *RTG2*, *or RTG3* in the *crd1*Δ mutant exacerbated the growth phenotype at elevated temperature in synthetic media (Figure 4.4). As discussed above, defects in the RTG pathway lead to glutamate auxotrophy, as RTG proteins regulate the expression of genes required for glutamate synthesis. We observed that supplementation of glutamate rescues the ts phenotype of the *crd1*Δ mutant at elevated temperature (Figure 4.5), which is consistent with the prediction that the RTG response in the CL mutant is

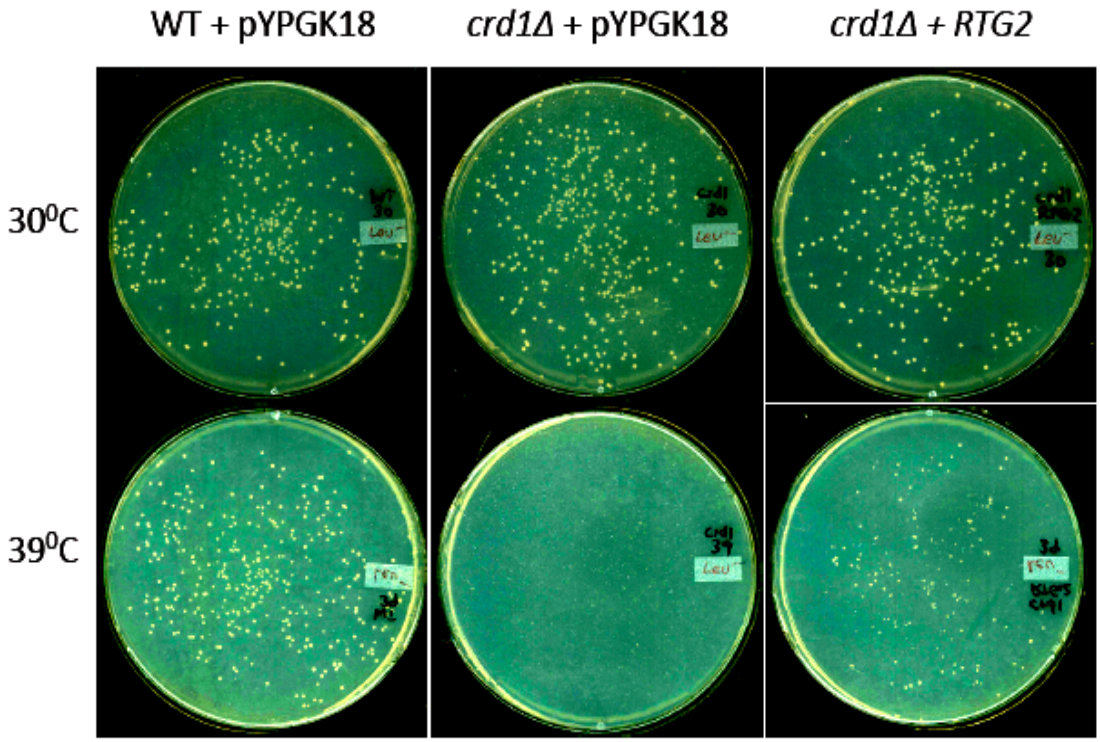


Figure 4.3: Overexpression of *RTG2* rescues temperature sensitivity of *crd1*Δ. Cells were grown in leucine deficient synthetic medium overnight at 30^0C, diluted, plated on synthetic media lacking leucine and incubated at 30^0C and 39^0C for 3-5 days.

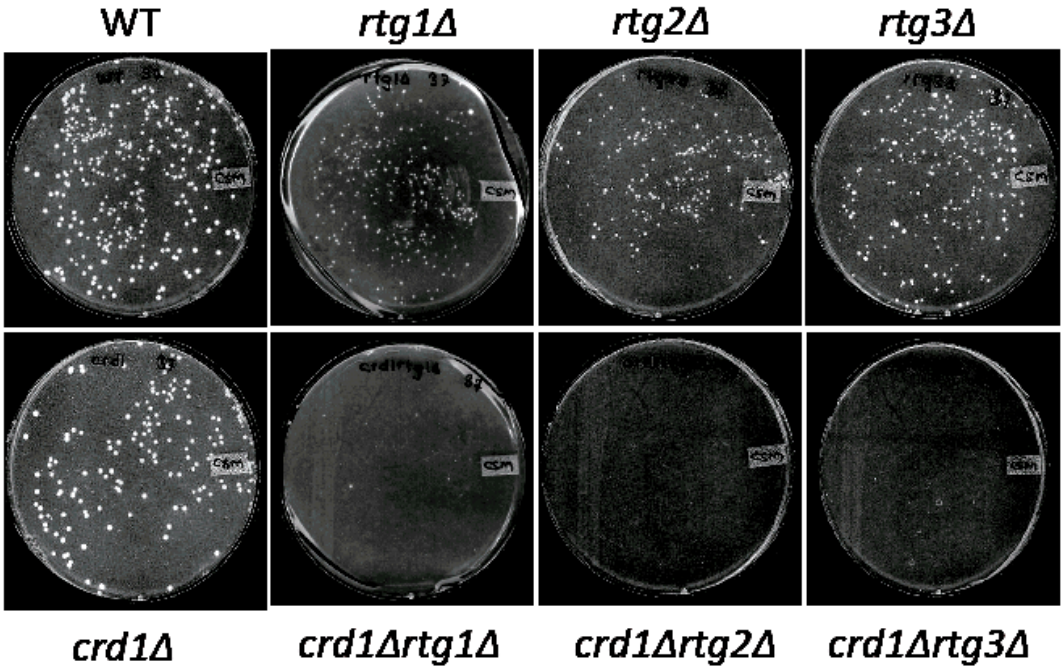


Figure 4.4: RTG genes are required for growth of *crd1*Δ in synthetic media without glutamate at elevated temperature. Cells were pre-cultured overnight in YPD at 30^0C, plated on synthetic media with glucose lacking glutamate and incubated at 37^0C for 3-5 days.

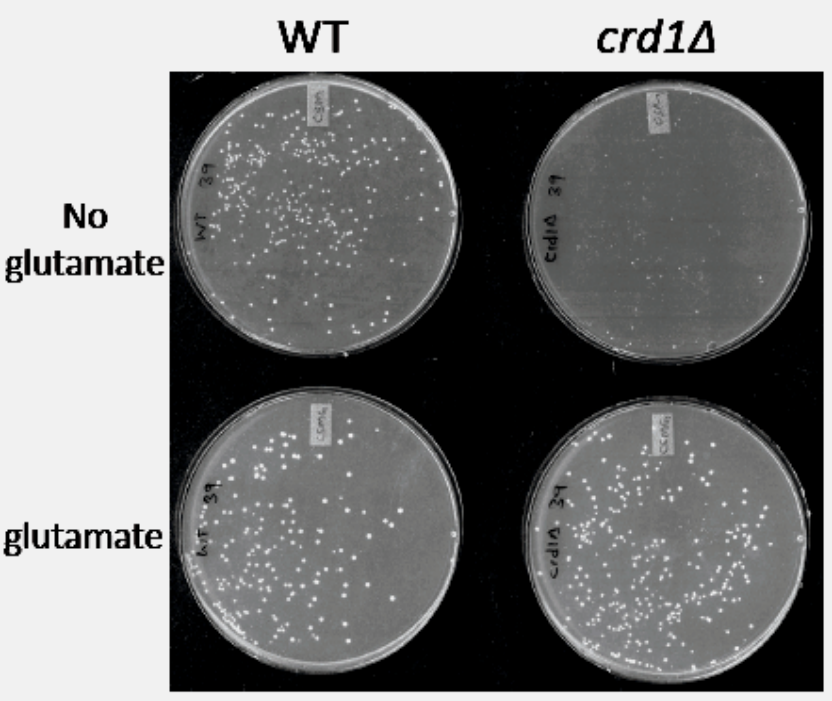


Figure 4.5: Glutamate supplementation rescues the ts phenotype of *crd1*Δ. Cells were pre-cultured overnight in YPD at 30^0C, washed and plated onto synthetic media with or without 0.05% glutamate and incubated at 39^0C for 3-5 days.

insufficient to alleviate mitochondrial dysfunction at elevated temperature.

Cells lacking CL cannot induce expression of *CIT2* at elevated temperature.

Previous studies have shown that loss of mitochondrial DNA (Epstein et al., 2001), decreased mitochondrial membrane potential (Miceli et al., 2011), and decreased activities of TCA cycle enzymes such as succinate dehydrogenase (Lin et al., 2011) lead to activation of the RTG pathway. Therefore, I expected that in *crd1*Δ cells, which exhibit similar mitochondrial defects, the RTG pathway would be activated and expression of the RTG target gene *CIT2* would be increased. I found that *CIT2* mRNA levels in *crd1*Δ cells, as compared to WT, were upregulated ~4 fold relative to WT at 30^0C (Figure 4.6). However, to my surprise, *CIT2* was not upregulated in *crd1*Δ at elevated temperature, the condition in which it exhibits severe mitochondrial defects (Figure 4.6). These results indicate that the RTG pathway is not activated at elevated temperature in the *crd1*Δ mutant. As the RTG response is strain specific, I checked if the defect in activation of the RTG pathway at elevated temperature could be explained by a reduced RTG response in the BY4742 strain utilized in this study. To this end, I compared *CIT2* mRNA levels in WT rho^0 and rho$^+$ cells. As expected, *CIT2* mRNA levels were ~5-fold upregulated in WT rho^0 compared to rho$^+$ cells, indicating that the BY4742 strain does exhibit an RTG response. Interestingly, *crd1*Δ rho^0 cells exhibited a greater increase in *CIT2* mRNA levels than WT at 30^0C probably due to the combinatorial effect of loss of mtDNA and decreased mitochondrial function

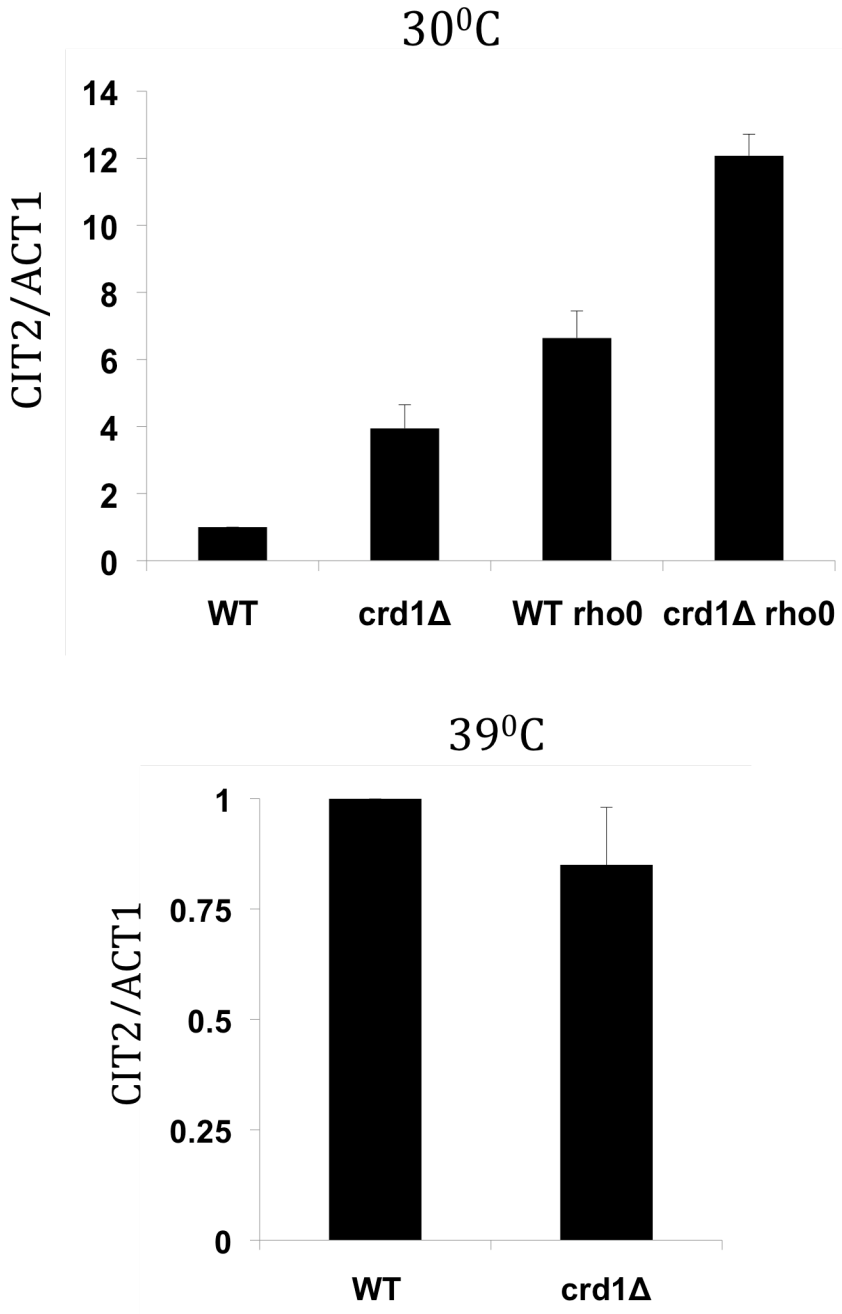


Figure 4.6: Quantitative measurement of CIT2 mRNA levels. Total RNA was extracted from cells grown to an A_{550} of 1.0 to 2.0 in YPD at 30°C and 39°C. mRNA levels of *CIT2* were determined by real-time PCR. Expression was normalized to the mRNA levels of the internal control *ACT1*. Values are mean ± S.E from two independent experiments with technical duplicates.

(Figure 4.6). Even though the retrograde pathway was activated in *crd1*Δ rho^0 cells at 30^{0}C, these cells exhibited a growth defect in synthetic media that was rescued by glutamate supplementation (Figure 4.7). Taken together, my results suggest that *crd1*Δ cells can activate the RTG pathway at 30^{0}C but not at the elevated temperature.

Deletion of negative regulator *BMH2* rescues glutamate auxotrophy of *crd1*Δ at elevated temperature

The loss of negative regulators of the RTG pathway leads to constitutive expression of the target genes and rescue of glutamate auxotrophy (Dilova et al., 2002). Therefore, I predicted that defective activation of the RTG pathway would be rescued by deletion of the negative regulators, include *MKS1*, *BMH1*, *BMH2* and *LST8*. Among these, *LST8* is an essential gene and the null mutant is not viable. As the other genes are not essential, I deleted *MKS1*, *BMH1*, and *BMH2* in the *crd1*Δ mutant. I observed that deletion of *BHM2* but not *MKS1* or *BMH1* rescued the ts phenotype of the *crd1*Δ mutant in synthetic media lacking glutamate (Figure 4.8). In contrast, deletion of *MKS1* exacerbated the *crd1*Δ mutant phenotype (Figure 4.8). A possible explanation for this finding is that in the BY4742 background, Mks1p may not be a negative regulator. Discrepancies regarding the role of *MKS1* in the RTG pathway have been reported (Dilova and Powers, 2006). While Mks1p was shown to be a negative regulator in some studies (Dilova et al., 2002; Sekito et al., 2002), Shamji et al., (2000) reported that RTG target gene expression

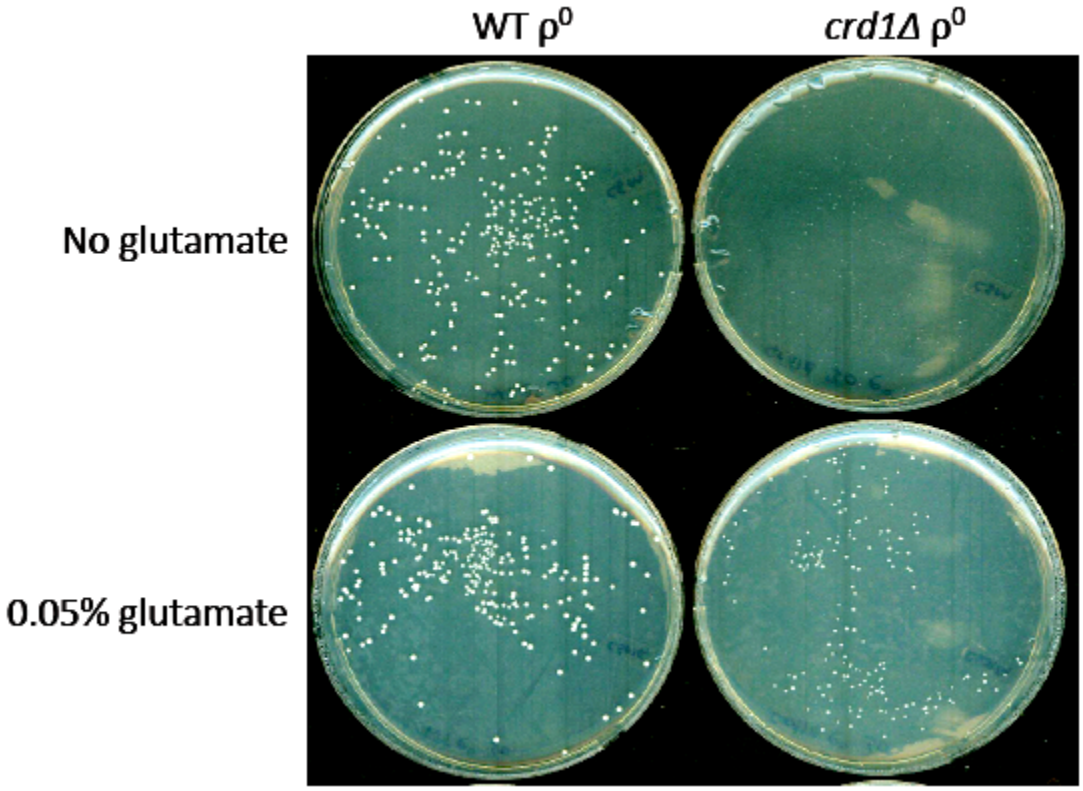


Figure 4.7: *crd1*Δ rho^0 exhibits glutamate auxotrophy at 30^{0}C. Cells were pre-cultured overnight in YPD at 30^{0}C. WT rho^0 and *crd1*Δ rho^0 cells were plated on synthetic media with or without 0.05% glutamate and incubated at 30^{0}C for 3-5 days.

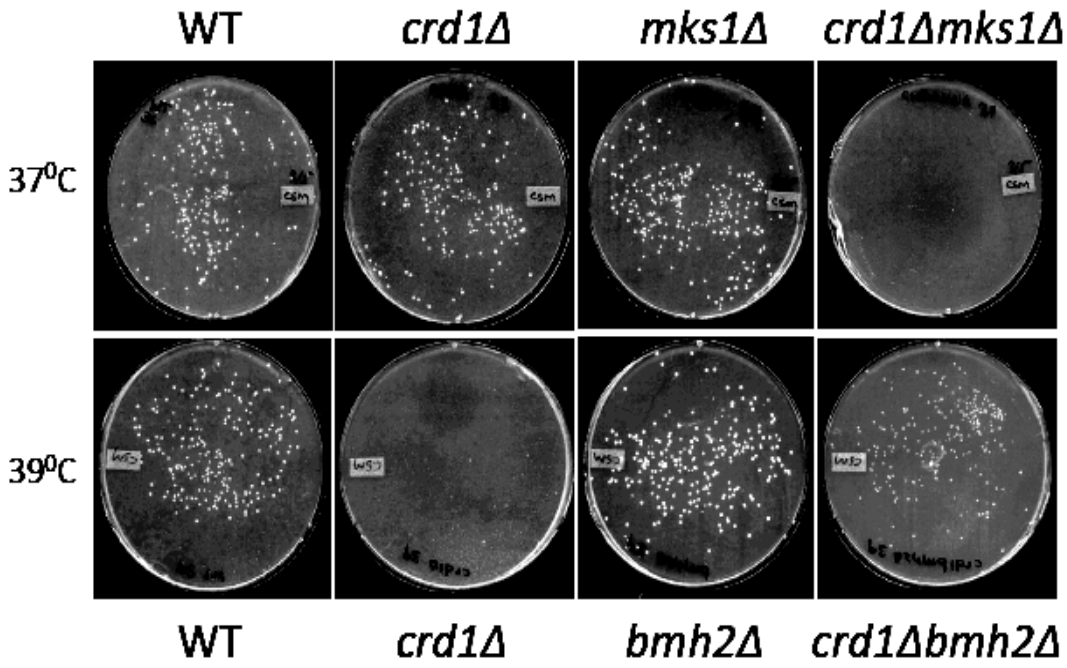


Figure 4.8: Deletion of *BMH2* suppresses the ts phenotype of *crd1*Δ. Cells were pre-cultured overnight in YPD at 30^0C, grown until an A_{550} of 1.0 in YPD at 30^0C, washed with water, plated onto synthetic media without glutamate and incubated at 37^0C and 39^0C as indicated for 3-5 days.

was actually impaired in the *mks1*Δ strain, suggesting that Mksp1 is a positive regulator of the RTG pathway. Consistent with an activator role, I observed that the *mks1*Δ mutant, similar to the *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ mutants, required glutamate for growth in the BY4742 strain used in the current study (data not shown). In addition, expression of the RTG target genes was impaired in *mks1*Δ (data not shown) In summary, deletion of the negative regulator *BMH2* rescued the glutamate auxotrophy of *crd1*Δ, while deletion of positive regulators exacerbated the phenotype of the *crd1*Δ mutant.

Cells lacking CL cannot utilize acetate or oleic acid as sole source of carbon

The glyoxylate cycle and β-oxidation pathways compensate for TCA cycle deficiencies by supplying intermediates, as discussed above (Figure 4.2). Oleic acid is broken down in peroxisomes by β-oxidation to supply acetyl-CoA to the TCA cycle, or to supply TCA cycle intermediates by the glyoxylate cycle. As mentioned, the RTG pathway is required for expression of *CIT2*, a glyoxylate cycle gene, and for peroxisomal genes required for β-oxidation in cells grown in oleic acid (Chelstowska and Butow, 1995). Therefore, RTG mutants cannot utilize acetate or oleic acid as a sole source of carbon. Consistent with our hypothesis that *crd1*Δ has defective RTG signaling, the *crd1*Δ mutant, similar to RTG mutants, exhibits decreased growth on acetate (Figure 4.9) or oleic acid media (Figure 4.10). The growth defects in acetate are indications of TCA and glyoxylate cycle deficiencies, whereas inability of

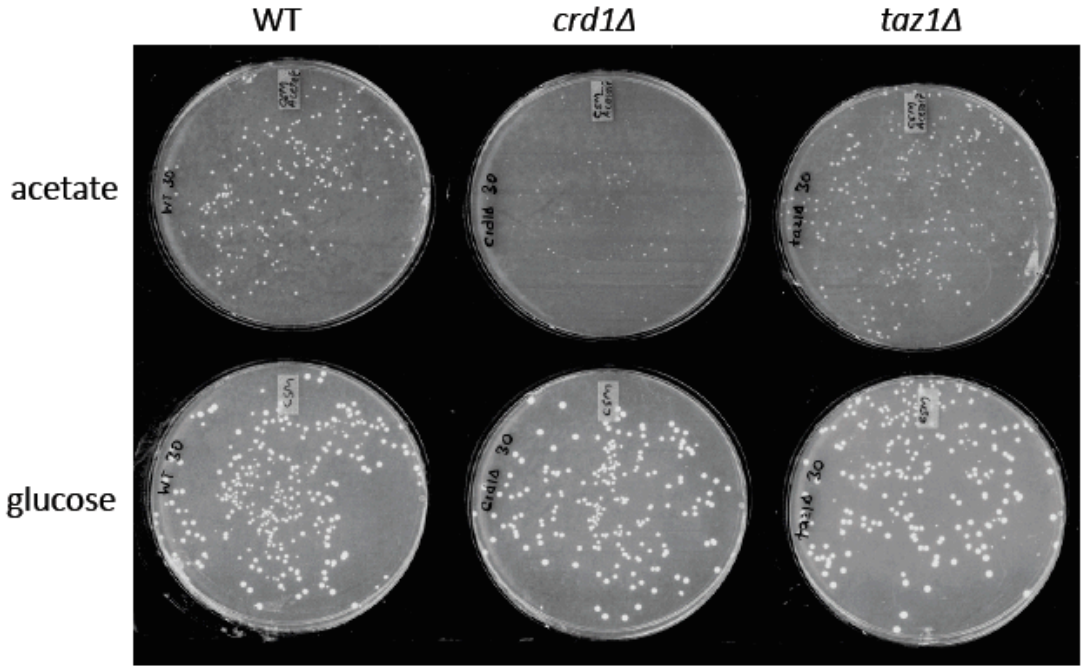


Figure 4.9: *crd1*Δ exhibits a growth defect on acetate. Cells were pre-cultured overnight in YPD. WT, *crd1*Δ, and *taz1*Δ cells were plated on synthetic media containing glucose and acetate as the sole source of carbon and incubated at 30^0C for 6 days.

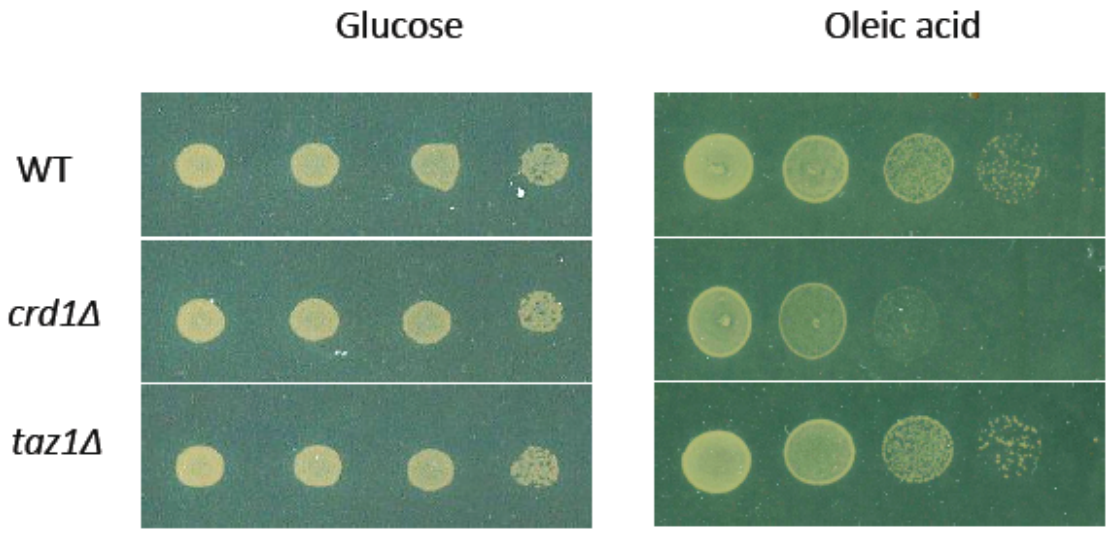


Figure 4.10: *crd1*Δ exhibits a growth defect on oleic acid. Cells were pre-cultured in YPD until stationary phase at 30^0C. Cells were counted and spotted on synthetic media containing glucose or oleic acid as the sole source of carbon and incubated at 30^0C for 5-7days.

*crd1*Δ cells to utilize oleic acid as a sole source of carbon (Figure 4.10) indicates that the mutant may have a defect in 1) β-oxidation, 2) mitochondrial uptake of acetyl-CoA generated by β-oxidation, or 3) uptake of TCA intermediates synthesized by the β-oxidation pathway and the glyoxylate cycle. Interestingly, the *taz1*Δ mutant grows normally (Figures 4.9 and 4.10) suggesting that decrease in CL, aberrant CL species or increased MLCL levels, as observed in *taz1*Δ, are sufficient to support growth on acetate or oleic acid media. These results are consistent with the hypothesis that cells lacking CL exhibit a defective RTG pathway.

Discussion

The experiments in this study support the hypothesis that the loss of CL leads to defective activation of the RTG pathway at elevated temperature. Consistent with an RTG defect, the RTG target gene *CIT2* is not upregulated, and *crd1*Δ cells exhibit glutamate auxotrophy at elevated temperature. Temperature sensitivity of the *crd1*Δ mutant was rescued by overexpression of *RTG2,* a positive regulator of the RTG pathway, and by deletion of *BMH2*, a negative regulator of the RTG pathway. Furthermore, *crd1*Δ cells exhibit growth defects on acetate or oleic acid media, indicating a defect in the glyoxylate cycle and β-oxidation pathways, which require the RTG pathway for activation. Taken together, these results indicate that perturbation of the TCA cycle in *crd1*Δ cells cannot be alleviated due to a defective activation of the RTG pathway.

Deletion of *BMH2*, a negative regulator of the RTG pathway rescued the glutamate auxotrophy of the *crd1*Δ mutant at elevated temperature (Figure 4.8). In addition to its role in the RTG pathway, Bmh2p, interacts with several phosphorylated proteins that regulate cell signaling pathways, stress response pathways, the cell cycle, and chitin synthesis at the cell wall (Kakiuchi et al., 2007). Interestingly, the Greenberg lab has shown that the *crd1*Δ mutant has defects in the stress response pathway, cell wall synthesis, and the cell cycle (Chen et al., 2010b; Zhong and Greenberg, 2005; Zhou et al., 2009). This suggests the possibility that Bmh2p might not only affect the

activation of the RTG pathway but also affect these other pathways, which are deficient in the *crd1*Δ mutant.

A decrease in the mitochondrial membrane potential is believed to be the signal for activation of the RTG pathway (Miceli et al., 2011), which is probably sensed by Rtg2p (Jazwinski and Kriete, 2012). Although a decreased membrane potential was observed in *crd1*Δ (Jiang et al., 2000) the RTG pathway was not triggered at elevated temperature. This suggests that the signal for activation of the RTG pathway is not relayed in *crd1*Δ cells, and that Rtg2p may be defective in the *crd1*Δ mutant. Consistent with this, overexpression of Rtg2p rescued the ts phenotype of *crd1*Δ cells (Figure 4.3).

In mammalian cells, NFκB is believed to be a master regulator of the RTG response that senses mitochondrial dysfunction (Liu and Butow, 2006), a role similar to Rtg2p in yeast. NFκB modulates the expression of nuclear encoded genes (Cogswell et al., 2003) via activation of the Myc-Max heterodimer, a homolog of the RTG1-RTG3 heterodimer (Jazwinski and Kriete, 2012). Thus, identifying the mechanism of RTG regulation in *crd1*Δ may facilitate understanding of CL function in NFκB mediated signaling in mammalian cells.

As discussed earlier, the TCA cycle is required for the biosynthesis of many metabolites, including amino acids such as glutamate, arginine, methionine and lysine. Recent studies from the Greenberg lab have shown that CL deficient cells are auxotrophic for methionine and lysine (Vinay Patil, unpublished) as well as for glutamate (Figure 4.6). Interestingly, amino acids have been used as supplements to treat BTHS patients, with a positive

outcome (Richard Kelly, unpublished). We anticipate that elucidating the metabolic deficiencies in *crd1*Δ cells will contribute to our understanding of similar deficiencies observed in BTHS. The restoration of deficient metabolites will offer potential new treatments for this disorder.

CHAPTER 5

FUTURE DIRECTIONS

The studies described in this thesis show exciting new roles of CL in diverse mitochondrial functions, including protein import, fusion and RTG pathway regulation. While my findings have shed some light on the mechanisms underlying the cellular functions of CL, I feel that they have uncovered just the tip of the iceberg. In this chapter, I suggest fascinating as well as challenging new directions for future studies that could lead to a better understanding of the mechanisms underlying CL function.

1. CL and mitochondrial protein import: CL plays vital roles in the mitochondrial inner membrane, including assembly of protein complexes, (Claypool, 2009; Claypool et al., 2006; Claypool et al., 2008; Pfeiffer et al., 2003), mitochondrial protein import, and membrane potential (Jiang et al., 2000; Kutik et al., 2008; Tamura et al., 2009). Based on the genetic interaction of CL mutants with the TOM, SAM and MDM complex mutants, I hypothesized that CL plays a role in outer membrane protein biogenesis. As CL is synthesized and is predominantly present in the mitochondrial inner membrane, it was exciting to identify a function of CL in the biogenesis of outer membrane protein complexes required for mitochondrial protein import (Chapter 2). Importantly, CL is required for import not only in yeast. We also showed a role of CL in assembly of outer membrane complexes in humans

(Gebert et al., 2009). This is the first finding in humans that links CL to mitochondrial protein import.

While we have established the function of CL in mitochondrial protein import at the outer as well as inner membrane (Chapter 2), the implication of defective protein import in CL deficient cells is unknown. A large-scale proteomic study to compare the mitochondrial proteins in *crd1*Δ and WT cells would identify the proteins that are not imported into mitochondria lacking CL. Although this may seem challenging, it is both doable (as there are only ~600 to 700 mitochondrial proteins in yeast) and promising, as it may greatly facilitate the identification of specific mitochondrial metabolic deficiencies observed in *crd1*Δ cells (Chapter 4). Furthermore, it will be fascinating to elucidate the role of a mitochondrial lipid in the regulation of basic metabolic pathways.

A complementary approach to determine the consequences of defective protein import in CL deficient cells is to identify suppressors of the genetic interactions identified between mitochondrial protein import mutants and CL mutants (Chapter 2). Suppressor mutations may fall into a number of categories, including those that increase mitochondrial protein import, that increase the amount of an essential protein that is limiting, and/or that have a decrease in the accumulation of a toxic product that leads to the defective growth phenotype.

2. CL and mitochondrial morphology: The study described in Chapter 2 utilized a genetic interaction screen to identify the functions of CL in

mitochondrial morphology. Genetic interaction was determined based on the phenotype of the double mutant (*crd1*Δ and mitochondrial morphology mutant). While several categories were uncovered, I draw future students' attention to two in particular, the UPS and GET complexes. An interesting finding pertinent to the regulation of CL synthesis is that *crd1*Δ is synthetically lethal with *ups1*Δ (Figure 2.3). Other studies have reported that the UPS complex (Ups1p and Ups2p) is required for regulating CL levels (Osman et al., 2009a; Tamura et al., 2009). Why is *crd1*Δ synthetically lethal with *ups1*Δ? How do UPS proteins regulate the levels of CL in the mitochondria? Answers to these intriguing questions would clarify how Ups1p regulates CL synthesis.

While UPS proteins are present in mitochondria, GET proteins (Get1p and Get2p) are present in the ER membrane. I showed that *crd1*Δ is synthetically sick with *get1*Δ and *get3*Δ, which suggests that CL shares functions with GET proteins. It was surprising to find genetic interaction of a mitochondrial lipid mutant with the ER mutants. The GET proteins are required for targeting proteins into the ER membrane (Schuldiner et al., 2008). It is interesting to speculate that CL may be involved in targeting proteins to the ER membrane, especially given the fact that ER and mitochondrial membranes are tightly connected (Kornmann et al., 2009).

Decreased levels of CL observed in the *get2*Δ mutant point to another interesting question. In addition to its role in protein targeting, does Get2p also play a role in lipid transfer from the ER to the mitochondria? The mechanism of lipid transfer from ER to mitochondria is not known. It is possible that GET proteins might transfer lipids from the ER to the mitochondria by creating

ERMES-like contact sites (Kornmann et al., 2009). Future work focusing on these questions may reveal very interesting connections between ER and mitochondria.

3. CL and mitochondrial fusion: Chapter 3 describes the function of CL in mitochondrial fusion in yeast. The loss of CL in yeast does not lead to a defect in the mitochondrial tubular structure, although *in vitro* studies reported that CL is required for the assembly and activity of the fusion protein Mgm1p. Based on earlier findings that the loss of both CL and PE is lethal (Gohil et al., 2005), I hypothesized that PE might compensate for CL in mitochondrial fusion. Indeed, my studies showed that loss of both PE and CL leads to mitochondrial fragmentation due to a fusion defect (Joshi et al., 2012). It will be fascinating to study if the loss of mitochondrial PE and CL leads to structural defects in other cellular organelles such as ER, vacuole, and peroxisomes. These findings would suggest that PE and CL might be required for cross talk between cellular organelles.

While the studies in Chapter 3 address a shared function of CL and mitochondrial PE, they do not answer the question of why the loss of both is lethal. Answering this question would identify essential mitochondrial as well as non-mitochondrial functions of these phospholipids. The “awesome power of yeast genetics” can be used to isolate suppressors of *crd1*Δ*psd1*Δ lethality, which would lead to the identification of essential functions that are missing in *crd1*Δ*psd1*Δ cells.

4. CL and the mitochondrial RTG pathway: In Chapter 4, I discussed a fascinating new role of CL in activation of the RTG pathway. While decreased mitochondrial function leads to activation of the RTG pathway in WT cells, *crd1*Δ cells, which have decreased mitochondrial function, fail to activate the RTG pathway at elevated temperature. This is the first report to show that a phospholipid be might required for activation of the RTG mediated signaling pathway. I reported in Chapter 4 that the key RTG target *CIT2* is not upregulated in *crd1*Δ cells (Figure 4.6B). Consistent with this, temperature sensitivity of *crd1*Δ is rescued by 1) overexpression of *RTG2* (Figure 4.3), a positive regulator of the RTG pathway, 2) deletion of *BMH2* (Figure 4.5), a negative regulator of the RTG pathway, and 3) supplementation with glutamate (Figure 4.6). All these findings indicate that activation of the RTG pathway is defective.

Why do cells lacking CL have a defect in activation of the RTG pathway? One possible reason involves the interplay between the positive and the negative regulators of the RTG pathway. Rtg3p phosphorylation may be perturbed in *crd1*Δ cells. Rtg2p activity may be defective in the mutant. Alternatively, protein levels of the negative regulators may be elevated in *crd1*Δ cells. A second possibility is that altered levels of TCA cycle intermediates in the CL mutants lead to decreased expression of *CIT2*. At least two possible mechanisms can alter the levels of TCA intermediates in *crd1*Δ cells. First, decreased activities of TCA cycle enzymes such as aconitase and succinate dehydrogenase (SDH), as seen in *crd1*Δ cells (Vinay Patil, unpublished) could lead to decrease TCA intermediates. Second,

decrease in TCA cycle intermediates in mitochondria may result from decreased activity of transporters present in mitochondrial membranes. Transporters such as Crc1p (carnitine), Sfc1p (succinate-fumarate), Ctp1p (citrate), and Odc1p (α-ketoglutarate) are required to shuttle metabolites across the mitochondrial membranes. CL might affect the activity of the transporters in the membrane, causing an accumulation of the TCA cycle intermediates in the mitochondria. An accumulation of metabolites such as succinate in the mitochondria has been shown to inhibit the expression of *CIT2* (Lin et al., 2011). It will be interesting to check if accumulation of other metabolites in the mitochondria has a similar effect on expression of *CIT2*. Based on these observations, I hypothesize that the loss of CL leads to defective shuttling of TCA cycle intermediates across the mitochondrial membranes due to decreased activity of metabolite transporters, culminating in feedback inhibition of *CIT2* expression.

Another interesting possibility is that the loss of CL can lead to decreased activity enzymes in the glyoxylate cycle, and β-oxidation pathway that cannot be alleviated by activation of the RTG pathway. Consistent glyoxylate and β-oxidation defects, the *crd1*Δ mutant is unable to utilize acetate or oleic acid as a sole source of carbon (Figure 4.9 and 4.10). The enzymes for these pathways are localized in peroxisomes. Interestingly, a significant amount of CL (7% of total phospholipids) is present in the peroxisomal membrane (Zinser et al., 1991) Therefore, CL deficiency may lead to decreased activities of enzymes in this organelle. It is possible that

blocks in these pathways could lead to metabolic deficiencies in *crd1*Δ cells that are not alleviated by activation of the RTG pathway.

I challenge my colleagues in the Greenberg lab to address these questions, the answers to which will facilitate our understanding of the role of CL in the mitochondrial RTG pathway and in pathways replenishing the TCA cycle.

5. What makes CL research exciting? A great deal remains to be learned about the uniqueness of CL. As discussed above, questions regarding the function of CL in mitochondrial protein import, mitochondrial morphology and the RTG pathway remain unanswered. What makes this study exciting? First, because these functions are conserved and have direct implications for human disorders, answering these questions will have important implications not just for BTHS, but also for other disorders in which CL plays a role. Second, we can use the simple yeast model system to elucidate the link between the functions of a lipid to regulation of basic metabolic pathways. Third, imagine that we identify metabolites that are deficient in CL mutants. Can we then use this knowledge to cure CL related disorders such as BTHS simply by supplementing metabolites as nutrients, saving patients from more harsh treatments? This is what excites me about CL research. I hope you are excited too. I wish all the success to the folks in the Greenberg lab for their future work. Cheers!

REFERENCES

Acehan, D., F. Vaz, R.H. Houtkooper, J. James, V. Moore, C. Tokunaga, W. Kulik, J. Wansapura, M.J. Toth, A. Strauss, and Z. Khuchua. 2011. Cardiac and skeletal muscle defects in a mouse model of human barth syndrome. *J Biol Chem*. 286:899-908.

Acehan, D., Y. Xu, D.L. Stokes, and M. Schlame. 2007. Comparison of lymphoblast mitochondria from normal subjects and patients with Barth syndrome using electron microscopic tomography. *Lab Invest*. 87:40-48.

Agsteribbe, E., A. Huckriede, M. Veenhuis, M.H. Ruiters, K.E. Niezen-Koning, O.H. Skjeldal, K. Skullerud, R.S. Gupta, R. Hallberg, O.P. van Diggelen, and et al. 1993. A fatal, systemic mitochondrial disease with decreased mitochondrial enzyme activities, abnormal ultrastructure of the mitochondria and deficiency of heat shock protein 60. *Biochem Biophys Res Commun*. 193:146-154.

Ardail, D., O. Gateau-Roesch, P. Louisot, R. Morelis, J.P. Privat, M. Egret-Charlier, and M. Ptak. 1990. Triggering of mannosyltransferase activity in inner mitochondrial membranes by dolichyl-monophosphate incorporation mediated through phospholipids or fatty acids. *Eur J Biochem*. 188:547-556.

Ban, T., J.A. Heymann, Z. Song, J.E. Hinshaw, and D.C. Chan. 2010. OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. *Hum Mol Genet*. 19:2113-2122.

Barth, K.H., S.W. Brusilow, S.L. Kaufman, and F.T. Ferry. 1981. Percutaneous transluminal angioplasty of homograft renal artery stenosis in a 10-year-old girl. *Pediatrics*. 67:675-677.

Barth, P.G., H.R. Scholte, J.A. Berden, J.M. Van der Klei-Van Moorsel, I.E. Luyt-Houwen, E.T. Van 't Veer-Korthof, J.J. Van der Harten, and M.A. Sobotka-Plojhar. 1983. An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes. *J Neurol Sci*. 62:327-355.

Barth, P.G., F. Valianpour, V.M. Bowen, J. Lam, M. Duran, F.M. Vaz, and R.J. Wanders. 2004. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. *Am J Med Genet A*. 126A:349-354.

Barth, P.G., C. Van den Bogert, P.A. Bolhuis, H.R. Scholte, A.H. van Gennip, R.B. Schutgens, and A.G. Ketel. 1996. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): respiratory-chain abnormalities in cultured fibroblasts. *J Inherit Metab Dis*. 19:157-160.

Barth, P.G., R.J. Wanders, and P. Vreken. 1999a. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome)-MIM 302060. *J Pediatr*. 135:273-276.

Barth, P.G., R.J. Wanders, P. Vreken, E.A. Janssen, J. Lam, and F. Baas. 1999b. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) (MIM 302060). *J Inherit Metab Dis*. 22:555-567.

Bauer, M.F., C. Sirrenberg, W. Neupert, and M. Brunner. 1996. Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell*. 87:33-41.

Beranek, A., G. Rechberger, H. Knauer, H. Wolinski, S.D. Kohlwein, and R. Leber. 2009. Identification of a cardiolipin-specific phospholipase encoded by the gene CLD1 (YGR110W) in yeast. *J Biol Chem*. 284:11572-11578.

Beyer, K., and M. Klingenberg. 1985. ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by 31P nuclear magnetic resonance. *Biochemistry*. 24:3821-3826.

Bione, S., P. D'Adamo, E. Maestrini, A.K. Gedeon, P.A. Bolhuis, and D. Toniolo. 1996. A novel X-linked gene, G4.5. is responsible for Barth syndrome. *Nature genetics*. 12:385-389.

Bleazard, W., J.M. McCaffery, E.J. King, S. Bale, A. Mozdy, Q. Tieu, J. Nunnari, and J.M. Shaw. 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat Cell Biol*. 1:298-304.

Bolender, N., A. Sickmann, R. Wagner, C. Meisinger, and N. Pfanner. 2008. Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Rep*. 9:42-49.

Bolhuis, P.A., G.W. Hensels, T.J. Hulsebos, F. Baas, and P.G. Barth. 1991. Mapping of the locus for X-linked cardioskeletal myopathy with neutropenia and abnormal mitochondria (Barth syndrome) to Xq28. *Am J Hum Genet*. 48:481-485.

Briones, P., M.A. Vilaseca, A. Ribes, A. Vernet, M. Lluch, V. Cusi, A. Huckriede, and E. Agsteribbe. 1997. A new case of multiple mitochondrial enzyme deficiencies with decreased amount of heat shock protein 60. *J Inherit Metab Dis*. 20:569-577.

Burgermeister, M., R. Birner-Grunberger, R. Nebauer, and G. Daum. 2004. Contribution of different pathways to the supply of phosphatidylethanolamine and phosphatidylcholine to mitochondrial membranes of the yeast Saccharomyces cerevisiae. *Biochim Biophys Acta*. 1686:161-168.

Cardonick, E.H., K. Kuhlman, E. Ganz, and L.T. Pagotto. 1997. Prenatal clinical expression of 3-methylglutaconic aciduria: Barth syndrome. *Prenat Diagn*. 17:983-988.

Chacinska, A., M. Lind, A.E. Frazier, J. Dudek, C. Meisinger, A. Geissler, A. Sickmann, H.E. Meyer, K.N. Truscott, B. Guiard, N. Pfanner, and P. Rehling. 2005. Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17. *Cell*. 120:817-829.

Chang, S.C., P.N. Heacock, C.J. Clancey, and W. Dowhan. 1998a. The PEL1 gene (renamed PGS1) encodes the phosphatidylglycero-phosphate synthase of Saccharomyces cerevisiae. *J Biol Chem*. 273:9829-9836.

Chang, S.C., P.N. Heacock, E. Mileykovskaya, D.R. Voelker, and W. Dowhan. 1998b. Isolation and characterization of the gene (CLS1) encoding cardiolipin synthase in Saccharomyces cerevisiae. *J Biol Chem*. 273:14933-14941.

Chelstowska, A., and R.A. Butow. 1995. RTG genes in yeast that function in communication between mitochondria and the nucleus are also required for expression of genes encoding peroxisomal proteins. *J Biol Chem*. 270:18141-18146.

Chen, D.C., B.C. Yang, and T.T. Kuo. 1992. One-step transformation of yeast in stationary phase. *Curr Genet*. 21:83-84.

Chen, H., M. Vermulst, Y.E. Wang, A. Chomyn, T.A. Prolla, J.M. McCaffery, and D.C. Chan. 2010a. Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell*. 141:280-289.

Chen, L., Q. Gong, J.P. Stice, and A.A. Knowlton. 2009. Mitochondrial OPA1, apoptosis, and heart failure. *Cardiovasc Res*. 84:91-99.

Chen, S., Q. He, and M.L. Greenberg. 2008a. Loss of tafazzin in yeast leads to increased oxidative stress during respiratory growth. *Mol Microbiol*. 68:1061-1072.

Chen, S., D. Liu, R.L. Finley, Jr., and M.L. Greenberg. 2010b. Loss of mitochondrial DNA in the yeast cardiolipin synthase crd1 mutant leads to up-regulation of the protein kinase Swe1p that regulates the G2/M transition. *J Biol Chem*. 285:10397-10407.

Chen, S., M. Tarsio, P.M. Kane, and M.L. Greenberg. 2008b. Cardiolipin mediates cross-talk between mitochondria and the vacuole. *Mol Biol Cell*. 19:5047-5058.

Claypool, S.M. 2009. Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. *Biochim Biophys Acta*. 1788:2059-2068.

Claypool, S.M., J.M. McCaffery, and C.M. Koehler. 2006. Mitochondrial mislocalization and altered assembly of a cluster of Barth syndrome mutant tafazzins. *J Cell Biol*. 174:379-390.

Claypool, S.M., Y. Oktay, P. Boontheung, J.A. Loo, and C.M. Koehler. 2008. Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol*. 182:937-950.

Cogswell, P.C., D.F. Kashatus, J.A. Keifer, D.C. Guttridge, J.Y. Reuther, C. Bristow, S. Roy, D.W. Nicholson, and A.S. Baldwin, Jr. 2003. NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. *J Biol Chem*. 278:2963-2968.

Collart, M.A., and S. Oliviero. 2001. Preparation of yeast RNA. *Curr Protoc Mol Biol*. Chapter 13:Unit13 12.

Cullis, P.R., and B. de Kruijff. 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim Biophys Acta*. 559:399-420.

Curran, S.P., D. Leuenberger, W. Oppliger, and C.M. Koehler. 2002. The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier. *EMBO J*. 21:942-953.

Daum, G. 1985. Lipids of mitochondria. *Biochim Biophys Acta*. 822:1-42.

Davey, K.M., J.S. Parboosingh, D.R. McLeod, A. Chan, R. Casey, P. Ferreira, F.F. Snyder, P.J. Bridge, and F.P. Bernier. 2006. Mutation of DNAJC19, a human homologue of yeast inner mitochondrial membrane co-chaperones, causes DCMA syndrome, a novel autosomal recessive Barth syndrome-like condition. *J Med Genet*. 43:385-393.

de Kruijff, B., A. Rietveld, N. Telders, and B. Vaandrager. 1985. Molecular aspects of the bilayer stabilization induced by poly(L-lysines) of varying size in cardiolipin liposomes. *Biochim Biophys Acta*. 820:295-304.

DeVay, R.M., L. Dominguez-Ramirez, L.L. Lackner, S. Hoppins, H. Stahlberg, and J. Nunnari. 2009. Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. *J Cell Biol*. 186:793-803.

Dilova, I., S. Aronova, J.C. Chen, and T. Powers. 2004. Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1.Rtg3p-dependent target genes. *J Biol Chem*. 279:46527-46535.

Dilova, I., C.Y. Chen, and T. Powers. 2002. Mks1 in concert with TOR signaling negatively regulates RTG target gene expression in S. cerevisiae. *Curr Biol*. 12:389-395.

Dilova, I., and T. Powers. 2006. Accounting for strain-specific differences during RTG target gene regulation in Saccharomyces cerevisiae. *FEMS Yeast Res*. 6:112-119.

Dorn, G.W., 2nd, C.F. Clark, W.H. Eschenbacher, M.Y. Kang, J.T. Engelhard, S.J. Warner, S.J. Matkovich, and C.C. Jowdy. 2011. MARF and Opa1 control mitochondrial and cardiac function in Drosophila. *Circ Res*. 108:12-17.

Dowhan, W. 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu Rev Biochem*. 66:199-232.

Duvezin-Caubet, S., R. Jagasia, J. Wagener, S. Hofmann, A. Trifunovic, A. Hansson, A. Chomyn, M.F. Bauer, G. Attardi, N.G. Larsson, W. Neupert, and A.S. Reichert. 2006. Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. *J Biol Chem*. 281:37972-37979.

Dzugasova, V., M. Obernauerova, K. Horvathova, M. Vachova, M. Zakova, and J. Subik. 1998. Phosphatidylglycerolphosphate synthase encoded by the

PEL1/PGS1 gene in Saccharomyces cerevisiae is localized in mitochondria and its expression is regulated by phospholipid precursors. *Current genetics*. 34:297-302.

Eilers, M., T. Endo, and G. Schatz. 1989. Adriamycin, a drug interacting with acidic phospholipids, blocks import of precursor proteins by isolated yeast mitochondria. *J Biol Chem*. 264:2945-2950.

Elgersma, Y., C.W. van Roermund, R.J. Wanders, and H.F. Tabak. 1995. Peroxisomal and mitochondrial carnitine acetyltransferases of Saccharomyces cerevisiae are encoded by a single gene. *EMBO J*. 14:3472-3479.

Epstein, C.B., J.A. Waddle, W.t. Hale, V. Dave, J. Thornton, T.L. Macatee, H.R. Garner, and R.A. Butow. 2001. Genome-wide responses to mitochondrial dysfunction. *Mol Biol Cell*. 12:297-308.

Fry, M., and D.E. Green. 1980. Cardiolipin requirement by cytochrome oxidase and the catalytic role of phospholipid. *Biochem Biophys Res Commun*. 93:1238-1246.

Fry, M., and D.E. Green. 1981. Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. *J Biol Chem*. 256:1874-1880.

Furt, F., and P. Moreau. 2009. Importance of lipid metabolism for intracellular and mitochondrial membrane fusion/fission processes. *Int J Biochem Cell Biol*. 41:1828-1836.

Gammie, A.E., L.J. Kurihara, R.B. Vallee, and M.D. Rose. 1995. DNM1, a dynamin-related gene, participates in endosomal trafficking in yeast. *J Cell Biol*. 130:553-566.

Gangloff, S.P., D. Marguet, and G.J. Lauquin. 1990. Molecular cloning of the yeast mitochondrial aconitase gene (ACO1) and evidence of a synergistic regulation of expression by glucose plus glutamate. *Mol Cell Biol*. 10:3551-3561.

Gebert, N., M. Gebert, S. Oeljeklaus, K. von der Malsburg, D.A. Stroud, B. Kulawiak, C. Wirth, R.P. Zahedi, P. Dolezal, S. Wiese, O. Simon, A. Schulze-Specking, K.N. Truscott, A. Sickmann, P. Rehling, B. Guiard, C. Hunte, B. Warscheid, M. van der Laan, N. Pfanner, and N. Wiedemann. 2011. Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane. *Mol Cell*. 44:811-818.

Gebert, N., A.S. Joshi, S. Kutik, T. Becker, M. McKenzie, X.L. Guan, V.P. Mooga, D.A. Stroud, G. Kulkarni, M.R. Wenk, P. Rehling, C. Meisinger, M.T. Ryan, N. Wiedemann, M.L. Greenberg, and N. Pfanner. 2009. Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome. *Curr Biol*. 19:2133-2139.

Gelperin, D., J. Weigle, K. Nelson, P. Roseboom, K. Irie, K. Matsumoto, and S. Lemmon. 1995. 14-3-3 proteins: potential roles in vesicular transport and Ras signaling in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 92:11539-11543.

Gohil, V.M., and M.L. Greenberg. 2009. Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand. *J Cell Biol*. 184:469-472.

Gohil, V.M., M.N. Thompson, and M.L. Greenberg. 2005. Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in Saccharomyces cerevisiae. *J Biol Chem*. 280:35410-35416.

Gonzalez, I.L. 2005. Barth syndrome: TAZ gene mutations, mRNAs, and evolution. *Am J Med Genet A*. 134:409-414.

Gonzalvez, F., and E. Gottlieb. 2007. Cardiolipin: setting the beat of apoptosis. *Apoptosis*. 12:877-885.

Gu, Z., F. Valianpour, S. Chen, F.M. Vaz, G.A. Hakkaart, R.J. Wanders, and M.L. Greenberg. 2004. Aberrant cardiolipin metabolism in the yeast taz1 mutant: a model for Barth syndrome. *Mol Microbiol*. 51:149-158.

Guan, K., L. Farh, T.K. Marshall, and R.J. Deschenes. 1993. Normal mitochondrial structure and genome maintenance in yeast requires the dynamin-like product of the MGM1 gene. *Curr Genet*. 24:141-148.

Guo, Z., D. Cromley, J.T. Billheimer, and S.L. Sturley. 2001. Identification of potential substrate-binding sites in yeast and human acyl-CoA sterol acyltransferases by mutagenesis of conserved sequences. *J Lipid Res*. 42:1282-1291.

Hammermeister, M., K. Schodel, and B. Westermann. 2010. Mdm36 is a mitochondrial fission-promoting protein in Saccharomyces cerevisiae. *Mol Biol Cell*. 21:2443-2452.

Hampton, R.Y., R.G. Gardner, and J. Rine. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase,

an integral endoplasmic reticulum membrane protein. *Mol Biol Cell*. 7:2029-2044.

Hartman, J.L.t., B. Garvik, and L. Hartwell. 2001. Principles for the buffering of genetic variation. *Science*. 291:1001-1004.

Hauff, K.D., and G.M. Hatch. 2006. Cardiolipin metabolism and Barth Syndrome. *Prog Lipid Res*. 45:91-101.

Herlan, M., C. Bornhovd, K. Hell, W. Neupert, and A.S. Reichert. 2004. Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. *J Cell Biol*. 165:167-173.

Herlan, M., F. Vogel, C. Bornhovd, W. Neupert, and A.S. Reichert. 2003. Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. *J Biol Chem*. 278:27781-27788.

Hermann, G.J., J.W. Thatcher, J.P. Mills, K.G. Hales, M.T. Fuller, J. Nunnari, and J.M. Shaw. 1998. Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. *J Cell Biol*. 143:359-373.

Hoch, F.L. 1992. Cardiolipins and biomembrane function. *Biochim Biophys Acta*. 1113:71-133.

Hoppins, S., J. Horner, C. Song, J.M. McCaffery, and J. Nunnari. 2009. Mitochondrial outer and inner membrane fusion requires a modified carrier protein. *J Cell Biol*. 184:569-581.

Hoppins, S., L. Lackner, and J. Nunnari. 2007. The machines that divide and fuse mitochondria. *Annu Rev Biochem*. 76:751-780.

Hostetler, K.Y., H. Van den Bosch, and L.L. Van Deenen. 1971. Biosynthesis of cardiolipin in liver mitochondria. *Biochim Biophys Acta*. 239:113-119.

Hostetler, K.Y., H. van den Bosch, and L.L. van Deenen. 1972. The mechanism of cardiolipin biosynthesis in liver mitochondria. *Biochim Biophys Acta*. 260:507-513.

Houtkooper, R.H., and F.M. Vaz. 2008. Cardiolipin, the heart of mitochondrial metabolism. *Cell Mol Life Sci*. 65:2493-2506.

Iverson, S.L., and S. Orrenius. 2004. The cardiolipin-cytochrome c interaction and the mitochondrial regulation of apoptosis. *Arch Biochem Biophys*. 423:37-46.

Jazwinski, S.M., and A. Kriete. 2012. The yeast retrograde response as a model of intracellular signaling of mitochondrial dysfunction. *Front Physiol*. 3:139.

Jiang, F., Z. Gu, J.M. Granger, and M.L. Greenberg. 1999. Cardiolipin synthase expression is essential for growth at elevated temperature and is regulated by factors affecting mitochondrial development. *Molecular microbiology*. 31:373-379.

Jiang, F., H.S. Rizavi, and M.L. Greenberg. 1997. Cardiolipin is not essential for the growth of Saccharomyces cerevisiae on fermentable or non-fermentable carbon sources. *Molecular microbiology*. 26:481-491.

Jiang, F., M.T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner, and M.L. Greenberg. 2000. Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J Biol Chem*. 275:22387-22394.

Joshi, A.S., M.N. Thompson, N. Fei, M. Huttemann, and M.L. Greenberg. 2012. Cardiolipin and Mitochondrial Phosphatidylethanolamine Have Overlapping Functions in Mitochondrial Fusion in Saccharomyces cerevisiae. *J Biol Chem*. 287:17589-17597.

Jung, U.S., and D.E. Levin. 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol Microbiol*. 34:1049-1057.

Kagan, V.E., V.A. Tyurin, J. Jiang, Y.Y. Tyurina, V.B. Ritov, A.A. Amoscato, A.N. Osipov, N.A. Belikova, A.A. Kapralov, V. Kini, Vlasova, II, Q. Zhao, M. Zou, P. Di, D.A. Svistunenko, I.V. Kurnikov, and G.G. Borisenko. 2005. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol*. 1:223-232.

Kakiuchi, K., Y. Yamauchi, M. Taoka, M. Iwago, T. Fujita, T. Ito, S.Y. Song, A. Sakai, T. Isobe, and T. Ichimura. 2007. Proteomic analysis of in vivo 14-3-3 interactions in the yeast Saccharomyces cerevisiae. *Biochemistry*. 46:7781-7792.

Khuchua, Z., Z. Yue, L. Batts, and A.W. Strauss. 2006. A zebrafish model of human Barth syndrome reveals the essential role of tafazzin in cardiac development and function. *Circ Res*. 99:201-208.

Kito, M., S. Aibara, M. Kato, and T. Hata. 1972. Differences in fatty acid composition among phosphatidylethanolamine, phosphatidylglycerol and cardiolipin of Escherichia coli. *Biochim Biophys Acta*. 260:475-478.

Kornmann, B., E. Currie, S.R. Collins, M. Schuldiner, J. Nunnari, J.S. Weissman, and P. Walter. 2009. An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science*. 325:477-481.

Kornmann, B., C. Osman, and P. Walter. 2011. The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. *Proc Natl Acad Sci U S A*. 108:14151-14156.

Koshkin, V., and M.L. Greenberg. 2000. Oxidative phosphorylation in cardiolipin-lacking yeast mitochondria. *Biochem J*. 347 Pt 3:687-691.

Koshkin, V., and M.L. Greenberg. 2002. Cardiolipin prevents rate-dependent uncoupling and provides osmotic stability in yeast mitochondria. *Biochem J*. 364:317-322.

Kozjak, V., N. Wiedemann, D. Milenkovic, C. Lohaus, H.E. Meyer, B. Guiard, C. Meisinger, and N. Pfanner. 2003. An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J Biol Chem*. 278:48520-48523.

Krayl, M., J.H. Lim, F. Martin, B. Guiard, and W. Voos. 2007. A cooperative action of the ATP-dependent import motor complex and the inner membrane potential drives mitochondrial preprotein import. *Mol Cell Biol*. 27:411-425.

Kuravi, K., S. Nagotu, A.M. Krikken, K. Sjollema, M. Deckers, R. Erdmann, M. Veenhuis, and I.J. van der Klei. 2006. Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in Saccharomyces cerevisiae. *J Cell Sci*. 119:3994-4001.

Kuroda, T., M. Tani, A. Moriguchi, S. Tokunaga, T. Higuchi, S. Kitada, and O. Kuge. 2011a. FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. *Mol Microbiol*. 80:248-265.

Kuroda, T., M. Tani, A. Moriguchi, S. Tokunaga, T. Higuchi, S. Kitada, and O. Kuge. 2011b. FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. *Mol Microbiol*.

Kutik, S., M. Rissler, X.L. Guan, B. Guiard, G. Shui, N. Gebert, P.N. Heacock, P. Rehling, W. Dowhan, M.R. Wenk, N. Pfanner, and N. Wiedemann. 2008. The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. *J Cell Biol*. 183:1213-1221.

Lecocq, J., and C.E. Ballou. 1964. On the Structure of Cardiolipin. *Biochemistry*. 3:976-980.

Lees, N.D., M. Bard, and D.R. Kirsch. 1999. Biochemistry and molecular biology of sterol synthesis in Saccharomyces cerevisiae. *Crit Rev Biochem Mol Biol*. 34:33-47.

Li, G., S. Chen, M.N. Thompson, and M.L. Greenberg. 2007. New insights into the regulation of cardiolipin biosynthesis in yeast: implications for Barth syndrome. *Biochim Biophys Acta*. 1771:432-441.

Liao, X., and R.A. Butow. 1993. RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus. *Cell*. 72:61-71.

Liao, X.S., W.C. Small, P.A. Srere, and R.A. Butow. 1991. Intramitochondrial functions regulate nonmitochondrial citrate synthase (CIT2) expression in Saccharomyces cerevisiae. *Mol Cell Biol*. 11:38-46.

Lill, R., and W. Neupert. 1996. Mechanisms of protein import across the mitochondrial outer membrane. *Trends Cell Biol*. 6:56-61.

Lin, A.P., S.L. Anderson, K.I. Minard, and L. McAlister-Henn. 2011. Effects of excess succinate and retrograde control of metabolite accumulation in yeast tricarboxylic cycle mutants. *J Biol Chem*. 286:33737-33746.

Liu, Z., and R.A. Butow. 1999. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. *Mol Cell Biol*. 19:6720-6728.

Liu, Z., and R.A. Butow. 2006. Mitochondrial retrograde signaling. *Annu Rev Genet*. 40:159-185.

Liu, Z., T. Sekito, M. Spirek, J. Thornton, and R.A. Butow. 2003. Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p. *Mol Cell*. 12:401-411.

Ludovico, P., F. Sansonetty, and M. Corte-Real. 2001. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology*. 147:3335-3343.

Lussier, M., A.M. Sdicu, E. Winnett, D.H. Vo, J. Sheraton, A. Dusterhoft, R.K. Storms, and H. Bussey. 1997. Completion of the Saccharomyces cerevisiae genome sequence allows identification of KTR5, KTR6 and KTR7 and definition of the nine-membered KRE2/MNT1 mannosyltransferase gene family in this organism. *Yeast*. 13:267-274.

Ma, L., F.M. Vaz, Z. Gu, R.J. Wanders, and M.L. Greenberg. 2004. The human TAZ gene complements mitochondrial dysfunction in the yeast taz1Delta mutant. Implications for Barth syndrome. *J Biol Chem*. 279:44394-44399.

Mariappan, M., A. Mateja, M. Dobosz, E. Bove, R.S. Hegde, and R.J. Keenan. 2011. The mechanism of membrane-associated steps in tail-anchored protein insertion. *Nature*. 477:61-66.

Mazzocco, M.M., A.E. Henry, and R.I. Kelly. 2007. Barth syndrome is associated with a cognitive phenotype. *J Dev Behav Pediatr*. 28:22-30.

McKenzie, M., M. Lazarou, D.R. Thorburn, and M.T. Ryan. 2006. Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. *J Mol Biol*. 361:462-469.

McMillin, J.B., and W. Dowhan. 2002. Cardiolipin and apoptosis. *Biochim Biophys Acta*. 1585:97-107.

Meeusen, S., J.M. McCaffery, and J. Nunnari. 2004. Mitochondrial fusion intermediates revealed in vitro. *Science*. 305:1747-1752.

Miceli, M.V., J.C. Jiang, A. Tiwari, J.F. Rodriguez-Quinones, and S.M. Jazwinski. 2011. Loss of mitochondrial membrane potential triggers the retrograde response extending yeast replicative lifespan. *Front Genet*. 2:102.

Mitra, K., C. Wunder, B. Roysam, G. Lin, and J. Lippincott-Schwartz. 2009. A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc Natl Acad Sci U S A*. 106:11960-11965.

Mozdy, A.D., J.M. McCaffery, and J.M. Shaw. 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J Cell Biol*. 151:367-380.

Nunnari, J., W.F. Marshall, A. Straight, A. Murray, J.W. Sedat, and P. Walter. 1997. Mitochondrial transmission during mating in Saccharomyces cerevisiae is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol Biol Cell*. 8:1233-1242.

Ong, S.B., S. Subrayan, S.Y. Lim, D.M. Yellon, S.M. Davidson, and D.J. Hausenloy. 2010. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation*. 121:2012-2022.

Osman, C., M. Haag, C. Potting, J. Rodenfels, P.V. Dip, F.T. Wieland, B. Brugger, B. Westermann, and T. Langer. 2009a. The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. *J Cell Biol*. 184:583-596.

Osman, C., M. Haag, F.T. Wieland, B. Brugger, and T. Langer. 2010. A mitochondrial phosphatase required for cardiolipin biosynthesis: the PGP phosphatase Gep4. *EMBO J*. 29:1976-1987.

Osman, C., C. Merkwirth, and T. Langer. 2009b. Prohibitins and the functional compartmentalization of mitochondrial membranes. *J Cell Sci*. 122:3823-3830.

Osman, C., D.R. Voelker, and T. Langer. 2011. Making heads or tails of phospholipids in mitochondria. *J Cell Biol*. 192:7-16.

Ostrander, D.B., M. Zhang, E. Mileykovskaya, M. Rho, and W. Dowhan. 2001. Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain. A yeast genetic model system for the study of anionic phospholipid function in mitochondria. *J Biol Chem*. 276:25262-25272.

Otsuga, D., B.R. Keegan, E. Brisch, J.W. Thatcher, G.J. Hermann, W. Bleazard, and J.M. Shaw. 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J Cell Biol*. 143:333-349.

Pangborn, M.C. 1948. Method of recovering and refining cardiolipin. No. 2,456,836. *Journal of the Patent Office Society. Patent Office Society (U.S*. 108:UNKNOWN.

Pfanner, N., E.A. Craig, and M. Meijer. 1994. The protein import machinery of the mitochondrial inner membrane. *Trends Biochem Sci*. 19:368-372.

Pfeiffer, K., V. Gohil, R.A. Stuart, C. Hunte, U. Brandt, M.L. Greenberg, and H. Schagger. 2003. Cardiolipin stabilizes respiratory chain supercomplexes. *J Biol Chem*. 278:52873-52880.

Pollack, M., and C. Leeuwenburgh. 2001. Apoptosis and aging: role of the mitochondria. *J Gerontol A Biol Sci Med Sci*. 56:B475-482.

Pray-Grant, M.G., D. Schieltz, S.J. McMahon, J.M. Wood, E.L. Kennedy, R.G. Cook, J.L. Workman, J.R. Yates, 3rd, and P.A. Grant. 2002. The novel SLIK histone acetyltransferase complex functions in the yeast retrograde response pathway. *Mol Cell Biol*. 22:8774-8786.

Rand, R.P., and S. Sengupta. 1972. Cardiolipin forms hexagonal structures with divalent cations. *Biochim Biophys Acta*. 255:484-492.

Rapaport, D. 2003. Finding the right organelle. Targeting signals in mitochondrial outer-membrane proteins. *EMBO reports*. 4:948-952.

Rapaport, D., M. Brunner, W. Neupert, and B. Westermann. 1998. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. *J Biol Chem*. 273:20150-20155.

Rissler, M., N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, and A. Chacinska. 2005. The essential mitochondrial protein Erv1 cooperates with Mia40 in biogenesis of intermembrane space proteins. *J Mol Biol*. 353:485-492.

Rujiviphat, J., G. Meglei, J.L. Rubinstein, and G.A. McQuibban. 2009. Phospholipid association is essential for dynamin-related protein Mgm1 to function in mitochondrial membrane fusion. *J Biol Chem*. 284:28682-28686.

Rytomaa, M., and P.K. Kinnunen. 1994. Evidence for two distinct acidic phospholipid-binding sites in cytochrome c. *J Biol Chem*. 269:1770-1774.

Schlame, M., S. Brody, and K.Y. Hostetler. 1993. Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. *European journal of biochemistry / FEBS*. 212:727-735.

Schlame, M., R.I. Kelley, A. Feigenbaum, J.A. Towbin, P.M. Heerdt, T. Schieble, R.J. Wanders, S. DiMauro, and T.J. Blanck. 2003. Phospholipid abnormalities in children with Barth syndrome. *J Am Coll Cardiol*. 42:1994-1999.

Schlame, M., and M. Ren. 2006. Barth syndrome, a human disorder of cardiolipin metabolism. *FEBS letters*. 580:5450-5455.

Schlame, M., and M. Ren. 2009. The role of cardiolipin in the structural organization of mitochondrial membranes. *Biochim Biophys Acta*. 1788:2080-2083.

Schlame, M., M. Ren, Y. Xu, M.L. Greenberg, and I. Haller. 2005. Molecular symmetry in mitochondrial cardiolipins. *Chem Phys Lipids*. 138:38-49.

Schlame, M., D. Rua, and M.L. Greenberg. 2000. The biosynthesis and functional role of cardiolipin. *Progress in lipid research*. 39:257-288.

Schlame, M., and B. Rustow. 1990. Lysocardiolipin formation and reacylation in isolated rat liver mitochondria. *The Biochemical journal*. 272:589-595.

Schlame, M., J.A. Towbin, P.M. Heerdt, R. Jehle, S. DiMauro, and T.J. Blanck. 2002. Deficiency of tetralinoleoyl-cardiolipin in Barth syndrome. *Annals of neurology*. 51:634-637.

Schmidt, M.R., N. Birkebaek, I. Gonzalez, and L. Sunde. 2004. Barth syndrome without 3-methylglutaconic aciduria. *Acta Paediatr*. 93:419-421.

Schuldiner, M., J. Metz, V. Schmid, V. Denic, M. Rakwalska, H.D. Schmitt, B. Schwappach, and J.S. Weissman. 2008. The GET complex mediates insertion of tail-anchored proteins into the ER membrane. *Cell*. 134:634-645.

Schuller, C., J.L. Brewster, M.R. Alexander, M.C. Gustin, and H. Ruis. 1994. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. *EMBO J*. 13:4382-4389.

Sedlak, E., and N.C. Robinson. 1999. Phospholipase A(2) digestion of cardiolipin bound to bovine cytochrome c oxidase alters both activity and quaternary structure. *Biochemistry*. 38:14966-14972.

Sekito, T., Z. Liu, J. Thornton, and R.A. Butow. 2002. RTG-dependent mitochondria-to-nucleus signaling is regulated by MKS1 and is linked to formation of yeast prion [URE3]. *Mol Biol Cell*. 13:795-804.

Sekito, T., J. Thornton, and R.A. Butow. 2000. Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p. *Mol Biol Cell*. 11:2103-2115.

Sesaki, H., C.D. Dunn, M. Iijima, K.A. Shepard, M.P. Yaffe, C.E. Machamer, and R.E. Jensen. 2006. Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and alternative topogenesis of Mgm1p. *J Cell Biol*. 173:651-658.

Sesaki, H., and R.E. Jensen. 1999. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J Cell Biol*. 147:699-706.

Sesaki, H., and R.E. Jensen. 2001. UGO1 encodes an outer membrane protein required for mitochondrial fusion. *J Cell Biol*. 152:1123-1134.

Sesaki, H., and R.E. Jensen. 2004. Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. *J Biol Chem*. 279:28298-28303.

Spencer, C.T., R.M. Bryant, J. Day, I.L. Gonzalez, S.D. Colan, W.R. Thompson, J. Berthy, S.P. Redfearn, and B.J. Byrne. 2006. Cardiac and clinical phenotype in Barth syndrome. *Pediatrics*. 118:e337-346.

Stefer, S., S. Reitz, F. Wang, K. Wild, Y.Y. Pang, D. Schwarz, J. Bomke, C. Hein, F. Lohr, F. Bernhard, V. Denic, V. Dotsch, and I. Sinning. 2011. Structural

basis for tail-anchored membrane protein biogenesis by the Get3-receptor complex. *Science*. 333:758-762.

Stojanovski, D., M. Rissler, N. Pfanner, and C. Meisinger. 2006. Mitochondrial morphology and protein import--a tight connection? *Biochim Biophys Acta*. 1763:414-421.

Su, X., and W. Dowhan. 2006. Translational regulation of nuclear gene COX4 expression by mitochondrial content of phosphatidylglycerol and cardiolipin in Saccharomyces cerevisiae. *Mol Cell Biol*. 26:743-753.

Sweeney, R.T., G.J. Davis, and J.A. Noonan. 2008. Cardiomyopathy of unknown etiology: Barth syndrome unrecognized. *Congenit Heart Dis*. 3:443-448.

Tamai, K.T., and M.L. Greenberg. 1990. Biochemical characterization and regulation of cardiolipin synthase in Saccharomyces cerevisiae. *Biochim Biophys Acta*. 1046:214-222.

Tamura, Y., T. Endo, M. Iijima, and H. Sesaki. 2009. Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. *J Cell Biol*. 185:1029-1045.

Terashima, H., N. Yabuki, M. Arisawa, K. Hamada, and K. Kitada. 2000. Up-regulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in response to cell wall damage caused by disruption of FKS1 in Saccharomyces cerevisiae. *Mol Gen Genet*. 264:64-74.

Tieu, Q., and J. Nunnari. 2000. Mdv1p is a WD repeat protein that interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial division. *J Cell Biol*. 151:353-366.

Tieu, Q., V. Okreglak, K. Naylor, and J. Nunnari. 2002. The WD repeat protein, Mdv1p, functions as a molecular adaptor by interacting with Dnm1p and Fis1p during mitochondrial fission. *J Cell Biol*. 158:445-452.

Trotter, P.J., J. Pedretti, and D.R. Voelker. 1993. Phosphatidylserine decarboxylase from Saccharomyces cerevisiae. Isolation of mutants, cloning of the gene, and creation of a null allele. *J Biol Chem*. 268:21416-21424.

Trotter, P.J., J. Pedretti, R. Yates, and D.R. Voelker. 1995. Phosphatidylserine decarboxylase 2 of Saccharomyces cerevisiae. Cloning and mapping of the gene, heterologous expression, and creation of the null allele. *J Biol Chem*. 270:6071-6080.

Trotter, P.J., and D.R. Voelker. 1995. Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast Saccharomyces cerevisiae. *J Biol Chem*. 270:6062-6070.

Truscott, K.N., P. Kovermann, A. Geissler, A. Merlin, M. Meijer, A.J. Driessen, J. Rassow, N. Pfanner, and R. Wagner. 2001. A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat Struct Biol*. 8:1074-1082.

Tuller, G., C. Hrastnik, G. Achleitner, U. Schiefthaler, F. Klein, and G. Daum. 1998. YDL142c encodes cardiolipin synthase (Cls1p) and is non-essential for aerobic growth of Saccharomyces cerevisiae. *FEBS letters*. 421:15-18.

Vaden, D.L., V.M. Gohil, Z. Gu, and M.L. Greenberg. 2005. Separation of yeast phospholipids using one-dimensional thin-layer chromatography. *Anal Biochem*. 338:162-164.

Vaena de Avalos, S., X. Su, M. Zhang, Y. Okamoto, W. Dowhan, and Y.A. Hannun. 2005. The phosphatidylglycerol/cardiolipin biosynthetic pathway is required for the activation of inositol phosphosphingolipid phospholipase C, Isc1p, during growth of Saccharomyces cerevisiae. *J Biol Chem*. 280:7170-7177.

Valianpour, F., V. Mitsakos, D. Schlemmer, J.A. Towbin, J.M. Taylor, P.G. Ekert, D.R. Thorburn, A. Munnich, R.J. Wanders, P.G. Barth, and F.M. Vaz. 2005. Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis. *J Lipid Res*. 46:1182-1195.

Valianpour, F., R.J. Wanders, H. Overmars, F.M. Vaz, P.G. Barth, and A.H. van Gennip. 2003. Linoleic acid supplementation of Barth syndrome fibroblasts restores cardiolipin levels: implications for treatment. *J Lipid Res*. 44:560-566.

van den Brink-van der Laan, E., J.A. Killian, and B. de Kruijff. 2004. Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. *Biochim Biophys Acta*. 1666:275-288.

van der Laan, M., A. Chacinska, M. Lind, I. Perschil, A. Sickmann, H.E. Meyer, B. Guiard, C. Meisinger, N. Pfanner, and P. Rehling. 2005. Pam17 is required for architecture and translocation activity of the mitochondrial protein import motor. *Mol Cell Biol*. 25:7449-7458.

van der Laan, M., M. Meinecke, J. Dudek, D.P. Hutu, M. Lind, I. Perschil, B. Guiard, R. Wagner, N. Pfanner, and P. Rehling. 2007. Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. *Nat Cell Biol*. 9:1152-1159.

van Roermund, C.W., E.H. Hettema, M. van den Berg, H.F. Tabak, and R.J. Wanders. 1999. Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in Saccharomyces cerevisiae and identification of a plasma membrane carnitine transporter, Agp2p. *EMBO J*. 18:5843-5852.

Vasilenko, I., B. De Kruijff, and A.J. Verkleij. 1982. Polymorphic phase behaviour of cardiolipin from bovine heart and from Bacillus subtilis as detected by 31P-NMR and freeze-fracture techniques. Effects of Ca2+, Mg2+, Ba2+ and temperature. *Biochim Biophys Acta*. 684:282-286.

Vaz, F.M., R.H. Houtkooper, F. Valianpour, P.G. Barth, and R.J. Wanders. 2003. Only one splice variant of the human TAZ gene encodes a functional protein with a role in cardiolipin metabolism. *J Biol Chem*. 278:43089-43094.

Vreken, P., F. Valianpour, L.G. Nijtmans, L.A. Grivell, B. Plecko, R.J. Wanders, and P.G. Barth. 2000. Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. *Biochemical and biophysical research communications*. 279:378-382.

Wang, F., A. Whynot, M. Tung, and V. Denic. 2011. The Mechanism of Tail-Anchored Protein Insertion into the ER Membrane. *Mol Cell*. 43:738-750.

Westermann, B., and W. Neupert. 2000. Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in Saccharomyces cerevisiae. *Yeast*. 16:1421-1427.

Wiedemann, N., N. Pfanner, and M.T. Ryan. 2001. The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. *EMBO J*. 20:951-960.

Willingham, M.C., and A.V. Rutherford. 1984. The use of osmium-thiocarbohydrazide-osmium (OTO) and ferrocyanide-reduced osmium methods to enhance membrane contrast and preservation in cultured cells. *J Histochem Cytochem*. 32:455-460.

Wong, E.D., J.A. Wagner, S.W. Gorsich, J.M. McCaffery, J.M. Shaw, and J. Nunnari. 2000. The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. *J Cell Biol*. 151:341-352.

Wong, E.D., J.A. Wagner, S.V. Scott, V. Okreglak, T.J. Holewinske, A. Cassidy-Stone, and J. Nunnari. 2003. The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J Cell Biol*. 160:303-311.

Xu, F.Y., H. McBride, D. Acehan, F.M. Vaz, R.H. Houtkooper, R.M. Lee, M.A. Mowat, and G.M. Hatch. 2010. The dynamics of cardiolipin synthesis post-mitochondrial fusion. *Biochim Biophys Acta*. 1798:1577-1585.

Xu, Y., M. Condell, H. Plesken, I. Edelman-Novemsky, J. Ma, M. Ren, and M. Schlame. 2006a. A Drosophila model of Barth syndrome. *Proc Natl Acad Sci U S A*. 103:11584-11588.

Xu, Y., A. Malhotra, M. Ren, and M. Schlame. 2006b. The enzymatic function of tafazzin. *J Biol Chem*. 281:39217-39224.

Xu, Y., J.J. Sutachan, H. Plesken, R.I. Kelley, and M. Schlame. 2005. Characterization of lymphoblast mitochondria from patients with Barth syndrome. *Lab Invest*. 85:823-830.

Zhang, J., Z. Guan, A.N. Murphy, S.E. Wiley, G.A. Perkins, C.A. Worby, J.L. Engel, P. Heacock, O.K. Nguyen, J.H. Wang, C.R. Raetz, W. Dowhan, and J.E. Dixon. 2011. Mitochondrial phosphatase PTPMT1 is essential for cardiolipin biosynthesis. *Cell Metab*. 13:690-700.

Zhang, M., E. Mileykovskaya, and W. Dowhan. 2005. Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. *J Biol Chem*. 280:29403-29408.

Zhong, Q., V.M. Gohil, L. Ma, and M.L. Greenberg. 2004. Absence of cardiolipin results in temperature sensitivity, respiratory defects, and mitochondrial DNA instability independent of pet56. *J Biol Chem*. 279:32294-32300.

Zhong, Q., and M.L. Greenberg. 2005. Deficiency in mitochondrial anionic phospholipid synthesis impairs cell wall biogenesis. *Biochem Soc Trans*. 33:1158-1161.

Zhong, Q., J. Gvozdenovic-Jeremic, P. Webster, J. Zhou, and M.L. Greenberg. 2005. Loss of function of KRE5 suppresses temperature sensitivity of mutants lacking mitochondrial anionic lipids. *Mol Biol Cell*. 16:665-675.

Zhong, Q., G. Li, J. Gvozdenovic-Jeremic, and M.L. Greenberg. 2007. Up-regulation of the cell integrity pathway in saccharomyces cerevisiae suppresses temperature sensitivity of the pgs1Delta mutant. *J Biol Chem*. 282:15946-15953.

Zhou, J., Q. Zhong, G. Li, and M.L. Greenberg. 2009. Loss of cardiolipin leads to longevity defects that are alleviated by alterations in stress response signaling. *J Biol Chem*. 284:18106-18114.

Zick, M., S. Duvezin-Caubet, A. Schafer, F. Vogel, W. Neupert, and A.S. Reichert. 2009. Distinct roles of the two isoforms of the dynamin-like GTPase Mgm1 in mitochondrial fusion. *FEBS Lett*. 583:2237-2243.

Zinser, E., C.D. Sperka-Gottlieb, E.V. Fasch, S.D. Kohlwein, F. Paltauf, and G. Daum. 1991. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. *J Bacteriol*. 173:2026-2034.

ABSTRACT

IDENTIFICATION OF CELLULAR FUNCTIONS OF CARDIOLIPIN AS PHYSIOLOGICAL MODIFIERS OF BARTH SYNDROME

by

AMIT S. JOSHI

December 2012

Advisor: Dr. Miriam L. Greenberg

Major: Biological Sciences

Degree: Doctor of Philosophy

Cardiolipin (CL) is an anionic phospholipid synthesized in the mitochondrial inner membrane. Perturbation of CL metabolism leads to Barth syndrome (BTHS), a life threatening genetic disorder. I utilized genetic, biochemical and cell biological approaches in yeast to elucidate the cellular functions of CL. Understanding the functions of CL is expected to shed light on the pathology and possible treatments for BTHS.

BTHS is caused by mutations in *TAZ1*, which encodes a CL remodeling enzyme called tafazzin. BTHS patients exhibit a wide range of clinical presentations, indicating that physiological modifiers influence the BTHS phenotype. A targeted synthetic lethality screen was performed to identify physiological modifiers of CL deficiency. Using this approach, synthetic genetic interactions of CL mutants were identified with genes encoding mitochondrial outer membrane proteins, specifically in the TOM, MDM and SAM complexes, which are involved in mitochondrial protein import, suggesting that CL plays a role in this process. Consistent with this, we

showed that CL is present in the outer mitochondrial membrane and is involved in assembly of outer membrane protein complexes in yeast as well as in BTHS lymphoblasts. In addition to this, we showed that CL mutants interacted with genes encoding mitochondrial protein import complexes of the inner membrane, including the TIM and PAM complexes. To identify the role of CL in maintaining mitochondrial morphology, a targeted synthetic lethality screen was performed to determine if CL mutants genetically interacted with mutants defective in this function. The *crd1*Δ mutant genetically interacted with genes required for mitochondrial fusion and fission, suggesting a common cellular function. In addition to genes involved in mitochondrial fusion, *crd1*Δ genetically interacted with the UPS and GET complex mutants suggesting shared cellular functions with these as well. Unlike the UPS complex, a role for the GET complex in CL metabolism is unknown. My studies indicated that levels of CL were decreased in the *get2*Δ mutant, thus identifying a novel role of Get2p in the regulation of CL levels in yeast. In summary, the genetic interaction studies identified functions that could be physiological modifiers of CL deficiency in yeast, and could possibly point to modifiers of the BTHS phenotype.

Based on the genetic interactions of CL mutants with mitochondrial fusion mutants, we tested if CL plays a role in mitochondrial fusion. Because the lack of CL does not lead to defects in the mitochondrial network in *Saccharomyces cerevisiae*, I hypothesized that PE may compensate for CL in the maintenance of mitochondrial tubular morphology and fusion. Previous studies have shown that CL and mitochondrial PE have overlapping functions,

and the loss of both is synthetically lethal. In the current study, we showed that the loss of both CL and mitochondrial PE exhibited highly fragmented mitochondria, loss of mitochondrial DNA, and reduced membrane potential, characteristic of fusion mutants. Deletion of *DNM1*, required for mitochondrial fission, restored the tubular mitochondrial morphology. Loss of CL and mitochondrial PE led to reduced levels of small and large isoforms of the fusion protein Mgm1p, possibly accounting for the fusion defect. Taken together, these data demonstrate *in vivo* that CL and mitochondrial PE are required to maintain tubular mitochondrial morphology and have overlapping functions in mitochondrial fusion.

Recent studies have shown that cells lacking CL exhibit decreased activities of the TCA cycle enzymes aconitase and succinate dehydrogenase. Consistent with this finding, we showed that *crd1*Δ cells exhibit a growth defect on acetate medium, consistent with a defect in the TCA and glyoxylate cycles. A defect in the TCA cycle, and decreased mitochondrial functions, leads to activation of the retrograde (RTG) pathway. While the *crd1*Δ mutant exhibits these mitochondrial defects, it fails to activate the RTG pathway, as the expression of *CIT2* in *crd1*Δ is not upregulated at elevated temperature. Consistent with the RTG defect, *crd1*Δ cells exhibit glutamate auxotrophy at elevated temperature. We also find that overexpression of *RTG2*, a positive regulator of the RTG pathway, and deletion of *BHM2*, a negative regulator of the RTG pathway, rescues the ts phenotype of *crd1*Δ. The RTG pathway is required for expression of genes that replenish TCA cycle metabolites. In addition to the RTG pathway, the β-oxidation pathway can also compensate

for the defect in the TCA cycle by replenishing intermediates such as acetyl-CoA. Interestingly, *crd1*Δ exhibits growth defects on oleic acid medium, suggesting that cells lacking CL have a defect in the β-oxidation pathway. Taken together, my studies suggest that CL mutants have defects in the TCA and glyoxylate cycles and in the β-oxidation pathway, which cannot be alleviated due to defective activation of the RTG pathway. Identifying the function of CL in RTG signaling and metabolic pathways will facilitate understanding of specific metabolic deficiencies in BTHS patients.

AUTOBIOGRAPHICAL STATEMENT

AMIT S. JOSHI

EDUCATION:

2005-2012: Ph.D. in Biology, Wayne State University, Detroit, USA

2002-2004: M.S in Biochemistry, Maharaja Sayajirao University, Baroda, India

1999-2002: B.S in Microbiology, Ramnarain Ruia College, University of Mumbai, Mumbai, India

HONORS AND AWARDS:

2010-2011: Graduate Research Enhancement Award, Wayne State University.

2009-2010: P.D. Smith Award for Research in Genetics, Wayne State University.

2008-2009: Outstanding Graduate Research Assistant Award, Wayne State University.

2007-2008: Second Prize at the Annual Biological Sciences Poster Day, Wayne State University.

PUBLICATIONS:

Joshi AS, Zhou J, Gohil VM, Chen S, Greenberg ML. 2009. Cellular functions of cardiolipin in yeast. *Biochim Biophys Acta* 1793 (1): 212-8.

Gebert N*, **Joshi AS***, Kutik S, Becker T, McKenzie M, Guan XL, Mooga VP, Stroud DA, Kulkarni G, Wenk MR, Rehling P, Meisinger C, Ryan MT, Wiedemann N, Greenberg ML, Pfanner N. Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome. Curr Biol. 2009 Dec 29; 19(24): 2133-9. (* Equal contributors)

Joshi AS, Thompson MN, Fei N, Hüttemann M and Greenberg ML. Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in *Saccharomyces cerevisiae*. J Biol Chem. 2012 May 18; 287(21): 17589-97.

Joshi AS, Fei N, Greenberg ML. A genetic screen to identify physiological modifiers exacerbating loss of cardiolipin in *S. cerevisiae*. (In preparation)