Cardiolipin Regulates Mitophagy Through The Pkc Pathway

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CARDIOLIPIN REGULATES MITOPHAGY THROUGH THE PKC PATHWAY

by

ZHENI SHEN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

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Approved by:

Advisor Date

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DEDICATION

To my loving parents, husband Yanhua Zhang, for all their love and support & my dear daughter Faith for bringing me so much happiness
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I would like to extend my deepest gratitude to my advisor Professor Miriam L. Greenberg for her continuous support and outstanding guidance throughout my work. She not only taught me numerous scientific knowledge and writing skills, but also taught me many virtues that will benefit me throughout my life. I can write pages to express how much I respect and appreciate her.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>CHAPTER 1 – INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2 – LOSS OF CARDIOLIPIN LEADS TO DEFECTIVE MITOPHAGY</td>
<td>19</td>
</tr>
<tr>
<td>Introduction</td>
<td>19</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>23</td>
</tr>
<tr>
<td>Results</td>
<td>32</td>
</tr>
<tr>
<td>Discussion</td>
<td>44</td>
</tr>
<tr>
<td>CHAPTER 3 – DEFECTIVE MITOPHAGY IN crd1Δ MAY RESULT FROM DECREASED MAPK PATHWAYS</td>
<td>47</td>
</tr>
<tr>
<td>Introduction</td>
<td>47</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>51</td>
</tr>
<tr>
<td>Results</td>
<td>59</td>
</tr>
<tr>
<td>Discussion</td>
<td>73</td>
</tr>
<tr>
<td>CHAPTER 4 - FUTURE DIRECTIONS</td>
<td>76</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>104</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>135</td>
</tr>
<tr>
<td>AUTOBIOGRAPHICAL STATEMENT</td>
<td>137</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1  Yeast strains and plasmids used in Chapter 2</td>
<td>26</td>
</tr>
<tr>
<td>Table 2.2  Real-time PCR primers used in Chapter 2</td>
<td>27</td>
</tr>
<tr>
<td>Table 3.1  Yeast strains used in Chapter 3</td>
<td>52</td>
</tr>
<tr>
<td>Table 3.2  Antibodies used in determining MAPK pathway activation</td>
<td>58</td>
</tr>
<tr>
<td>Table 4.1  Deletion strains used in the mini SGA</td>
<td>82</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>CL synthesis and remodeling pathway in Saccharomyces cerevisiae.</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Deletion of UTH1 rescues (A) the vacuole morphology defect of crd1Δ at 37°C, (B) the vacuole acidification defect of crd1 at 37°C</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Deletion of autophagy/mitophagy genes does not rescue crd1Δ growth defect at 37°C</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>crd1Δ is (A) synthetically lethal with atg8Δ and atg18Δ at 36°C, (B) synthetically sick with atg32Δ at 36°C</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Insertion of GFP into the IDH1 gene locus</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Delivery of mitochondria to the vacuole in response to increased temperature is inhibited in crd1Δ</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Increased temperature triggers increased upregulation of ATG8 expression in crd1Δ cells</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Decreased mitophagy in crd1Δ cells</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Decreased mitophagy in crd1Δ cells expressing Idh1-GFP</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>The PKC and HOG pathways regulate different stages of mitophagy.</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Construction of the pYPGK18-PTP2 and pYPGK18-PTP3 overexpression plasmids</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Deletion of HOG pathway genes is synthetically lethal with CRD1 deletion</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Overexpression of the negative regulators of the HOG pathway is synthetically lethal with CRD1 deletion</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>(A) Decreased Hog1p phosphorylation in the crd1Δ mutant, (B) Translocation of activated Hog1p is not affected in crd1Δ</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Heat stress does not (A) trigger phosphorylation in the BY4742 background, (B) induce the translocation of GFP tagged Hog1p into the nucleus in the BY4742 background</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>(A) The PKC pathway is decreased in crd1Δ, (B) Upregulation of the PKC pathway rescues the crd1Δ growth defect</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Upregulation of the PKC pathway rescues the delivery of mitochondria into vacuole in crd1Δ after induction of mitophagy</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Upregulation of the PKC pathway rescues mitophagy in crd1Δ</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>200 mM NaCl rescues crd1Δ temperature sensitivity</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>1 M sorbitol induces Hog1p activation in crd1Δ at elevated temperature</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Deletion of ANP1, PTC7, OSM1 or DOG2 does not abrogate the rescue of crd1Δ temperature sensitivity by sorbitol</td>
</tr>
</tbody>
</table>
Figure 4.3  Deletion of \textit{ATG8} does not rescue the vacuole enlargement triggered by elevated temperature in \textit{crd1Δ}.........................................................85
Figure 4.4  Rapamycin triggers enlargement of vacuoles in both WT and \textit{crd1Δ}, which is not rescued by deletion of \textit{ATG8} .........................................................87
Figure 4.5  The FAB pathway and its regulation.........................................................89
Figure 4.6  Deletion of \textit{FIG4} partially rescues \textit{crd1Δ} temperature sensitivity ....91
Figure 4.7  \textit{crd1Δ} exhibits normal PI3P localization at the vacuolar membrane....93
Figure 4.8  Normal vacuolar localization of \textit{PI(3,5)P_2} in \textit{crd1Δ}.................................94
Figure 4.9  Overexpression of \textit{FAB1} and \textit{VAC7} does not rescue \textit{crd1Δ} temperature sensitivity .............................................................................96
Figure 4.10 Deletion of \textit{ENA1} does not affect \textit{crd1Δ} temperature sensitivity ....99
Figure 4.11 Inadequate \textit{ENA1} upregulation in \textit{crd1Δ} compared to WT, at elevated temperature ..........................................................101
Figure 4.12 Overexpression of \textit{ENA1} does not rescue \textit{crd1Δ} temperature sensitivity ..........................................................102
CHAPTER 1

INTRODUCTION

Parts of this chapter have been submitted for publication to the journal *BioMed Research International*.

Cardiolipin (CL), the signature phospholipid of mitochondrial membranes, is crucial for mitochondrial function and is involved in various cellular processes outside of the mitochondria. The importance of CL in cardiovascular health is underscored by the life-threatening genetic disorder Barth syndrome (BTHS), which manifests clinically as cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation. In addition to BTHS, CL is linked to various cardiovascular diseases (CVDs), including cardiomyopathy, atherosclerosis, myocardial ischemia-reperfusion injury, heart failure and Tangier disease. The link between CL and CVDs may possibly be explained by the physiological roles of CL in pathways that have cardioprotective function, such as autophagy/mitophagy and the mitogen-activated protein kinase (MAPK) pathways. My dissertation work focuses on elucidating how CL influences mitophagy and MAPK pathways. This knowledge may contribute to our understanding of the function of this important lipid, and may ultimately identify novel therapeutic strategies to treat CVDs and improve heart performance.

1. CL and CL synthesis
CL contains two phosphatidyl moieties joined by a central glycerol backbone, forming a dimeric structure (De Bruijn, 1966). Thus, unlike other phospholipids that contain two fatty acyl chains linked to glycerol, CL has four acyl chains. Considering the potential combinations of fatty acyl groups on CL, a large number of CL species may be possible. However, in most organisms and tissues, the fatty acyl composition of CL is unique and specific. For example, bacterial CL contains both saturated and monounsaturated fatty acyl chains that are composed of 14–19 carbons (Kito et al., 1972). In eukaryotes, CL mainly contains monounsaturated and diunsaturated fatty acyl chains that consist of 16–18 carbons (Schlame et al., 1993). In mammals, CL acyl species vary in different tissues, but the most abundant species in the heart is tetralinoleoyl CL. In yeast, the predominant fatty acyl species of CL are oleic acid and palmitoleic acid (Schlame et al., 1993). While CL plays critical roles in mitochondrial biogenesis, fusion and fission, respiration and protein import (Joshi et al., 2009), it is also involved in various cellular processes outside of the mitochondria. These include, but are not limited to cell wall biogenesis (Zhong et al., 2005), vacuole homeostasis (Chen et al., 2008b), ageing (Zhou et al., 2009), the cell cycle (Chen et al., 2010), and apoptosis (Li et al., 2015).

Unlike mitochondrial membrane lipids that are synthesized in the endoplasmic reticulum, de novo synthesis of CL occurs exclusively in the inner membrane of the mitochondria (Hostetler et al., 1972) in a series of well-characterized steps that are conserved from yeast to higher eukaryotes (Tian et al., 2012). The first step in the CL biosynthetic pathway is the conversion of phosphatidic acid (PA) to CDP-diacylglycerol
(CDP-DAG), which is catalyzed in the inner membrane by CDP-DAG synthase encoded by TAM41 (Deprez et al., 2002; Kutik et al., 2008; Tamura et al., 2013). The PGS1-encoded phosphatidylglycerolphosphate (PGP) synthase catalyzes transfer of the phosphatidyl group from CDP-DAG to a glycerol-3-phosphate molecule to generate PGP (Chang et al., 1998b). PGP is subsequently dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase, encoded by PTPMT1 in mammals (Xiao et al., 2011; Zhang et al., 2011) and GEP4 in yeast (Osman et al., 2010). The final step in the biosynthetic pathway is carried out by CL synthase, encoded by hCLS1 in human cells (Chen et al., 2006; Houtkooper et al., 2006; Lu et al., 2006) and by CRD1 in yeast (Chang et al., 1998a; Jiang et al., 1997; Tuller et al., 1998). In this step, a second phosphatidyl group is added to PG from another CDP-DAG molecule, generating CL (Hostetler et al., 1972; Houtkooper et al., 2006; Malhotra et al., 2009).

The acyl composition of CL varies in different tissues, and the CL remodeling process is responsible for the CL acyl group exchange following CL de novo synthesis. CL remodeling may occur through two mechanisms (Ye et al., 2014b). In the two-step mechanism, CL is first deacylated to monolyso-CL (MLCL) by phospholipases (Lands, 1960). In yeast, the only CL-specific phospholipase is encoded by CLD1 (Beranek et al., 2009), while in mammals, several phospholipases are reported to have CL-hydrolyzing activities, including iPLA2β, iPLA2γ, cPLA2, and sPLA2 (Buckland et al., 1998; Dennis et al., 2011; Hsu et al., 2013). MLCL is reacylated to remodeled CL by the transacylase tafazzin, encoded by the tafazzin gene (TAZ/G4.5) located on Xq28 in human cells (Bione et al., 1996) and by TAZ1 in yeast (Gu et al., 2004; Vaz et al.,
Acyltransferases encoded by *ALCAT1* (Cao et al., 2004) and *MLCLAT1* (Taylor and Hatch, 2009) have also been described in mammalian cells. In the one-step mechanism, CL remodeling occurs by direct transacylation (Xu et al., 2003; Yamashita et al., 1997). Mutations in tafazzin perturb CL remodeling and cause the life-threatening genetic disorder Barth syndrome (BTHS) (Barth PG et al., 1983), which is discussed below.

The CL synthesis and remodeling pathway in my research model, *Saccharomyces cerevisiae*, is shown in Fig. 1.1.

## 2. Relationship between CL and CVD

### 2.1 CL and cardiomyopathy

#### 2.1.1 Barth syndrome

The most direct link between CVD and CL is seen in BTHS, an X-linked genetic disorder of CL remodeling caused by tafazzin mutations. BTHS manifests clinically as cardiomyopathy, skeletal myopathy, neutropenia, and growth retardation (Barth et al., 1999). More than 160 mutations in the tafazzin gene have been identified in BTHS patients (Chen et al., 2002; D'Adamo et al., 1997; Hijikata et al., 2015). These mutations result in loss of function of tafazzin, leading to decreased cellular CL, increased MLCL, and altered CL fatty acyl composition (Schlame et al., 2003; Schlame et al., 2002; Valianpour et al., 2002). Total CL is decreased to about 80% in BTHS platelets and skeletal muscle and 20% in cardiac tissue (Schlame et al., 2002). CL species vary in different tissues. Tetralinoleoyl-CL (L4-CL) is the most abundant
Fig. 1.1 Cardiolipin synthesis and remodeling pathway in *Saccharomyces cerevisiae*. Cited from Ye et al. 2014b. Cited from Ye et al. 2014b. The first reaction of CL de novo synthesis is the conversion of phosphatidic acid (PA) to CDP-diaclyglycerol (CDP-DAG) by the mitochondrial CDP-DAG synthase Tam41. The committed step of CL synthesis is catalyzed by Pgs1, which converts CDP-DAG to phosphatidylglycerolphosphate (PGP). PGP is subsequently dephosphorylated to phosphatidylglycerol (PG) by the GEP4-encoded PGP phosphatase. CL synthase, encoded by CRD1, condenses PG and CDP-DAG to form CL. CL synthesized de novo has primarily saturated acyl chains (CLSAT). CLSAT is deacylated by the CL-specific phospholipase Cld1 to monolysocardiolipin (MLCL), which is reacylated by tafazzin (the TAZ1 gene product) to CL containing more unsaturated acyl chains (CLUNSAT). All the CL biosynthetic enzymes are localized in the mitochondrial inner membrane (IM), whereas tafazzin is localized in the outer face of the (IM) and the inner face of the outer membrane (OM). IMS: intermembrane space.
CL species in heart, skeletal muscle and most other tissues, whereas acyl species such as arachidonic and docosahexaenoic acids are found in brain (Cheng et al., 2008; Houtkooper and Vaz, 2008; Schlame and Ren, 2006). L4-CL is absent in BTHS, while increases in other CL species are present (Schlame et al., 2003; Schlame et al., 2002; Valianpour et al., 2002).

Understanding the cellular function and molecular mechanisms of CL remodeling may provide better treatment options for BTHS patients (Ye et al., 2014b). Tafazzin deficiency results in decreased CL, increased MLCL, and altered CL species, any of which may cause the pathology in BTHS. Recent findings in yeast indicate that deletion of Cld1p-mediated deacylation rescues growth and lifespan defects in tafazzin deficient cells (Baile et al., 2013; Ye et al., 2014a). Because the cld1 mutation restored CL levels without generating remodeled CL, these findings suggest that decreased total CL and/or increased MLCL, but not decreased remodeled CL, causes the defects associated with tafazzin deficiency. Inhibiting CL deacylation may, thus, be a potential strategy to treat BTHS patients.

2.1.2 Dilated cardiomyopathy with ataxia (DCMA) syndrome

DCMA syndrome is an autosomal recessive genetic disorder that is characterized by early onset dilated cardiomyopathy with conduction defects, non-progressive cerebellar ataxia, testicular dysgenesis, growth failure, and 3-methylglutaconic aciduria (Davey et al., 2006). These clinical manifestations are similar to phenotypes found in BTHS. Patients with DCMA have a common mutation, a G→C
base substitution within a splice site of the DNAJC19 gene (Davey et al., 2006). DNAJC19 protein localizes to the mitochondria and shares sequence and location similarity with yeast Tim14, an essential subunit of the TIM23 complex (D'Silva et al., 2003; Mokranjac et al., 2003). TIM23 is required for the import of protein precursors from the cytoplasm into the mitochondrial matrix and inner membrane (Rehling et al., 2003). This suggests that the DCMA phenotype may result from defective mitochondrial protein import. Interestingly, loss of CL also leads to defective mitochondrial protein import (Eilers et al., 1989; Endo et al., 1989; Endo and Schatz, 1988; Gebert et al., 2009; Jiang et al., 2000). Therefore, it is interesting to speculate that defective mitochondrial protein import may be common to DCMA and BTHS. A recent study suggests that CL may play a role in the pathogenesis of DCMA (Richter-Dennerlein et al., 2014). DNAJC19 protein is reported to form the PHB/DNAJC19 complex with prohibitin, a ring-like scaffold protein located in the mitochondrial inner membrane. The PHB/DNAJC19 complex modulates CL remodeling by regulating tafazzin activity. Depletion of DNAJC19 does not affect CL or MLCL levels but alters the acyl chain composition of CL (Richter-Dennerlein et al., 2014). However, if the cause of DCMA is due to defective protein import, altered CL fatty acyl species, or a combination of the two remains unknown.

2.1.3 Diabetic cardiomyopathy

Diabetes is a metabolic disease characterized by increased levels of glucose in the blood over a prolonged period. It is due to either poor insulin production (type I) or
insulin resistance with β-cell dysfunction (type II) (He and Han, 2014). Diabetic complications are characterized by a group of diseases derived from microvascular and macrovascular damage, including diabetic cardiomyopathy, myonecrosis, stroke, peripheral vascular disease, nephropathy, retinopathy and encephalopathy (Nathan, 1993). Diabetes doubles the risk of CVD, of which diabetic cardiomyopathy is the leading cause of mortality. Diabetic cardiomyopathy is characterized by altered lipid composition and mitochondrial dysfunction in the diabetic myocardium (Han et al., 2000). In the early stages of pathological development in the type II diabetic mouse model, a sharp decrease in total cardiac CL is observed (Han et al., 2005). In addition to a decrease in the whole cell CL content, there is also a shift from the predominant fatty acyl species, L4-CL (18:2) to longer and polyunsaturated fatty acids, due to aberrant CL remodeling (Han et al., 2007). Strikingly, these alterations are similar to changes observed in the type I model of diabetes. In type II diabetic mice, treatment with the antidiabetic drug rosiglitazone restored total CL, L4-CL, and polyunsaturated CL levels (Pan et al., 2006). Impairment of CL synthesis plays a causal role in mitochondrial dysfunction (Koshkin and Greenberg, 2002; Mileykovskaya and Dowhan, 2014a; Pfeiffer et al., 2003), and mitochondrial dysfunction is associated with the pathogenesis of diabetic CVD, especially the sequential events following silent myocardial ischemia in diabetics (Sack, 2009). Thus, the sharp decrease in total cardiac CL and the altered CL fatty acyl species in the early stages of diabetic pathogenesis may play a key role in the progression of this disease.
2.2 CL is associated with other CVDs

In addition to its role in cardiomyopathy, CL has been linked to other CVDs, including atherosclerosis, Tangier disease, myocardial ischemia-reperfusion and heart failure.

Atherosclerosis is a form of arteriosclerosis in which an artery wall thickens due to chronic invasion and further accumulation of white blood cells (WBC), remnants of dead cells, cholesterol, and triglycerides (Ross, 1993). Oxidized CL (oxCL) was found to accumulate both in rabbit and human atherosclerotic lesions (Tuominen et al., 2006), and in the aortic root of mice fed a high fat diet (Zhong et al., 2014). Increased anti-oxCL IgG (Lopez et al., 2003; Marai et al., 2008; Türkoğlu et al., 2008) and IgM (Su et al., 2013; Türkoğlu et al., 2008) antibodies are associated with atherosclerosis development. oxCL is recognized as a natural antigen that stimulates pro-inflammatory effects in the artery and promotes formation of atherosclerotic plaques (Bochkov et al., 2010; Marai et al., 2008). However, some studies purport that autoantibodies to oxCL may serve a protective role against the onset and development of atherosclerosis (Frostegård et al., 2014; Su et al., 2006). The seemingly contradictory findings regarding the role of anti-oxCL antibodies in atherosclerosis may reflect the influence of other factors, including age, gender and existing diseases. The anti-coagulation protein annexin A5 has been reported to bind to and inhibit the pro-inflammatory effects of oxCL (Wan et al., 2014), providing the basis of a promising therapeutic strategy for oxCL-positive atherosclerosis.

Tangier disease (TD) is another genetic disorder that may be linked to CL. TD is
a disorder of cholesterol efflux and lipid metabolism characterized by a nearly complete absence of plasma high-density lipoproteins (HDLs), atherosclerosis, peripheral neuropathy and an increased risk for developing CVD (Fredrickson, 1964; Oram, 2000). The genetic cause of TD is mutations of the ABCA1 gene, which is located on chromosome 9 (Rust et al., 1999). ABCA1 encodes a highly conserved ATP binding cassette transporter that belongs to a subfamily of ABC transporters. The ABCA subfamily is involved in lipoprotein metabolism and lipid transport across the plasma membrane (Knight, 2004). Researchers propose that a physical interaction between apoA-I and ABCA1 results in the formation of a phospholipid-apoA-I complex that promotes cholesterol efflux (Wang et al., 2001). Three phospholipids, including CL and lysoCL 1 and 2 (LC₁ and LC₂), which together contribute only a small fraction of the total cellular phospholipid content, were found to be enriched up to five-fold in TD fibroblasts compared to wild type (WT) cells (Fobker et al., 2001). This finding suggests that phospholipid and cholesterol efflux may be co-regulated and, therefore, dually impaired in TD cells. Additionally, it is possible that increased CL may play an as yet uncharacterized regulatory role in cholesterol trafficking and efflux.

In addition to the above disorders, the CL profile is altered in both myocardial ischemia-reperfusion injury and heart failure. Myocardial ischemia occurs when the myocardium does not receive sufficient blood flow, resulting in cell death and further irreversible injury (Carden and Granger, 2000). Restoration of circulation in ischemic myocardium exacerbates the injury (Carden and Granger, 2000). In the early stages of myocardial ischemia, there is an increase in reactive oxygen species (ROS).
During and post ischemia-reperfusion, ROS is thought to trigger lipid peroxidation as well as damage to cellular macromolecules and the electron transport chain, which together lead to apoptosis, necrosis and tissue damage (Ferrari et al., 1991; Kalogeris et al., 2014; Kloner et al., 1989). Unsaturated CL acyl species in the mitochondrial inner membrane that are close to the site of ROS generation are vulnerable to oxidative damage. Consistent with this, total CL was decreased and peroxidized CL was increased in the rat heart during ischemia-reperfusion (Paradies et al., 1999). A study of ischemia-reperfusion in rabbit heart reported that reduction of total CL was due in large part to a significant decrease in CL in the subsarcolemmal mitochondria, whereas CL in the interfibrillar mitochondria was unchanged (Lesnefsky et al., 2001). The levels of all other phospholipids remained unaffected in the same study. Decreased CL was shown to have an impact on electron transport chain complexes I (Paradies et al., 2004), III (Petrosillo et al., 2003), and IV (Paradies et al., 1999). The enzyme activities of these complexes in mitochondria from the ischemic rat heart was restored by the addition of exogenous CL, but not by other phospholipids or peroxidized CL (Paradies et al., 1999). In summary, a feedback loop appears to be formed, in which CL is damaged by ischemia-reperfusion-induced ROS, and damaged CL leads to impairment of electron transport chain complexes, resulting in the generation of more ROS. In addition, the fact that membranes containing peroxidized CL are more permeable to apoptosis factors (Korytowski et al., 2011) may suggest a route through which ROS triggers apoptosis and tissue damage.

Heart failure (HF) occurs when the heart is not able to contract efficiently enough
to pump blood to meet the body’s needs. The major clinical symptoms of HF include edema, shortness of breath and lack of energy. HF is usually the end stage of CVD, including cardiomyopathy, heart attack, cardiac valvular disease, atrial fibrillation, and high blood pressure (McMurray and Pfeffer). In both the spontaneously hypertensive HF rat (SHHF) and human HF patients, decreased tafazzin mRNA levels were observed, concomitant with compensatory increases in the activity of phosphatidylglycerol phosphate synthase and MLCL acyltransferase (Saini-Chohan et al., 2009). However, studies of the CL profile in HF are controversial. While most studies report a significant reduction of total CL and L4-CL in human HF (Chatfield et al.; Le et al., 2014; Sparagna et al., 2007) and in the rat HF model (Reibel et al., 1986; Sparagna et al., 2007), one study reported an unchanged CL profile in a rat model with intracoronary microembolization-induced HF (Rosca et al., 2011). It is likely that different HF pathogenesis mechanisms lead to varying degrees of CL profile change and mitochondrial damage.

3 CL plays a role in cellular events and pathways that are important for maintaining cardiovascular health

3.1 Autophagy/mitophagy

Autophagy refers to the cellular process in which cytoplasmic contents are delivered to the lysosome or vacuole for degradation. Autophagy is further classified into selective and nonselective autophagy (Nair and Klionsky, 2005). Various types of selective autophagy have been identified, including mitophagy, pexophagy, lipophagy, nucleophagy, lysophagy, reticulophagy/ER-phagy and ribophagy (Okamoto, 2014).
Mitophagy is the selective degradation of mitochondria by autophagy (Wang and Klionsky, 2011). Mitophagy and autophagy are generally not distinguished in studies of CVD and will be discussed together here.

In the heart, autophagy is an important housekeeping process and is essential for maintaining cardiac health (Moyzis et al., 2015). Autophagic activity declines with age, and decreased or impaired autophagy leads to accumulation of proteins and damaged mitochondria, contributing to cardiac aging (Linton et al.). In addition, deletion of ATG5, the gene encoding a protein that regulates phagophore expansion, is known to result in cardiomyopathy in mice (Nakai et al., 2007).

As discussed above, decreased CL causes impairment of the electron transport chain complexes I (Paradies et al., 2004), III (Petrosillo et al., 2003) and IV (Paradies et al., 1999), resulting in mitochondrial dysfunction. In response to mitochondrial damage, mitophagy increases as an adaptive and protective strategy to eliminate damaged mitochondria (Frank et al., 2012; Narendra et al., 2008). Therefore, I undertook the study in this thesis to investigate if the loss of CL leads to altered mitophagy.

3.2 CL and MAPK pathways

3.2.1 PKC pathway

Protein kinase C (PKC) is a family of protein kinases that regulate the function of other proteins through specific phosphorylation of hydroxyl groups on threonine and serine residues. Human cells have fifteen PKC isozymes (Mellor and Parker,
Over-stimulation of PKCα, PKCβ, PKCδ or PKCε results in hypertrophy of cardiomyocytes through activation of the extracellular signal-related kinase (ERK) pathway (Steven P. Marso and Stern, 2003). However, during ischemia preconditioning, PKCα, PKCδ, PKCε and PKCη have been shown to translocate to the active membrane pool and perform cardioprotective functions (Steven P. Marso and Stern, 2003). Activation of PKCδ results in intracellular pH changes and viability protection; activation of PKCη protects against myocardial stunning; activation of both PKCδ and PKCη provides global myocardial protection against necrosis, acidosis, and myocardial stunning (Meldrum et al.). Blocking the phosphatidylinositol-specific phospholipase C (PI-PLC)-induced translocation of PKCα, PKCε, and PKCη during ischemia impairs myocardial recovery (Munakata M et al., 2002). Therefore, PKC isozymes have dual functions in the pathogenesis and progression of CVD. However, unlike other PKC isozymes that affect different CVDs, PKCη is mainly reported to play a cardioprotective role during ischemia.

Yeast has only one PKC (Pkc1p). Human PKCη is the only human PKC isozyme that can complement the defects caused by deletion of PKC1 in yeast through activation of the same protein kinase cascade (Nomoto S et al., 1997). This suggests that PKCη shares both functional and structural homology with Pkc1p. A previous study in yeast showed that loss of PG, the precursor of CL, leads to defects in the activation of the PKC pathway (Zhong et al., 2007). Extrapolating from the finding in yeast that the CL precursor is required for PKC pathway activation, the cardioprotective role of PKCη activation during ischemia preconditioning may be dependent on CL. In yeast,
PKC activation is also required for mitophagy (Mao et al., 2011), another cardioprotective process. Thus, to investigate how CL plays a role in maintaining cardiovascular health, it is necessary to determine if CL is required for PKC pathway activation.

3.2.2 HOG pathway

The high osmolarity glycerol (HOG) pathway is a highly conserved MAPK pathway that mediates the cellular response to hyperosmotic shock. In yeast, the HOG pathway consists of two stress-sensing branches, SLN1 and SHO1 branches (Saito and Tatebayashi, 2004). Both branches are cascades that lead to phosphorylation of Hog1p, the yeast homolog of the mammalian MAPK p38, which functions in the inflammatory and stress responses (Raingeaud et al., 1995). Phosphorylation activates Hog1p and promotes translocation into the nucleus. The phosphorylated protein (pHog1p) induces transcription of GPD1, and GPP2, which encode glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase, respectively (Rep et al., 2000). These enzymes are involved in the production of glycerol, the cytosolic accumulation of which counteracts osmotic stress. Activated Hog1p also regulates intracellular ion homeostasis by phosphorylating the C-termini of Nha1p, the Na+/H+ antiporter on the plasma membrane, and Tok1p, the potassium channel on the plasma membrane (Proft and Struhl, 2004). In addition to regulating glycerol biogenesis and ion homeostasis, pHog1p regulates the transcription of about 600 genes, resulting in cellular adaptation to various stresses (O'Rourke and Herskowitz, 2004).
A previous study showed that CL mutants exhibit vacuole defects that are characterized by enlarged vacuoles and loss of vacuole acidification (Chen et al., 2008b). The vacuole defects were rescued by deletion of *NHX1*, which encodes the Na+/H+ and K+/H+ exchanger, suggesting that ion homeostasis is perturbed in CL mutants. The vacuole defects were also rescued by supplementation with 1 M sorbitol, a HOG pathway stimulant, suggesting that osmoregulation may be perturbed in CL mutants. Because the HOG pathway functions in both osmoregulation and intracellular ion homeostasis, it is necessary to determine if the HOG pathway is perturbed in CL mutants.

### 4 Saccharomyces cerevisiae as my research model

As discussed above, CL biosynthesis is highly conserved from yeast to humans. The yeast *taz1Δ* mutant exhibits respiratory and metabolic deficiencies similar to those in human BTHS cells (Chen et al., 2008a; Gu et al., 2004; Ma et al., 2004). In addition, the availability of null mutants for each step of CL synthesis facilitates studies to elucidate CL function, which are not easily carried out in higher eukaryotes. For example, the finding that growth and respiratory defects of the yeast *taz1* mutant are rescued by deletion of *CLD1*, which restores CL/MLCL levels without generating remodeled CL, suggests that CL/MLCL levels are more important for mitochondrial function than CL acyl composition (Baile et al., 2014; Ye et al., 2014a). This study could not be carried out in mammalian cells, as CL phospholipases have not been characterized in mammals.
Most of the studies described in this thesis utilize the crd1Δ null mutant, in which the gene for CL synthase is deleted. The crd1Δ mutant has no CL and accumulates the CL precursor, PG. I also utilized the yeast genome deletion collection, which facilitated me to carry out genetic interaction studies. The genetic and molecular tools of the yeast model enabled me to carry out my analysis of the role of CL in mitophagy and the MAPK pathways.

5 Project outline

The goal of my doctoral research was to investigate the role of CL in cellular events and pathways that are required for maintaining cardiovascular health. I specifically focused on the role of CL in mitophagy, and the mechanism underlying this connection.

The study described in Chapter 2 shows that the loss of CL leads to defective mitophagy. Synthetic lethality with autophagy/mitophagy mutants suggested that mitophagy may be deficient in crd1Δ. Microscopic examination of mitophagy revealed decreased translocation of GFP-tagged mitochondrial protein into the vacuole of crd1Δ cells. This was confirmed by immunoblotting detection of free GFP, which was generated by cleavage of GFP-tagged mitochondrial protein after it was delivered into the vacuole by mitophagy. These findings indicated that mitophagy is decreased in CL deficient cells.

Chapter 3 describes a possible mechanism underlying defective mitophagy in crd1Δ. It is known that the PKC pathway is required for early stages of mitophagy and
the HOG pathway is required for later stages (Mao et al., 2011). My studies showed that \textit{crd1Δ} growth defects are exacerbated by downregulation of the HOG pathway and rescued by upregulating the PKC pathway, suggesting that these MAPK pathways may be defective in CL mutants. Consistent with this, Western blot experiments showed decreased phosphorylation of Slt2p and Hog1p in \textit{crd1Δ}, indicating defective activation of the PKC and HOG pathways. Interestingly, upregulation of PKC rescued defective mitophagy in \textit{crd1Δ}. These results suggest that the mechanism underlying defective mitophagy in \textit{crd1Δ} cells is defective MAPK function.

Although the findings of my dissertation research shed light on the role of CL in pathways known to be important for maintaining cardiovascular health, the story is far from over. Chapter 4 describes exciting experiments that can be done to elucidate the mechanism underlying the role of CL in mitophagy and the MAPK pathways. In addition, follow up experiments are proposed to continue preliminary studies of the role of CL in vacuole homeostasis.
CHAPTER 2
LOSS OF CARDIOLIPIN LEADS TO DEFECTIVE MITOPHAGY

Introduction

Cardiolipin (CL) is the signature lipid of mitochondrial membranes. It plays an important role in mitochondrial function through interacting with various mitochondrial membrane proteins, including electron transport chain (ETC) complex proteins that are components of complex I (Fry and Green, 1981; Mileykovskaya and Dowhan, 2014b), complex III (Fry and Green, 1981; Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005), complex IV (Pfeiffer et al., 2003; Zhang et al., 2002; Zhang et al., 2005), complex V (Gohil et al., 2004), cytochrome c (Ranieri et al., 2015) and transporter proteins such as the ADP-ATP carrier (Beyer K and M., 1985), pyruvate carrier (Paradies and Ruggiero, 1988) and phosphate carrier (Kadenbach et al., 1982). Loss of CL leads to impaired mitochondrial function, including defective respiration (Schlame and Ren, 2006), decreased mitochondrial membrane potential (Jiang et al., 2000) and impaired mitochondrial protein import (Gebert et al., 2009; Jiang et al., 2000). Additional cellular functions of CL outside of mitochondria are continuously being explored.

In studies described in this chapter, I investigated the role of CL in mitophagy. Mitophagy is the selective degradation of mitochondria through autophagy (Wang and Klionsky, 2011). Autophagy is the cellular process in which cytoplasmic contents are delivered into the lysosome or vacuole for degradation. According to the different ways by which cargos are delivered to the lysosome/vacuole for degradation, autophagy is
classified as macroautophagy, microautophagy, and chaperone-mediated autophagy (Shintani and Klionsky, 2004). Autophagy is further classified as selective or nonselective, depending on whether specific cargo is selected (Nair and Klionsky, 2005). Various types of selective autophagy have been identified (Okamoto, 2014), among which mitophagy is the selective degradation of mitochondria (Wang and Klionsky, 2011). In the heart, autophagy is essential for maintaining cardiac health (Moyzis et al., 2015). Impairment of the process by deletion of \( \text{ATG5} \) has been demonstrated to cause cardiomyopathy in mice (Nakai et al., 2007), linking abnormal autophagy to cardiomyopathy.

Cardiomyocytes have a remarkably high mitochondrial density that comprises about 30% of the total intracellular volume (Maack and O'Rourke, 2007). This allows cardiomyocytes to produce ATP quickly to satisfy the high demand for energy. Even subtle alterations in mitochondrial function or membrane potential can cause a significant change in cardiomyocyte energy production and further harm cardiovascular health. In response to mitochondrial damage, mitophagy increases as an adaptive and protective strategy (Frank et al., 2012; Narendra et al., 2008). Therefore, I carried out studies to determine if loss of CL, which perturbs numerous mitochondrial functions, leads to altered mitophagy.

Genetic interaction analysis is a very powerful approach to study gene function. Rescue of a mutant phenotype by deletion of another gene identifies suppression of the mutant phenotype. Exacerbation of a mutant phenotype to lethality by deletion of another gene is defined as synthetic lethality, which suggests that the two genes may
work in redundant pathways. I predicted that genetic interaction analysis between autophagy/mitophagy genes and CRD1 would shed light on the role of CL in mitophagy.

Although mitophagy occurs via both macro- and micro-autophagy (Kanki et al., 2009c; Kiššová et al., 2007; Nowikovsky et al., 2007; Okamoto et al., 2009), degradation of mitochondria via macro-autophagy is the dominant process when mitophagy is triggered by nitrogen starvation or glucose deprivation (Kiššová et al., 2007). During macro-mitophagy, mitochondria are recognized as the cargo that is brought to the phagophore assembly site (PAS), where the double-membrane of the phagophore expands and closes to form a mature mitophagosome that contains mitochondria. Macro-mitophagy adapts the general autophagy core machinery, which involves various autophagy-related (ATG) proteins, including the Atg1p/Atg13p complex, Atg9p, Atg18p-Atg2p complex, Atg8p conjugation system (Atg3p, Atg4p, Atg7p, and Atg8p), and the Atg12 ubiquitin-like conjugation system (Atg5p, Atg7p, Atg10p, Atg12p, and Atg16p) (Köfinger et al., 2015; Reggiori et al., 2005; Reggiori et al., 2004; Sakoh-Nakatogawa et al., 2015; Suzuki et al., 2001; Suzuki et al., 2007; Xie and Klionsky, 2007). Atg8p is the yeast homolog of mammalian LC3, and it is conjugated to phosphatidylethanolamine (PE) (Kirisako et al., 1999). Atg8p-PE coats the mitophagosome membrane during membrane elongation. Phosphatidylinositol 3-phosphate (PI3P) is another important lipid that constitutes the mitophagosome membrane (Gillooly et al., 2000). Atg18p binds to PI3P and regulates the retrieval of Atg9p from PAS, and normal transport of Atg9p between PAS and the peripheral site is essential in PI3P synthesis on the mitophagosome membrane (Reggiori et al.,
Thus, Atg8p and Atg18p are essential proteins in the general autophagy core machinery.

The mitochondrial outer membrane protein Atg32p plays a key role in selecting mitochondria as the specific cargo. When mitophagy is triggered, Atg32p is phosphorylated (Aoki et al., 2011). Then it interacts with Atg11p, which directs mitochondria to the PAS, where an Atg32–Atg11–Atg8 initiator complex is formed (Aoki et al., 2011; Farré et al., 2013). When an intact mitophagosome is formed and delivered to the vacuole, the mitophagosome outer membrane fuses with the vacuole membrane and releases the mitochondria wrapped by the mitophagosome inner membrane into the vacuole lumen for degradation.

In this chapter, I investigated if loss of CL in the crd1Δ mutant leads to altered mitophagy. Deletion of autophagy/mitophagy genes exacerbated the crd1Δ growth defect. Mito-GFP marker tracking experiments and Western detection of free GFP, the mitophagy product, both showed inhibited mitophagy in crd1Δ compared to WT. These findings suggest that CL plays a novel role in mitophagy.
Methods and materials

Yeast strains and growth media

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 2.1. Yeast extract peptone dextrose (YPD) medium contained yeast extract (1%), peptone (2%), and glucose (2%). Synthetic complete (SC) medium contained lab-made vitamin-free yeast nitrogen base without amino acids (Difco protocol, 0.17%), ammonium sulfate (0.5%), glucose (2%), vitamins, and the following amino acids: 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). Synthetic drop-out medium contained all ingredients mentioned above except the amino acid used as the selective marker. Synthetic lactate (SL) medium contained lab-made vitamin free yeast nitrogen base without amino acids (Difco protocol, 0.17%), ammonium sulfate (0.5%), and lactate (2%). Solid medium contained agar (2%).

Construction of deletion mutants and strains that express GFP-tagged mitochondrial proteins

Deletion mutants were constructed as follows. The entire open reading frame of the target gene was replaced by a *KanMX4* cassette via homologous recombination in the wild type (WT) strain. The *KanMX4* cassette was amplified by polymerase chain reaction (PCR), using the pUG6 plasmid, which contains the *KanMX4* cassette, as the
The amplification primers consisted of 50 nucleotides that were identical to either the upstream or the downstream flanking region of the target gene at the 5’ end and 21 nucleotides that annealed to the KanMX4 gene at the 3’ end. For construction of the single mutants with the target mitophagy gene deleted, the PCR product was transformed into the WT strain by electroporation. For construction of the double mutants with both CRD1 and the target mitophagy gene deleted, the PCR product was transformed into the crd1Δ strain by electroporation. Transformants were selected on YPD plates with 200 μg/ml G418. Deletion of the target gene was confirmed by PCR using primers that amplify the original target gene.

To monitor mitophagy in both WT and crd1Δ strains, the mitochondrial matrix protein Idh1p in both strains was tagged by GFP as previously described (Kanki et al., 2009a). The pFA6a-GFP (S65T)-HIS3MX6 plasmid (kindly provided by Dr. Daniel Klionsky, University of Michigan) was used as the PCR template to amplify a DNA fragment that encodes GFP with HIS3 as the selective marker. The primers consisted of 50 nucleotides that were identical to either the upstream or the downstream flanking region of the IDH1 ending code (TAA) at the 5’ end and 20 nucleotides that annealed to the GFP sequence on the plasmid at the 3’ end. The sequences of these primers are 5’-CTTCTACTGACTTCAGCCTGGTAAAATCATCAACAAATTATCATGCCACCGATCCGCCGGGTTAATTAA-3’ and 5’-AAATTTGAACACACTTAAGATGAGAACAAAAAAAAGGGGAATTGTTTTCAGAATTCGAGCTCGTTTAAAC-3’. The PCR product was then transformed into both WT and crd1Δ cells in the BY4742 and FGY genetic backgrounds by electroporation, and inserted into the end of the IDH1 locus.
via homologous recombination. The transformants that express IDH1-GFP were selected on SC his' medium. Correct GFP insertion was confirmed by PCR analysis, using confirmation primers 5'-ATGCTGTCTTCGAACCAGGTT-3’ and 5’-AGTTCATCCATGCCATGTGT-3’.

Another approach to construct strains containing GFP-tagged mitochondria was to transform the pCu416-IDP1-GFP plasmid that expresses IDP1-GFP (kindly provided by Dr. Hagai Abeliovich, Hebrew University) into WT and crd1Δ, by electroporation. Transformants carrying the plasmid encoding IDP1-GFP were selected on SC ura medium.

**Single colony formation analysis**

Cells were pre-cultured in YPD at 30°C to the mid-log phase. The absorbance at 550 nm (A550) of the liquid culture was determined using a spectrophotometer (Beckman). Cell aliquots were centrifuged and pellets were resuspended in YPD to adjust the A550 value to 10 units per ml. 10 μl of the above sample were added to 990 μl of water. 10 μl of the 100X diluted sample were placed on a hemocytometer for cell counting using light microscopy at a magnification of 1000 X. Based on cell number, cell aliquots with A550 of 10 were adjusted again and serial diluted to 2000 cells/ml, from which 100 μl suspension that contained about 200 cells were plated on a YPD plate and incubated at 30°C, 36°C or 37°C for 3 days. The ability of single cells to form colonies was evaluated at 30°C, 36°C or 37°C.
Table 2.1 Yeast strains and plasmids used in Chapter 2

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics or genotype</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>FGY3 (WT)</td>
<td>MAT a, ura3-52, lys2-801, ade2-101, trp1Δ1, his3Δ200, leu2Δ1</td>
<td>Jiang et al., 1997</td>
</tr>
<tr>
<td>FGY2 (crd1Δ)</td>
<td>MAT a, ura3-52, lys2-801, ade2-101, trp1Δ1, his3Δ200, leu2Δ1, crd1Δ::URA3</td>
<td>Jiang et al., 1997</td>
</tr>
<tr>
<td>FGY3 atg8Δ</td>
<td>Derivative of FGY3, atg8Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>FGY2 atg8Δ</td>
<td>Derivative of FGY2, atg8Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>FGY3 atg18Δ</td>
<td>Derivative of FGY3, atg18Δ::KanMX4</td>
<td>This study</td>
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<tr>
<td>FGY3 atg32Δ</td>
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<tr>
<td>FGY2 atg32Δ</td>
<td>Derivative of FGY2, atg32Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>FGY3 atg21Δ</td>
<td>Derivative of FGY3, atg21Δ::TRP1</td>
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<td>This study</td>
</tr>
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<td>FGY3-ΔIDH1-GFP</td>
<td>Derivative of FGY3, in which GFP is inserted into the IDH1 gene locus</td>
<td>This study</td>
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<td>FGY2-ΔIDH1-GFP</td>
<td>Derivative of FGY2, in which GFP is inserted into the IDH1 gene locus</td>
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<tr>
<td>BY4742</td>
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<td>Euroscarf*</td>
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<td>Gohil et al., 2005</td>
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<td>BY4742-ΔIDH1-GFP</td>
<td>Derivative of BY4742, in which GFP is inserted into the IDH1 gene locus</td>
<td>This study</td>
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<td>BY4742 crd1Δ-ΔIDH1-GFP</td>
<td>Derivative of BY4742 crd1Δ::KanMX4, in which GFP is inserted into the IDH1 gene locus</td>
<td>This study</td>
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<td>pFA6a-GFP[S65T]-His3MX6</td>
<td>Derivative of pFA6a-His3MX6</td>
<td>Kanki et al., 2009a</td>
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<tr>
<td>pCu416-IDP1-GFP</td>
<td>Derivative of pCu416, expresses GFP-tagged IDP1 from CUP1 promoter</td>
<td>Journo et al., 2009</td>
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</tbody>
</table>

* Euroscarf, European Saccharomyces cerevisiae Archive for Functional analysis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tr>
<td></td>
<td>Reverse</td>
<td>CGAATTGAGAGTTGCCCCAGAAG</td>
</tr>
<tr>
<td>ATG8</td>
<td>Forward</td>
<td>ACCTACCCTAGGGCAATTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCGTCCTTTATCCTTGTGTTC</td>
</tr>
</tbody>
</table>
**FM 4-64 and quinacrine staining**

FM 4-64 staining was performed as previously described (Conboy and Cyert, 2000) with minor revisions. Cell aliquots equivalent to an $A_{550}$ of 1.0 were harvested by centrifugation, resuspended in 100 μl YPD, and incubated at 37°C or 39°C with 0.16 mM FM4-64 for 15 min. Cells were pelleted and washed once with 37°C YPD or 39°C YPD and then resuspended in 500 μl YPD of the same temperature for 30 min. The cell suspension was centrifuged and the supernatants were discarded. The cell pellets were resuspended in 100 μl YPD, from which 3 μl were applied to the microscope slide and observed using epifluorescence microscopy using the filter for red fluorescence.

Quinacrine staining was performed as previously described (Roberts et al., 1991), with minor revisions. Cell aliquots equivalent to an $A_{550}$ of 1.0 were harvested by centrifugation and resuspended in 500 μl YPD (containing 2 mM quinacrine and buffered to pH 7.6 with 0.5M Na2HPO4) prewarmed to 37°C or 39°C. After a 5 min incubation, cells were pelleted and washed once with 500 μl 2% glucose that had been buffered to pH 7.6 with 0.5M Na2HPO4. The cell suspension was pelleted again and resuspended in 100 μl 2% glucose at pH 7.6, from which 3 μl were applied to a microscope slide and observed using epifluorescence microscopy using the filter for green fluorescence.

**Detection of mitophagy using fluorescence microscopy**

Microscopic analyses were performed using an Olympus BX41 epifluorescence
microscope. Images were captured by an Olympus Q-Color3 digital charge-coupled
device camera operated by QCapture2 software. All pictures were taken at a
magnification of 1000X. To induce mitophagy, cells expressing \textit{IDH1-GFP} were
cultured in SC medium to the mid-log phase, pelleted and resuspended in 5 ml SL
medium with a starting $A_{550}$ of 0.5. They were then cultured for 18 hours and observed
using epifluorescence microscopy. An increase in the signal of mito-GFP in the vacuole
indicated induction of mitophagy. In addition, cells were also treated with elevated
temperature. Both WT-\textit{IDH1-GFP} and \textit{crd1}Δ-\textit{IDH1-GFP} were cultured in YPD medium
to the mid-log phase at 30˚C and then switched to 39˚C for 8 hours. Cells were then
observed using epifluorescence microscopy, using the filter for green fluorescence.

\textbf{Western blot}

Whole cell extracts were prepared as previously described (Kanki et al., 2009a)
with minor changes. Cell pellets equivalent to 1.5 $A_{550}$ units were collected and
resuspended in 1 ml 10\% trichloroacetic acid (TCA). Samples were incubated for 10
min on ice and proteins were pelleted by centrifugation at 4˚C, 15,000 rpm for 10 min.
The pellets were washed twice with 1 ml of ice-cold acetone and air-dried at room
temperature. The air-dried cell pellets were resuspended in 75 μl sample buffer, which
consisted of 150 mM Tris-HCl with pH 8.8, 6\% SDS, 25\% glycerol, 6 mM EDTA, and
disrupted by vortexing for 3 min in the presence of 75 μl acid-washed glass beads.
Following this step, the samples were heated at 100˚C for 3 min and then centrifuged
at 4˚C, 7,000 rpm for 30 s to pellet the glass beads. 18 μl of the supernatants that
contained the proteins were mixed with 6 μl 4X loading dye (80% bromophenol blue and 20% β-mercaptoethanol) and loaded into a 12% SDS-polyacrylamide gel for electrophoresis. The proteins were then transferred to a PVDF membrane with 0.2 μm pores following the standard semidry Western blot transfer procedure. After blotting in TBST-milk (TBS containing 1% TWEEN 20 and 0.5% milk), the membranes were incubated in TBST-milk with mouse anti-YFP monoclonal antibody (JL-8, Clontech) at a 1:3000 dilution at 4°C overnight. After washing with TBST (TBS containing 1% TWEEN 20) for 4 times, 8 min per time, the membranes were incubated in 0.5% TBST-milk with the secondary antibody, HRP-conjugated goat anti-mouse IgG (Santa Cruz), at a 1:5000 dilution for 1 h at room temperature. After washing with TBST 4 more times, 8 min per time, the membrane was covered with Pierce™ ECL Plus Western Blotting Substrate reagent. To detect the signal of GFP, the HyBlot ES® Autoradiography film was exposed to the PVDF membrane and then developed. Idh1-GFP and cleaved GFP were detected as bands of 68 kDa and 28 kDa, respectively.

**Real-Time PCR (RT-PCR)**

Both WT-\textit{IDH1-GFP} and \textit{crd1Δ-IDH1-GFP} strains in the FGY background were cultured in 10 ml YPD medium to the mid-log phase at 30°C and then switched to 39°C for 8 hours. Cells were harvested and total RNA was extracted using the RNeasy Mini kit (QIAGEN). This procedure removed possible contaminating genomic DNA. cDNAs were synthesized using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) following the manufacturer’s protocol. RT-PCR was performed
in a 96-well plate using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). Duplicates for each sample were included. The primers used for RT-PCR are listed in Table 2.2. *ACT1* was used as the internal control. The mRNA level of the target gene was normalized to *ACT1* mRNA levels. PCR reactions were performed at 95°C for 10 min for denaturation and then 40 cycles that consisted of 30 s at 95°C and 60 s at 56°C.
Results

Deletion of autophagy/mitophagy genes exacerbates the temperature sensitivity of \textit{crd1}\textsubscript{Δ}

Previous studies have shown that loss of CL leads to impaired mitochondrial function, including defective respiration (Schlame and Ren, 2006), decreased mitochondrial membrane potential (Jiang et al., 2000) and impaired mitochondrial protein import (Gebert et al., 2009; Jiang et al., 2000). It is known that mitophagy increases as an adaptive and protective strategy in response to damage of mitochondria to eliminate damaged organelles (Frank et al., 2012; Narendra et al., 2008). A previous study in our lab found that, at elevated temperature, CL mutants exhibit not only growth defects but also vacuole defects characterized by enlarged vacuoles and loss of vacuole acidification (Chen et al., 2008b). The mechanism underlying the vacuole defects in the CL mutants are unknown. Because increased autophagy leads to a large influx of membrane and contents to the vacuole, resulting in increased vacuole size (Baba M et al., 1994; Baba et al., 1995; Takeshige K et al., 1992), I originally hypothesized that loss of CL might lead to increased mitophagy to eliminate the damaged mitochondria at elevated temperature. In addition, my preliminary study found that deletion of \textit{UTH1}, a gene that is involved in cell wall biogenesis and may play a role in mitophagy, rescued both the vacuole defects and the temperature sensitivity of \textit{crd1}\textsubscript{Δ} (Fig. 2.1), seeming to support my hypothesis that the vacuole defects in the CL mutants may be due to increased mitophagy.
Fig. 2.1  (A) Deletion of *UTH1* rescues the vacuole morphology defect of *crd1Δ* at 37°C. Cells were pre-cultured at 30 °C to the early log phase and then transferred to 37°C for 8 hours. FM 4-64 staining was performed and cells were observed using fluorescence microscopy.  (B) Deletion of *UTH1* rescues vacuole acidification defect of *crd1Δ* at 37°C. Cells were pre-cultured at 30 °C to the early log phase and then transferred to 37°C for 8 hours. Quinacrine staining was performed and cells were observed using fluorescence microscopy.
To test this hypothesis, I decided to determine if there was genetic interaction between \textit{CRD1} and autophagy/mitophagy genes. The rationale for this is that, deletion of autophagy/mitophagy genes should block excessive mitophagy and rescue the phenotype of \textit{crd1Δ}. I constructed single mutants of mitophagy genes in the FGY background, including \textit{atg8Δ}, \textit{atg18Δ}, \textit{atg21Δ}, \textit{atg32Δ}, double mutants of \textit{crd1Δ} and \textit{atg8Δ}, \textit{atg18Δ}, \textit{atg21Δ}, \textit{atg32Δ}, and the triple mutant \textit{crd1Δatg18Δatg21Δ}. Temperature sensitivity of these strains was examined. Surprisingly, deletion of autophagy/mitophagy genes did not rescue (Fig. 2.2) but rather exacerbated the temperature sensitivity of \textit{crd1Δ} (Fig. 2.3). Compared to deletion of the mitophagy-specific gene \textit{ATG32}, deletion of general autophagy genes, including \textit{ATG8} and \textit{ATG18}, caused more severe exacerbation of \textit{crd1Δ} temperature sensitivity. These findings suggested that mitophagy may be decreased in \textit{crd1Δ}.

\textbf{Delivery of mitochondria to the vacuole in response to increased temperature is inhibited in \textit{crd1Δ}}

\textit{WT-IDH1-GFP} and \textit{crd1Δ-IDH1-GFP} strains in the FGY background were constructed as described in the ‘Methods and materials’ section (Fig. 2.4). These strains express an endogenous \textit{Idh1} protein that is tagged with GFP at the C terminus and is localized in mitochondria. After culture in YPD medium to the mid-log phase at 30°C, \textit{WT-IDH1-GFP} and \textit{crd1Δ-IDH1-GFP} were switched to 39°C for 8 hours. Cells were then observed using epifluorescence microscopy. Accumulation of GFP was observed in the vacuole of WT cells, suggesting that elevated temperature
Fig. 2.2 Deletion of autophagy/mitophagy genes does not rescue crd1Δ growth defect at 37°C. Cells were pre-cultured in YPD liquid at 30°C to the mid-log phase. About 200 cells of each strain were plated on YPD and incubated at the indicated temperature for 3 days.
Fig. 2.3 (A) *crd1Δ* is synthetically lethal with *atg8Δ* and *atg18Δ* at 36°C. (B) *crd1Δ* is synthetically sick with *atg32Δ* at 36°C. Cells were precultured in YPD liquid at 30°C to the mid-log phase. About 200 cells of each strain were plated on SC complete plates and incubated at the indicated temperature for 3 days.
Fig. 2.4 Insertion of GFP into the IDH1 gene locus. Strains expressing an endogenous Idh1 protein tagged with GFP at the C terminus were constructed as described in the ‘Methods and materials’ section.
induced delivery of Idh1-GFP tagged mitochondria into the vacuole of WT cells for degradation. However, translocation of $crd1\Delta$ mitochondria into the vacuole was not observed, suggesting that mitophagy was inhibited in $crd1\Delta$ cells (Fig. 2.5).

A recent study reported that transcription of ATG8 positively regulates the size of the autophagosome (Backues et al., 2012) and reflects the extent of autophagy induction (Gasch et al., 2000; Kirisako et al., 1999). When autophagy is triggered, expression of ATG8 is induced more than 10-fold (Gasch et al., 2000; Kirisako et al., 1999). Expression of ATG8 was determined in both WT and $crd1\Delta$ cells at 30˚C and after switching to 39˚C for 8 hours. ATG8 mRNA levels in WT and $crd1\Delta$ were comparable at 30˚C. Interestingly, ATG8 expression increased 3-fold in WT cells at 39˚C compared to that at 30˚C, while it increased 6.7-fold in $crd1\Delta$ cells at 39˚C compared to that at 30˚C (Fig. 2.6). Expression of ATG8, the essential gene for mitophagy and non-selective autophagy, was upregulated in both WT and $crd1\Delta$ at elevated temperature. However, the increase was greater in $crd1\Delta$ than in WT cells, suggesting that, in $crd1\Delta$, there is a greater need to initiate mitophagy, or that non-selective autophagy may be upregulated to compensate for the decrease of mitophagy.

**Induction of mitophagy is inhibited in $crd1\Delta$ cells**

Based on the finding that the delivery of mitochondria into the vacuole is inhibited in $crd1\Delta$ compared to WT in response to elevated temperature, I predicted that mitophagy is decreased in $crd1\Delta$ compared to WT. Because elevated temperature has not been previously shown to trigger mitophagy, and mitophagy has not been studied
Fig. 2.5 Delivery of mitochondria to the vacuole in response to increased temperature is inhibited in \textit{crd1Δ}. WT and \textit{crd1Δ} cells expressing an endogenous Idh1 protein that is tagged with GFP at the C terminus were cultured in YPD medium to the mid-log phase at 30°C, shifted to 39°C for 8 hours and observed using fluorescence microscopy.
Fig. 2.6 Increased temperature triggers increased upregulation of *ATG8* expression in *crd1Δ* cells. WT and *crd1Δ* cells were cultured in YPD medium to the mid-log phase at 30°C and divided into two groups: one group was kept growing in 30°C for 8 hours while the other group was shifted to 39°C for 8 hours. *ATG8* expression was determined by RT-PCR.
in the FGY genetic background, I constructed WT-IDH1-GFP and crd1Δ-IDH1-GFP strains in the BY4742 background, which is widely used in mitophagy studies (Kanki et al., 2009b). WT-IDH1-GFP and crd1Δ-IDH1-GFP cells were cultured in SC medium to the mid-log phase at 30˚C, harvested, washed twice and resuspended in SL medium, which has been shown to induce mitophagy (Wu and Tu, 2011). After 18 hours, cells were observed using epifluorescence microscopy. Similar to the result seen in the FGY genetic background, accumulation of GFP was observed in the vacuole of WT but not crd1Δ cells, suggesting that mitophagy is inhibited in crd1Δ (Fig 2.7 A). To exclude the possibility that the observed differences were specific to the Idh1-GFP protein, I constructed WT and crd1Δ strains that have the GFP-tagged mitochondrial protein Idp1p exogenously expressed from the pCu416-IDP1-GFP plasmid. As seen in IDH1-GFP strains (Fig 2.7 B), accumulation of Idp1-GFP was observed in the vacuole of the WT but not crd1Δ cells, suggesting that mitophagy was inhibited in crd1Δ cells.

To confirm the microscopic finding, the release of free GFP from Idh1-GFP was determined in WT-IDH1-GFP and crd1Δ-IDH1-GFP strains by Western blot. Cells were cultured in SC medium to the mid-log phase at 30˚C, harvested, washed twice and resuspended in SL medium. After 5, 6 or 7 hours, proteins were extracted as described in the ‘Methods and materials’ section. As seen in Fig. 2.8, 6 hours after transferring to SL medium, a GFP band was detectable in WT cells. In contrast, the signal from crd1Δ cells was barely detectable. Taken together, these findings indicate that mitophagy is inhibited in crd1Δ.
Fig. 2.7 Decreased mitophagy in \textit{crd1}\Delta\ cells. WT and \textit{crd1}\Delta\ cells expressing an endogenous \textit{Idh1-GFP} (A) or exogenous \textit{Idp1-GFP} (B) were cultured in SC medium to the mid-log phase at 30°C and shifted to SL for 18 hours. Cells were observed using fluorescence microscopy.
Fig. 2.8 Decreased mitophagy in crd1Δ cells expressing Idh1-GFP. WT and crd1Δ cells carrying the GFP-tagged IDH1 gene were pre-cultured in SC medium to the mid-log phase at 30°C and shifted to SL medium for 5, 6 or 7 hours. Aliquots were taken at the indicated times. Immunoblotting was done with anti-YFP antibody and the positions of full-length Idh1-GFP and free GFP are indicated.
Discussion

In this chapter, I addressed the question of how loss of CL in the \textit{crd1}Δ mutant affects mitophagy. My findings indicate that CL deficiency leads to decreased mitophagy. This conclusion is based on the findings that 1) deletion of autophagy/mitophagy genes exacerbated \textit{crd1}Δ growth defects, 2) GFP-tagged mitochondria were not detected in the vacuole of \textit{crd1}Δ in response to mitophagy induction, and 3) in \textit{crd1}Δ, there was decreased level of free GFP, which is cleaved from Idh1-GFP after the mitochondria are delivered to the vacuole for degradation in response to mitophagy induction.

The finding of decreased mitophagy seemed to contradict my preliminary finding that deletion of \textit{UTH1} rescued both vacuole defects and temperature sensitivity in \textit{crd1}Δ. \textit{UTH1} was first linked to mitophagy by the finding that the \textit{uth1}Δ mutant was resistant to rapamycin, the inducer of autophagy (Camougrand et al., 2003). This was further supported by the report of Kissova et al. (Kissová et al., 2004), which showed loss of colocalization of mitochondria and vacuole in \textit{uth1}Δ after rapamycin treatment. However, Western analysis of degradation of mitochondria as determined by free GFP was not carried out in this study. More recently, Uth1p was found on the mitochondrial inner membrane, not in the outer membrane as previously predicted, and is dispensable for post-log-phase and rapamycin-induced mitophagy (Welter et al., 2013). Based on this recent finding, rescue of \textit{crd1}Δ by deletion of \textit{UTH1} is unrelated to mitophagy but more likely due to rescue of cell wall biogenesis defects, as CL mutants exhibit defects in cell wall biogenesis and in maintenance of cell integrity (Zhong et al.,
Interestingly, compared to deletion of the mitophagy specific gene, deletion of general autophagy genes that are required in both nonselective autophagy and mitophagy exacerbated crd1Δ growth defects to a more severe extent. As seen in Fig. 2.3 A, crd1Δ is synthetically lethal with atg8Δ and atg18Δ. In contrast, crd1Δ is synthetically sick with atg32Δ, as seen in Fig. 2.3 B. The explanation for this may be that CL is specifically involved in mitophagy, not nonselective autophagy. Thus, nonselective autophagy may function normally in crd1Δ, and damaged mitochondria may be delivered into vacuole for degradation as cargo in the nonselective autophagosome. If nonselective autophagy is also blocked by deletion of ATG8 or ATG18, cells will not be able to get rid of damaged mitochondria, resulting in cell death. If mitophagy is further impaired by deletion of ATG32, damaged mitochondria may be eliminated by nonselective autophagy, resulting in a sick but not lethal phenotype.

The possibility that nonselective autophagy in crd1Δ may not be compromised may explain the greater increase in ATG8 gene expression in crd1Δ cells compared to WT as seen in Fig. 2.6. Non-optimal growth temperatures likely result in a higher rate of formation of aberrant mitochondria and respiratory-deficient petite mutants. Furthermore, yeast mitochondrial protein synthesis is more thermolabile than cytoplasmic protein synthesis (Walker, 1998). It has been reported that exposure to elevated temperature induces mitochondrial swelling (Ma et al., 2004). These factors may account for increased ATG8 expression and mitophagy at elevated temperature in WT. When CL mutants are exposed to elevated temperature, mitochondrial swelling...
may be more pronounced than in WT cells (Ma et al., 2004). Thus, it is reasonable to speculate that increased \textit{ATG8} expression in \textit{crd1}\textsuperscript{Δ} is necessary to increase mitophagy in order to eliminate damaged mitochondria. Another possibility is that \textit{crd1}\textsuperscript{Δ} cells upregulate nonselective autophagy to a greater extent than WT cells to compensate for decreased mitophagy.

In summary, studies in this chapter suggest that loss of CL may lead to decreased mitophagy, which may account at least in part for the temperature sensitivity of the \textit{crd1}\textsuperscript{Δ} mutant. Previous studies indicate that the PKC and HOG MAPK pathways are required for mitophagy (Mao et al., 2011). To understand the underlying mechanism of decreased mitophagy in \textit{crd1}\textsuperscript{Δ}, I carried out targeted genetic interaction analysis between \textit{CRD1} and the genes of the MAPK pathways, which is described in Chapter 3. The findings in this chapter provide novel insights into the cellular functions of CL outside of mitochondria.
CHAPTER 3

DEFECTIVE MITOPHAGY IN crd1Δ MAY RESULT FROM DECREASED MAPK PATHWAYS

Introduction

The studies described in Chapter Two suggest that perturbation of CL synthesis leads to defective mitophagy. Mitophagy is regulated by different pathways from those that control nonselective autophagy. Nonselective autophagy is regulated by at least four signaling pathways, including the Ras/cAMP-dependent protein kinase A (PKA) (Budovskaya et al., 2004; Zhou et al., 2009), the target of rapamycin (TOR) (Li et al., 2012; Yorimitsu et al., 2007), Sch9 (Li et al., 2012; Yorimitsu et al., 2007) and Pho85 pathways. Mitophagy is specifically regulated by two MAPK pathways: the PKC and HOG pathways (Li et al., 2012; Mao et al., 2011). The studies described in this chapter were undertaken to determine if these MAPK pathways are affected by the loss of CL, and if defective mitophagy in crd1Δ is due to altered PKC and HOG pathways.

PKC is a MAPK pathway that regulates cell wall integrity in yeast. Cell wall integrity signaling is induced in response to various environmental stimuli, including heat stress, hypo-osmotic shock, mating pheromone, and agents that cause cell wall stress (Herskowitz, 1995; Levin and Errede, 1995). Wsc1p and Mid2p are plasma membrane sensors that trigger Pkc1p, which conveys the signal downstream to Bck1p, Mkk1p/ Mkk2p and Slt2p through a kinase cascade (Heinisch et al., 1999) (Fig. 3.1). In response to induction of mitophagy, Slt2p is dually phosphorylated on threonine and tyrosine residues (Mao et al., 2011). Deletion of the PKC pathway genes BCK1, MKK1/MKK2, and SLT2 leads to defective mitophagy, suggesting the involvement of
the PKC pathway in mitophagy. However, deletion of *RLM1* and *SWI4*, genes encoding the downstream effectors of phosphorylated Slt2p (pSlt2p), does not alter mitophagy in WT cells. In addition, pSlt2p remains in the cytosol during mitophagy, suggesting that, other than conveying the signal downstream, pSlt2p may directly play a role in a relatively early stage of mitophagy (Fig. 3.1). As described in Chapter 2, mitophagy is inhibited in *crd1Δ*, but the mechanism underlying the inhibition is not understood. The finding that loss of PG, the precursor of CL, leads to defective activation of the PKC pathway (Zhong et al., 2007) provides a clue to the potential mechanism underlying inhibited mitophagy in *crd1Δ*. It is necessary to investigate if CL is required for PKC pathway activation and further regulation of mitophagy.

The HOG pathway is a MAPK pathway that mediates the cellular response to osmotic stress. It consists of two stress sensing branches, SLN1 and SHO1 (Saito and Tatebayashi, 2004). While both branches are triggered by osmotic stress, only the SHO1 signaling is triggered by heat stress (Winkler et al., 2002). Sln1p and Sho1p are transmembrane osmosensors located within the plasma membrane. In response to osmotic stress, Sln1p conveys the signal downstream to Ypd1p, Ssk1p and Ssk2p/Ssk22p. In response to either osmotic or heat stress, Sho1p conveys the signal to Cdc42p, Ste20p and Ste11p/Ste50p. The MAPKKKS Ssk2p/Ssk22p and Ste11p trigger the same downstream MAPKK, Pbs2p. The MAPK Hog1p is dually phosphorylated by activated Pbs2p (Fig. 3.1). When Hog1p is phosphorylated, it translocates into the nucleus and induces transcription of up to 600 genes for cellular adaptation to stress, including the genes that function in the synthesis of glycerol.
Fig. 3.1 The PKC and HOG pathways regulate different stages of mitophagy. Revised from Mao et al. 2011.
(O’Rourke and Herskowitz, 2004). Alternatively, phosphorylated Hog1p (pHog1p) remains in the cytosol under certain conditions, including high temperature and mitophagy induction (Mao et al., 2011; Winkler et al., 2002). Deletion of HOG1, PBS2 and SSK1, leads to defective mitophagy, suggesting the involvement of the SLN1 signaling of the HOG pathway in mitophagy. However, deletion of SKO1, HOT1 and SMP1, genes encoding the downstream effectors of pHog1p, does not alter mitophagy in WT cells (Mao et al., 2011). In addition, Hog1 is phosphorylated and remains in the cytosol after induction of mitophagy, suggesting that pHog1p may directly play a role in the cytosol at a relatively late stage of mitophagy (Fig. 3.1). As described in Chapter 2, mitophagy is inhibited in crd1Δ. Thus, it is necessary to investigate if the HOG pathway is perturbed in crd1Δ.

In the studies described in this chapter, I investigated the effect of CL deficiency on the HOG and PKC pathways. This study suggests that both pathways are defective when there is a loss of CL. Interestingly, upregulation of the PKC pathway rescues mitophagy in crd1Δ, indicating that decreased mitophagy in crd1Δ may result from an impaired PKC pathway.
Methods and materials

Yeast strains and growth media

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 3.1. Yeast extract peptone dextrose (YPD) medium, synthetic complete (SC) medium, synthetic drop-out medium (SC ura<sup>-</sup>, SC leu<sup>-</sup>) and synthetic lactate (SL) medium were described in Chapter Two. YPDS medium was YPD medium supplemented with 1 M sorbitol. Sporulation medium contained potassium acetate (1%), glucose (0.05%), and essential amino acids. Solid medium contained agar (2%) for solidification.

Construction of deletion mutants

An established MAT<sup>a</sup> crd1<sup>Δ</sup> mutant with CRD1 replaced by the dominant selectable marker URA3 was used as the query strain. Single mutants that have a single HOG pathway gene deleted were obtained from the deletion collection generated in the BY4741 (MAT<sup>a</sup>) background (Research Genetics). The gene mutated in each strain was replaced by a kanamycin (geneticin) resistance marker (KanMX4). After crd1<sup>Δ</sup> was mated with each of these deletion strains, diploids were selected on SC met<sup>+</sup> lys<sup>+</sup> double drop-out medium, and sporulation was induced on sporulation medium. Double mutant meiotic progeny were selected on SC ura<sup>-</sup> drop-out medium with 200 mg/liter geneticin, as described (Tong et al., 2001). Growth of the double mutants was examined at various temperatures to identify genetic interactions between CRD1 and the HOG pathway genes.
Table 3.1 Yeast strains used in Chapter 3

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics or genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742 (WT)</td>
<td>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0</td>
<td>Euroscarf*</td>
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<tr>
<td>VGY1 (BY4742 crd1Δ)</td>
<td>Derivative of BY4742, crd1Δ::URA3</td>
<td>Gohil et al., 2005</td>
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<td>BY4742 sho1Δ</td>
<td>Derivative of BY4742, sho1Δ:: KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>VGY1sho1Δ</td>
<td>Derivative of BY4742, crd1Δ::URA3, sho1Δ::KanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>BY4742 ssk1Δ</td>
<td>Derivative of BY4742, ssk1Δ:: KanMX4</td>
<td>This study</td>
</tr>
<tr>
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<td>Derivative of BY4742, crd1Δ::URA3, ssk1Δ::KanMX4</td>
<td>This study</td>
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<tr>
<td>BY4742 ste50Δ</td>
<td>Derivative of BY4742, ste50Δ::KanMX4</td>
<td>This study</td>
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<td>VGY1ste50Δ</td>
<td>Derivative of BY4742, ste50Δ::KanMX4</td>
<td>This study</td>
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<td>BY4742 hog1Δ</td>
<td>Derivative of BY4742, hog1Δ::KAN</td>
<td>This study</td>
</tr>
<tr>
<td>VGY1hog1Δ</td>
<td>Derivative of BY4742, crd1Δ::URA3, hog1Δ::KanMX4</td>
<td>This study</td>
</tr>
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<td>Derivative of BY4742, in which IDH1 gene is chromosomally tagged with GFP</td>
<td>This study</td>
</tr>
<tr>
<td>BY4742 crd1Δ-IDH1-GFP</td>
<td>Derivative of BY4742 crd1Δ::KanMX4, in which IDH1 gene is chromosomally tagged with GFP</td>
<td>This study</td>
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<td>pYPGK18</td>
<td>2μm, LEU2</td>
<td>Vaz et al., 2003</td>
</tr>
<tr>
<td>pYPGK18-PTP2</td>
<td>Derivative of pYPGK18, expresses PTP2 from PGK1 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pYPGK18-PTP3</td>
<td>Derivative of pYPGK18, expresses PTP3 from PGK1 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pYCp50</td>
<td>CEN, URA3</td>
<td>Helliwell et al., 1998</td>
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<tr>
<td>pYCp50-PKC1^K18STOP</td>
<td>Derivative of pYCp50, expresses PKC1^K18STOP from PGK1 promoter</td>
<td>Helliwell et al., 1998</td>
</tr>
<tr>
<td>pRS352</td>
<td>2μm, URA3</td>
<td>Helliwell et al., 1998</td>
</tr>
<tr>
<td>pRS352-BCK1-20</td>
<td>Derivative of pRS352, expresses BCK1-20 from PGK1 promoter</td>
<td>Helliwell et al., 1998</td>
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<td>pPS1739-HOG1-GFP</td>
<td>CEN, URA3, HOG1-GFP</td>
<td>Ferrigno et al., 1998</td>
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Plasmid construction

To construct the *PTP2* overexpression plasmid, a sequence of 2250 base pairs that contains the entire open reading frame of *PTP2* was amplified from yeast genomic DNA using KpnI-tagged forward primer *PTP2fr-f* (5’-GG GTACCATTGATGGATCGCATAGCACAG-3’) and XbaI-tagged reverse primer *PTP2fr-r* (5’- CGC TCTAGA TTAACAAGGTAACGCGTTTATC -3’). After being cut by the restriction enzyme KpnI and XbaI, the PCR product was ligated downstream of the *PGK1* promoter on the pYPGK18 (2um, *LEU2*) plasmid (Fig 3.2).

To construct the *PTP3* overexpression plasmid, a sequence of 2751 base pairs that contains the entire open reading frame of *PTP3* was amplified from yeast genomic DNA using EcoRI-tagged forward primer *PTP3fr-f* (5’-CCGGAATTGGACATGAAGGACAGTGTAGACTGC-3’) and BamHI-tagged reverse primer *PTP3fr-r* (5’-CGCGGATCCGCTAATATTGCGGAATTCTTTC-3’). After being cut by the restriction enzyme KpnI and XbaI, the PCR product was ligated downstream of the *PGK1* promoter on the pYPGK18 (2um, *LEU2*) plasmid (Fig 3.2).

Single colony formation and spotting

Single colony formation experiments were performed as described in Chapter Two.

Spotting experiments were performed as follows. Cells were pre-cultured in SC drop-out liquid medium at 30°C to the mid-log phase. The $A_{550}$ of the cells was determined using a spectrometer (Beckman). Cell aliquots were centrifuged and pellets were resuspended in SC leu− medium adjusted to an $A_{550}$ of 10 units per ml. A
Fig. 3.2 Construction of the pYPGK18-PTP2 and pYPGK18-PTP3 overexpression plasmids. The detailed procedure was described in the ‘Methods and materials’ section.
100X diluted sample was added onto a hemocytometer for cell counting using light microscopy. Based on the cell counting result, cell aliquots were adjusted to $2 \times 10^8$ cells/ml. The cell aliquots were then diluted in a 10X serial dilution. Cells were spotted on SC drop-out plates with the most diluted spot containing 2000 cells and the plates were incubated at experimental temperatures for 2 days. The appearance of visible growth at various temperatures was evaluated.

**Fluorescence and microscopic analysis**

Microscopic analysis was performed using an Olympus BX41 epifluorescence microscope. Images were captured by an Olympus Q-Color3 digital charge-coupled camera operated by QCapture2 software. All pictures were taken at 1000x magnification.

The method used to detect mitophagy by monitoring the translocation of GFP-tagged mitochondria was described in Chapter Two. The same procedure was performed in BY4742 WT-\textit{IDH1-GFP} and \textit{crd1A-IDH1-GFP} strains that were transformed with either pYcp50 empty vector or pYcp50::\textit{PKC1}^{R398}, which overexpresses a constitutively activated Pkc1p.

To visualize the translocation of phosphorylated Hog1p, WT and \textit{crd1A} cells in the BY4742 background were transformed with the pPS1739 plasmid, which expresses \textit{HOG1-GFP} (kindly provided by Dr. Pamela Silver, Harvard Medical School). Transformants were cultured in SC ura' liquid medium and grown to an $A_{550}$ of 1.0. The cells were treated with different conditions (39°C or 0.5 M NaCl) and observed using
epifluorescence microscopy using a green fluorescence filter. Translocation of phosphorylated Hog1p was determined by the accumulation of GFP inside the nucleus.

**Western blot**

Western blot analysis to detect Idh1-GFP and cleaved free GFP was described in Chapter Two.

To detect Slt2p and pSlt2p by Western blot, whole cell extracts were prepared as previously described (Hoppins et al., 2011) with minor changes. Mid-log phase cells were diluted to an $A_{550}$ of 0.3 and grown at 30°C or 39°C. After culture at different temperatures for 2 hours, cells at an $A_{550}$ of 1 were collected, washed with 1 ml ice-cold sterile water and resuspended in 1 ml 0.255 M NaOH BME buffer, which consisted of 0.255 M NaOH, 1% β-mercaptoethanol, and 0.86 ml sterile distilled water. The samples were incubated for 10 min on ice and 138 μl 72% TCA were added. The samples were incubated on ice for another 10 min and then pelleted by centrifugation at 4°C, 15000 rpm for 10 min. The pellets were washed once with 500 μl of ice-cold acetone, spun down at 4°C, 15000 rpm for 5 min and then air-dried at room temperature. The air-dried cell pellets were resuspended in 50 μl MURB buffer, which consisted of 100 mM MES (PH 7.0), 1% SDS, 3 M urea, 10% β–ME and 35 μl ddH2O. 9 μl of each sample were mixed with 3 μl 4X loading dye (80% bromophenol blue and 20% β-mercaptoethanol) and loaded into a 10% SDS-polyacrylamide gel for electrophoresis. The transferring and blotting steps were described in Chapter Two.
The primary and secondary antibodies used to recognize dually phosphorylated pSlt2p and total Slt2p are listed in Table 3.2.

To detect Hog1p and pHog1p by Western blot, whole cell extracts were prepared as described (Li et al., 2012). The primary and secondary antibodies used to recognize phosphorylated pHog1p and total Hog1p are listed in Table 3.2.
Table 3.2 Antibodies used in determining MAPK pathway activation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSlt2p</td>
<td>anti-phospho-p44/42 MAPK (Thr202/Tyr204) mouse IgG (Cell signaling)</td>
<td>HRP-conjugated goat anti-mouse IgG (Santa Cruz)</td>
<td>2 bands ~ 55 kDa</td>
</tr>
<tr>
<td>Slt2p</td>
<td>anti-Slt2p goat IgG (Santa Cruz)</td>
<td>HRP-conjugated donkey anti-goat IgG (Santa Cruz)</td>
<td>1 band ~ 55 kDa</td>
</tr>
<tr>
<td>pHog1p</td>
<td>anti-phospho-p38 rabbit IgG (antibody 3D7; Cell Signaling)</td>
<td>HRP-conjugated goat anti-rabbit IgG (Santa Cruz)</td>
<td>1 band ~ 49 kDa</td>
</tr>
<tr>
<td>Hog1p</td>
<td>anti-Hog1p goat IgG (antibody yC-20, Santa Cruz)</td>
<td>HRP-conjugated donkey anti-goat IgG (Santa Cruz)</td>
<td>1 band ~ 49 kDa</td>
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</table>
Results

Down-regulation of the HOG pathway exacerbates the crd1Δ growth defect.

In Chapter Two, I reported that, under mitophagy inducing conditions, crd1Δ exhibited decreased mitophagy compared to WT. The HOG pathway is an upstream regulator of mitophagy. Deletion of the HOG pathway genes HOG1 and PBS2 leads to defective mitophagy (Mao et al., 2011). pHog1p appears to stay in the cytosol to play a role in a relatively late stage of mitophagy. Thus, I investigated the possibility that the HOG pathway is perturbed in crd1Δ.

For this purpose, I determined if there is genetic interaction between CRD1 and HOG pathway genes. The yeast deletion collection facilitated construction of double mutants (Tong et al., 2001). crd1Δ was mated with sho1Δ, ssk1Δ, ste50Δ and hog1Δ mutants. Tetrads were obtained subsequent to sporulation, and haploids were acquired via tetrad dissection. Haploids containing mutations in both CRD1 and one of the four HOG pathway genes were selected by uracil prototrophy (crd1Δ mutation) and geneticin resistance (HOG pathway mutation). crd1Δ in the BY4742 background cannot form colonies from single cells plated on YPD at 40°C, which is a permissive temperature for WT (Jiang et al., 1999; Zhong et al., 2004). At 38°C and 39°C, crd1Δ cells form smaller colonies than WT. Based on this growth phenotype, genetic interaction was assessed at elevated temperature. Growth of double mutants at 40°C was considered rescue of the crd1Δ growth defect, while inability to grow at 38°C or 39°C indicated exacerbation of the crd1Δ growth defect.

While crd1Δ cells formed colonies at 39°C, crd1Δssk1Δ did not. crd1Δste50Δ,
crd1Δsho1Δ, crd1Δhog1Δ did not form colonies even at 38˚C (Fig. 3.3), suggesting that mutants of SSK1, STE50, SHO1, and HOG1 genetically interact with crd1Δ. To determine if down-regulation of the HOG pathway leads to exacerbation of the crd1Δ growth defect, PTP2 and PTP3, which negatively regulate the HOG pathway, were overexpressed in crd1Δ. Serial diluted crd1Δ cells containing empty vector or overexpressing PTP2 or PTP3 were spotted on leu- plates and incubated at 30˚C - 38˚C. The growth defect of crd1Δ was exacerbated by overexpression of PTP2 and PTP3 (Fig. 3.4). These data suggest that downregulation of the HOG pathway leads to exacerbation of the crd1Δ growth defect, and the HOG pathway may be perturbed in crd1Δ.

Loss of CL leads to decreased Hog1p phosphorylation without perturbing activated Hog1p translocation

Downregulation of the HOG pathway leads to exacerbation of the crd1Δ growth defect, suggesting that the HOG pathway may be altered in crd1Δ cells. To determine if Hog1p phosphorylation is perturbed in crd1Δ, I induced Hog1p activation with 0.5 M NaCl for 5 min, and analyzed Hog1p phosphorylation by Western blot, using a monoclonal antibody against dually phosphorylated p38, as described (Li et al., 2012; Zhou et al., 2009). Compared to WT cells, Hog1p activation was decreased in crd1Δ in response to osmotic stress (Fig. 3.5 A). To determine if translocation of activated Hog1p was perturbed in crd1Δ, WT and crd1Δ cells were transformed with the pPS1739 plasmid containing HOG1-GFP. The cells were cultured in SC ura- liquid
Fig. 3.3 Deletion of HOG pathway genes is synthetically lethal with \textit{CRD1} deletion. Cells were pre-cultured in liquid YPD at 30°C to the mid-log phase. 200 cells of each strain were plated on YPD plates and incubated at 38°C or 39°C for 3 days.
Fig. 3.4 Overexpression of the negative regulators of the HOG pathway is synthetically lethal with CRD1 deletion. Cells were precultured in liquid SC leu- at 30°C to the mid-log phase. Cell aliquots were adjusted to 2x10⁶ cells/ml and then diluted in a 10X serial dilution. Cells were spotted on SC leu- plates with the most diluted spot containing 2000 cells, and the plates were incubated at 30°C, 36°C, 37°C or 38°C for 2 days.
medium to an $A_{590}$ of 1 and treated with 0.5 M NaCl for 5 min. Fluorescence microscopy showed no difference between WT and $crd1\Delta$ in the translocation of activated Hog1p after NaCl treatment (Fig. 3.5 B).

**Heat stress does not activate Hog1p in WT and does not rescue decreased Hog1p phosphorylation in $crd1\Delta$**

Both branches of the HOG pathway are triggered by activation of the corresponding membrane-bound sensors (Sho1p or Sln1p) in response to osmotic stress. A previous publication has shown that heat stress triggers the HOG pathway via the SHO1 but not the SLN1 signaling (Winkler A, 2002). Surprisingly, Hog1p phosphorylation was not observed in WT or $crd1\Delta$ in response to heat stress (Fig. 3.6 A). The translocation of Hog1-GFP in response to heat stress was also not observed. WT and $crd1\Delta$ cells containing the pPS1739 plasmid were pre-cultured in liquid YPD at 30°C to the mid-log phase, switched to 39°C for 10 min and observed using epifluorescence microscopy. Consistent with the Western blot result, the translocation of Hog1-GFP into the nucleus was not observed in either WT or $crd1\Delta$ (Fig. 3.6 B), suggesting that heat stress does not trigger Hog1p activation or translocation in the BY4742 background.

**Upregulation of the PKC pathway rescues the $crd1\Delta$ growth defect**

As mentioned above, a previous study in yeast demonstrated that activation of the PKC pathway is decreased in $pgs1\Delta$ (Zhong et al., 2007). Deletion of $PGS1$ causes loss of both PG and CL. Thus, I investigated if there is decreased activation of PKC
Fig. 3.5 (A) Decreased Hog1p phosphorylation in the crd1Δ mutant. Cells were pre-cultured in liquid YPD at 30˚C to the mid-log phase and treated with 0.5 M NaCl for 5 min. Dually phosphorylated Hog1p and total Hog1p protein were detected by Western blot, as described previously (Zhou et al., 2009). (B) Translocation of activated Hog1p is not affected in crd1Δ. WT and crd1Δ were transformed with the plasmid pPS1739, from which Hog1p fused to GFP was expressed. Cells were grown in SC ura− to an A550 of 1, treated with 0.5 M NaCl for 5 min and then observed using epifluorescence microscopy.
Fig. 3.6 (A) Heat stress does not trigger phosphorylation in the BY4742 background. Cells were pre-cultured in YPD at 30°C to the mid-log phase and switched to 39°C for 5 min. Dually phosphorylated Hog1p and total Hog1p proteins were detected by Western blot, as described previously (Zhou et al., 2009).

(B) Heat stress does not induce the translocation of GFP tagged Hog1p into the nucleus in the BY4742 background. WT and crd1Δ were transformed with the plasmid pPS1739, on which HOG1 is fused to GFP. Cells were grown in SC ura- to an A550 of 1, switched to 39°C, and observed using epifluorescence microscopy.
pathway in \textit{crd1Δ}, which has PG but not CL. For this purpose, I examined the activation state (dual phosphorylation) of Slt2p in response to elevated temperature in isogenic WT, \textit{crd1Δ} and \textit{pgs1Δ} cells in the BY4742 background by Western blot. Because \textit{pgs1Δ} in the FGY background requires sorbitol for survival at even 30°C, to exclude any influence caused by sorbitol, I also determined the Slt2p phosphorylation in \textit{pgs1Δ} in this background, supplemented with 1 M sorbitol. Cells were pre-cultured in YPD at 30°C to the mid-log phase, diluted to an \textit{A}_{550} of 0.3, and grown at 30°C or 39°C for 2 hours. Whole cell extracts were prepared, and dually phosphorylated Slt2p and total Slt2p proteins were detected by Western blot, as described in the ‘Methods and materials’ section. Dual phosphorylation of Slt2p in WT was detected after the cells were cultured for 2 hours at 39°C. Slt2p dual phosphorylation was decreased in \textit{crd1Δ} and almost absent in \textit{pgs1Δ} and \textit{pgs1Δ} supplemented with sorbitol, which indicates decreased PKC pathway activation in the CL mutants (Fig. 3.7 A). To upregulate the PKC pathway, \textit{crd1Δ} was transformed with a vector expressing constitutively activated Bck1 (\textit{BCK1-20}) or Pkc1 (\textit{PKC}^{R398P}). Consistent with the Western blot result, spotting experiments demonstrated that \textit{crd1Δ} temperature sensitivity was rescued by plasmids expressing constitutively activated Bck1 or Pkc1 (Fig. 3.7 B), suggesting that upregulation of the PKC pathway rescues the \textit{crd1Δ} growth defect.

\textbf{Upregulation of the PKC pathway rescues defective mitophagy in \textit{crd1Δ}}

As mentioned above, activation of the PKC and HOG pathways are required for
**Fig 3.7 (A)** The PKC pathway is decreased in crd1Δ. Cells were pre-cultured in liquid YPD at 30°C to the mid-log phase, diluted to an A_650_ of 0.3, and treated with increased temperature or sorbitol for 2 hours. Whole cell extracts were prepared and dually phosphorylated Slt2p and total Slt2p protein were detected by Western blot. **(B)** Upregulation of the PKC pathway rescues the crd1Δ growth defect. Cells containing overexpression plasmids that upregulate the PKC pathway (BCK1-20 and PKC1^{R398}) were cultured in SC ura- medium at 30°C to the mid-log phase. Cells were diluted, spotted on SC ura- plates, and incubated at 30°C or 38°C for 2 days.
mitophagy (Mao et al., 2011). Therefore, I investigated a possible causal relationship between decreased MAPK pathways and inhibited mitophagy in crd1Δ.

Upregulation of the PKC pathway was achieved in WT-IDH1-GFP and crd1Δ-IDH1-GFP strains in the BY4742 background by transfecting the cells with plasmid carrying the PCK1<sup>R398</sup> gene, which encodes a constitutively activated Pkc1p. WT-IDH1-GFP and crd1Δ-IDH1-GFP cells containing either empty vector or vector expressing PCK1<sup>R398</sup> were pre-cultured in liquid SC ura<sup>-</sup> medium, harvested, washed twice, and resuspended in SL medium at a starting A<sub>550</sub> of 0.5. After 18 hours, cells were observed using epifluorescence microscopy. As predicted, accumulation of GFP was observed in the vacuole of WT-IDH1-GFP cells containing either the empty vector or vector expressing PCK1<sup>R398P</sup>. GFP accumulation was not observed in crd1Δ-IDH1-GFP cells containing the empty vector. Interestingly, accumulation of GFP was observed in crd1Δ-IDH1-GFP cells containing the vector that expresses the PCK1<sup>R398P</sup> gene, suggesting that mitochondria were delivered into the vacuole of this strain (Fig. 3.8).

The release of free GFP, a byproduct of mitophagy, was determined by Western blot of WT-IDH1-GFP and crd1Δ-IDH1-GFP strains containing either an empty vector or a vector expressing PCK1<sup>R398</sup>. Cells were cultured in liquid SC ura<sup>-</sup> medium to the mid-log phase at 30°C, harvested, washed twice and resuspended in SL medium at a starting A<sub>550</sub> of 0.5. After 6 hours, proteins were extracted as described previously. Consistent with the finding of the microscopic examination experiment, the Western blot showed a strong free GFP band in WT cells containing either the empty vector or
Fig. 3.8 Upregulation of the PKC pathway rescues the delivery of mitochondria into vacuole in *crd1Δ* after induction of mitophagy.

WT-IDH1-GFP and *crd1Δ*-IDH1-GFP cells containing either an empty vector or a vector expressing *PKC1*<sub>R398P</sub> were cultured in SC ura<sup>-</sup> medium to the mid-log phase at 30°C, washed and shifted to SL for 18 hours. Cells were then observed with fluorescence microscopy.
the vector expressing $PKC1^{R398P}$, but only a very faint free GFP band in $crd1\Delta-IDH1$-GFP containing the empty vector, indicating that mitophagy is defective in $crd1\Delta$ cells. However, a strong free GFP band that is comparable to that detected in WT-ev and WT-PKC strains was observed in $crd1\Delta-IDH1$-GFP cells containing the vector that expresses $PKC1^{R398P}$, suggesting that PKC rescued mitophagy in this strain (Fig. 3.9).

The HOG pathway stimulants rescues the $crd1\Delta$ growth defect

A constitutively activated HOG pathway causes lethality, and cannot be used to study mitophagy in $crd1\Delta$. Although it is difficult to test if there is direct causal relationship between a decreased HOG pathway and defective mitophagy in $crd1\Delta$, indirect evidence suggests that upregulating the HOG pathway may rescue the $crd1\Delta$ growth defect. A previous study demonstrated that the addition of 1 M sorbitol, a HOG pathway stimulant, rescued $crd1\Delta$ growth and vacuole defects (Chen et al., 2008b). Our present study established that 200 mM NaCl, another HOG pathway activator, similarly rescued $crd1\Delta$ temperature sensitivity (Fig. 3.10). However, more direct evidence is needed to conclude that a decreased HOG pathway inhibits mitophagy in $crd1\Delta$. 
Fig. 3.9 Upregulation of the PKC pathway rescues mitophagy in *crd1Δ*. WT-IDH1-GFP and *crd1Δ-IDH1-GFP* cells containing either an empty vector or a vector expressing *PKC1^{R398P}* were cultured in SC ura' medium to the mid-log phase at 30°C, washed and shifted to SL. Samples were taken before shift to SL medium and at 6 hours post-shift. Immunoblotting was done with an anti-GFP antibody and the positions of full-length Idh1-GFP and free GFP are as indicated.
**Fig. 3.10** 200 mM NaCl rescues *crd1Δ* temperature sensitivity. WT and *crd1Δ* cells in the FGY background were pre-cultured in YPD liquid at 30°C to the mid-log phase. 200 cells were plated on a YPD plate with or without 200 mM NaCl. Plates were then incubated at 37.5°C for 3 days.


Discussion

In this chapter, I addressed the hypothesis that loss of CL leads to perturbation of the HOG and PKC pathways, which causes defective mitophagy in \textit{crd1}\(\Delta\). Consistent with this hypothesis, the \textit{crd1}\(\Delta\) mutant exhibited decreased phosphorylation of Hog1p in the presence of osmotic stress and decreased phosphorylation of Slt2p in the presence of heat stress, suggesting defective activation of both the HOG and PKC pathways in \textit{crd1}\(\Delta\). As expected, \textit{pgs1}\(\Delta\) treated with heat stress also exhibited largely decreased phosphorylation of Slt2p. I demonstrated that upregulation of the PKC pathway rescued the growth defect in \textit{crd1}\(\Delta\), as well as the accumulation of GFP labeled mitochondria and cleaved GFP in the vacuole of \textit{crd1}\(\Delta\)-\textit{IDH1-GFP}. This suggests that upregulation of the PKC pathway rescued mitophagy in \textit{crd1}\(\Delta\), supporting the hypothesis that defective mitophagy in \textit{crd1}\(\Delta\) may be due to defective activation of the PKC pathway.

Based on the previous findings that \textit{crd1}\(\Delta\) exhibits defective activation of the HOG pathway, which is required for mitophagy, upregulation of this pathway may rescue mitophagy in \textit{crd1}\(\Delta\). However, constitutive activation of the HOG pathway causes cell lethality. It was previously shown that 1 M sorbitol, a HOG pathway stimulant, rescues the \textit{crd1}\(\Delta\) growth and vacuole defects (Chen et al., 2008b). In addition, I found that 200 mM NaCl, which also stimulates the HOG pathway, rescued \textit{crd1}\(\Delta\) temperature sensitivity (Fig. 3.8). These findings provide a clue that upregulating the HOG pathway may rescue the \textit{crd1}\(\Delta\) growth defect. However, sorbitol and NaCl also regulate cellular ion homeostasis, which may account for rescue of \textit{crd1}\(\Delta\) defects.
Although ion homeostasis may be perturbed in \textit{crd1}\textDelta, it would not be the sole cause of the growth defect, because upregulation of the PKC pathway, which does not affect ion regulation, rescued the \textit{crd1}\textDelta growth defect.

As mentioned in Chapter Two, there was very little free GFP detected by Western blot in whole cell extracts from \textit{crd1}\textDelta-\textit{IDH1-GFP} after induction of mitophagy. Consistent with this, the phosphorylation of Slt2p and Hog1p upon induction by heat or osmotic stress was decreased but not fully blocked. Slt2p and Hog1p phosphorylation was detectable after stimulation, which might explain the faint band of free GFP detected in \textit{crd1}\textDelta-\textit{IDH1-GFP} cells 7 hours after mitophagy induction (Fig. 2.8). If the HOG pathway is fully blocked, the late stage of mitophagy may be disrupted. Upregulation of the PKC pathway, which regulates the early stage of mitophagy, may not rescue mitophagy in \textit{crd1}\textDelta under these conditions.

Because \textit{crd1}\textDelta is synthetically lethal with mutants of SLN1 and SHO1 branches, decreased activation of both branches may occur in \textit{crd1}\textDelta. To address this possibility, Hog1p activation in double mutants with deletion of \textit{CRD1} and a gene from either branch should be compared to that of \textit{crd1}\textDelta. For example, if \textit{sho1}\textDelta exacerbates the Hog1p activation defect in \textit{crd1}\textDelta, this would suggest that CL may be required for activation of SLN1 signaling.

Translocation of pHog1p into the nucleus was not affected in \textit{crd1}\textDelta cells. Surprisingly, elevated temperature did not induce phosphorylation or the translocation of Hog1p into the nucleus. This conflicts with the finding of Winkler, who reported that heat stress induced Hog1p activation (Winkler et al., 2002), but it may be explained by
the fact that our studies were performed using strains from different backgrounds. Heat
activation of the HOG pathway is likely to be strain dependent.

In summary, the studies described in this chapter suggest that the loss of CL may
lead to decreased PKC and HOG pathways, and that a defective PKC pathway may
account for impaired mitophagy in the \textit{crd1}\Delta mutant. The findings in this chapter
provide novel insights into the cellular function of CL. It is tempting to speculate that
upregulation of the PKC pathway may be a potential treatment strategy for BTHS
patients.
CHAPTER 4

FUTURE DIRECTIONS

The studies described in this thesis provide novel insights into the cellular functions of CL beyond the confines of mitochondria, including mitophagy and the PKC and HOG pathways. While my studies showed that loss of CL leads to defective mitophagy, which may be due to decreased function of MAPK pathways, much remains to be unveiled. To elucidate the mechanism linking defective mitophagy and MAPK pathways in CL-deficient cells, as well as the mechanism underlying the role of CL in vacuolar morphology and acidification, future studies should address the following questions:

1. **Does nonselective autophagy function normally in crd1Δ?**

   In Chapter 2, I showed that CL deficiency leads to decreased levels of mitochondria and cleaved GFP in the vacuole, suggesting defective mitophagy in crd1Δ cells. However, deletion of ATG8 or ATG18, the general autophagy genes required for both nonselective autophagy and mitophagy, exacerbated the crd1Δ growth defect to a greater extent than deletion of the mitophagy specific gene, ATG32, (Fig. 2.3). I hypothesize that CL is specifically required for mitophagy, not for nonselective autophagy. Accordingly, if nonselective autophagy is unaffected in crd1Δ, damaged mitochondria can be enclosed in nonselective autophagosomes and delivered to the vacuole for degradation. However, if nonselective autophagy in crd1Δ
is blocked by deletion of ATG8 or ATG18, the cell will not be able to remove damaged mitochondria. This would result in cell death, consistent with synthetic lethality of crd1Δ and atg8Δ. This hypothesis is further supported by the finding that expression of ATG8 in crd1Δ is two-fold higher than in WT at 39°C (Fig. 2.6), suggesting that nonselective autophagy is upregulated in crd1Δ.

If nonselective autophagy is functional in crd1Δ cells, stimulation by rapamycin might be expected to rescue crd1Δ growth defects. To test this possibility, rapamycin was added to YPD plates and temperature sensitivity of crd1Δ and WT in the FGY background was examined at 36.5°C and 37°C. WT cells were unable to grow at elevated temperature in the presence of rapamycin concentrations greater than 1 nM. 0.01-1 nM rapamycin did permit growth of WT cells, while 0.5 nM rapamycin induced a slight rescue of crd1Δ temperature sensitivity at elevated temperature (data not shown). Future experiments are necessary to explain why only 0.5 nM rapamycin rescued crd1Δ growth defects. Rapamycin is an antifungal antibiotic, which inhibits the target of rapamycin complex 1 (TOR1). In addition to negatively regulating autophagy and transcription of stress responsive genes, TOR1 positively regulates ribosome biogenesis, global translation initiation, and nutrient import. Thus, the effect of rapamycin is like a double-edged sword: it increases autophagy while inhibiting ribosome biogenesis, global translation initiation, and nutrient import, resulting in cell lethality. With additional growth defects, crd1Δ may be more sensitive to rapamycin than WT. The toxicity of 0.5-1 nM rapamycin may overwhelm the benefits of increased nonselective autophagy induced by these concentrations in crd1Δ. crd1Δ may tolerate
rapamycin less than 0.5 nM. However, levels of nonselective autophagy triggered by these concentrations may be inadequate to remove the majority of damaged mitochondria. 0.5 nM rapamycin may be an optimal concentration that can trigger enough nonselective autophagy to remove the majority of the damaged mitochondria.

To definitively ascertain if nonselective autophagy is functional in CL deficient cells, direct monitoring of nonselective autophagy in *crd1*Δ is required. An engineered protein Pho8Δ60 has been designed for this purpose (Noda and Klionsky, 2008). Pho8Δ60 lacks the N-terminal transmembrane domain of Pho8 (alkaline phosphatase) that targets the endoplasmic reticulum; thus, the mutated protein remains in the cytosol and is only delivered to the vacuole through nonspecific autophagy. After entering the vacuole lumen, the alkaline phosphatase activity of Pho8Δ60 is proteolytically activated. Therefore, enzyme activity of Pho8Δ60 reflects the level of nonspecific autophagy. To determine if nonselective autophagy is upregulated to compensate for defective mitophagy in *crd1*Δ cells, the Pho8Δ60 assay should be performed.

2. **Which branch of the HOG pathway is defective in *crd1*Δ?**

   As described in Chapter 3, loss of CL leads to decreased Hog1p phosphorylation without perturbing activated Hog1p translocation. However, it is not known if decreased Hog1p phosphorylation is due to perturbation of the SHO1 or SLN1 branch, or perhaps to both.

   If loss of CL leads to defective SHO1 signaling, downregulating SLN1 signaling
will exacerbate decreased Hog1p activation in \( crd1 \Delta \). In this situation, with 0.5 M NaCl, \( crd1 \Delta ssk1 \Delta \) (\( SSK1 \) is required for SLN1 signaling) should have less Hog1p activation than \( crd1 \Delta \), while \( crd1 \Delta sho1 \Delta \) should have comparable Hog1p activation with \( crd1 \Delta \).

If loss of CL leads to defective SLN1 signaling, downregulating SHO1 signaling will exacerbate the decreased Hog1p activation in \( crd1 \Delta \). In this situation, with 0.5 M NaCl, \( crd1 \Delta sho1 \Delta \) should have less Hog1p activation than \( crd1 \Delta \), while \( crd1 \Delta ssk1 \Delta \) should have comparable Hog1p activation with \( crd1 \Delta \). However, if CL is required for activation of both branches, then the Hog1p activation in both \( crd1 \Delta sho1 \Delta \) and \( crd1 \Delta ssk1 \Delta \) will be less than that of \( crd1 \Delta \).

In summary, comparing Hog1p activation in \( crd1 \Delta \), \( crd1 \Delta sho1 \Delta \) and \( crd1 \Delta ssk1 \Delta \) by Western blot should help to identify which HOG branch is defective in \( crd1 \Delta \).

3. **How does sorbitol rescue \( crd1 \Delta \) growth and vacuole defects?**

A previous study in our lab showed that 1 M sorbitol restores both growth and vacuole defects in \( crd1 \Delta \) by a mechanism not yet identified (Chen et al., 2008b). As shown in Fig. 4.1, 1 M sorbitol upregulates the HOG pathway at 39°C, which might be the mechanism underlying rescue of \( crd1 \Delta \) temperature sensitivity and vacuole defects by sorbitol. However, the possibility that 1 M sorbitol restores the defects of \( crd1 \Delta \) via stimulating other osmotic regulatory pathways is not excluded. To address this question, my previous labmate Shuliang Chen and I carried out a targeted screen of osmotic regulatory mutants to identify those that abrogated sorbitol-mediated rescue of \( crd1 \Delta \). Double mutants of \( CRD1 \) and the genes shown in Table 4.1 were constructed.
Fig. 4.1 1 M sorbitol induces Hog1p activation in crd1Δ at elevated temperature. Cells were pre-cultured in YPD at 30˚C to mid-log phase and switched to 39˚C for 5 min and at the same time treated with either 0.5 M NaCl (positive control) or 1 M sorbitol. The dually phosphorylated Hog1p and total Hog1p proteins were detected by Western blot, as described previously (Zhou et al., 2009).
Failure of sorbitol to rescue the double mutant indicated that the query gene is required for sorbitol-mediated rescue of \textit{crd1Δ}.

A search of the \textit{Saccharomyces} Genome Database identified 57 nonessential genes that are involved in the HOG or other osmotic stress pathways (Table 4.1). A targeted synthetic genetic array (SGA) was performed as described (Zhou et al., 2009). Single mutants of the stress response genes were mated with \textit{crd1Δ}, diploids were sporulated, and progeny containing mutations in both \textit{CRD1} and each of the query genes were selected by geneticin resistance (mutation of the query gene) and uracil prototrophy (\textit{CRD1} mutation). Double mutants were confirmed by PCR.

Four of the double mutants were tested, including \textit{crd1Δanp1Δ}, \textit{crd1Δptc7Δ}, \textit{crd1Δosm1Δ} and \textit{crd1Δdog2Δ}. All were rescued by 1 M sorbitol at elevated temperature (Fig.4.2). This indicates that the corresponding genes are not required for sorbitol rescue. 53 double mutants remain to be tested. The screen for double mutants that cannot grow on YPD or YPD supplemented with 1 M sorbitol at elevated temperature is an interesting direction to pursue in future studies.

4. \textbf{What is the mechanism underlying the vacuole defects in \textit{crd1Δ} ?}

A previous study in our lab found that, at elevated temperature, CL mutants exhibit enlarged vacuoles and loss of vacuole acidification (Chen et al., 2008b). The observed decrease in V-ATPase activity and proton pumping in \textit{crd1Δ} may explain the loss of acidification of the vacuole. However, the mechanism underlying the enlarged vacuole morphology remains unknown. The following studies aimed to address the
Table 4.1. Deletion strains used in the mini SGA

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Fig. 4.2 Deletion of ANP1, PTC7, OSM1 or DOG2 does not abrogate the rescue of crd1Δ temperature sensitivity by sorbitol. Cells were pre-cultured in liquid YPD at 30°C to the mid-log phase. Approximately 200 cells of each strain were plated on YPD or YPDS plates and incubated at 39.5°C for 3 days.
potential mechanism of vacuole morphology defects in *crd1Δ*.

### 4.1 Increased nonselective autophagy may not be the cause of vacuole enlargement

As described in Chapter 2, elevated temperature triggers increased expression of the general autophagy gene, *ATG8*, in both *crd1Δ* and WT (Fig. 2.6). In addition, at 39°C, *ATG8* expression is two-fold greater in *crd1Δ* than in WT. Although mitophagy is defective in *crd1Δ*, nonselective autophagy may not be affected, as discussed in Chapter 2. Because autophagy is the source of a large influx of lipid membrane and cellular contents to the vacuole (Baba M et al., 1994; Baba et al., 1995; Takeshige K et al., 1992), it is reasonable to speculate that increased nonselective autophagy may be the cause of vacuole enlargement in *crd1Δ*.

If enlargement of the vacuole in *crd1Δ* at elevated temperature is due to increased nonselective autophagy, deletion of the nonselective autophagy genes should rescue the vacuole morphology defect of *crd1Δ*. To address this possibility, the effect of deleting nonselective autophagy genes on vacuole morphology of *crd1Δ* cells was determined. As seen in Fig. 4.3, *crd1Δatg8Δ* exhibited enlarged vacuoles similar to *crd1Δ*, indicating that blocking nonselective autophagy does not rescue the vacuole enlargement triggered by elevated temperature in *crd1Δ*.

In addition, if increased nonselective autophagy causes vacuole enlargement, triggering nonselective autophagy should lead to enlarged vacuoles in WT and *crd1Δ* at optimal temperature. To address this hypothesis, rapamycin, a stimulant of
Fig. 4.3 Deletion of ATG8 does not rescue the vacuole enlargement triggered by elevated temperature in crd1Δ. Cells were pre-cultured at 30 °C to the early log phase and then transferred to 37°C at a starting A550 of 0.5 for 8h. FM4-64 staining was performed and cells were observed using fluorescence microscopy for vacuole morphology.
nonselective autophagy, was used to trigger nonselective autophagy in WT and crd1Δ at 30°C. FM 4-64 staining was performed to observe vacuole morphology. As seen in Fig. 4.4, both crd1Δ and WT exhibited enlarged vacuoles, raising the possibility that nonselective autophagy triggered by rapamycin results in enlarged vacuoles in even WT. However, rapamycin has downstream effects other than autophagy. If the vacuole enlargement triggered by rapamycin in crd1Δ and WT was due to increased nonselective autophagy, but not to other downstream effects of rapamycin, deletion of the nonselective autophagy genes in these strains should rescue vacuole morphology. However, with rapamycin, atg8Δ and crd1Δatg8Δ exhibited enlarged vacuoles similar to that in crd1Δ and WT, indicating that blocking autophagy does not rescue vacuole enlargement triggered by rapamycin. Thus, the vacuole enlargement induced by rapamycin is probably not due to increased nonselective autophagy. In addition to triggering nonselective autophagy via the TOR1 pathway (Li et al., 2012; Yorimitsu et al., 2007), rapamycin may be involved in other cellular processes that regulate vacuole size.

4.2 Is the vacuole defect caused by perturbation of the FAB pathway in crd1Δ?

The FAB pathway regulates the synthesis of phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), which affects the size and acidification of the vacuole. Interestingly, the fab1Δ mutant, in which PI(3,5)P₂ is undetectable, displays growth and vacuole defects that are similar to those in crd1Δ, including decreased growth at elevated temperature, vacuole enlargement and loss of
Fig. 4.4 Rapamycin triggers enlargement of vacuoles in both WT and crd1Δ, which is not rescued by deletion of ATG8. Cells were pre-cultured at 30 °C to the early log phase and then transferred to medium containing 10nM rapamycin at a starting A550 of 0.5 for 8h. FM4-64 staining was performed and cells were observed using fluorescence microscopy for vacuole morphology.
vacuole acidification (Bonangelino et al., 2002; Gary et al., 1998; Yamamoto et al., 1995). Thus, Chen et al. suggested that perturbation of the FAB pathway in CL deficient cells may explain the vacuole defects. Chen determined that mRNA levels of the FAB pathway genes were not affected in crd1Δ cells (Chen et al., 2008b). However, FAB1 is not transcriptionally regulated (Gary et al., 1998). Therefore, non-transcriptional regulation of the FAB pathway may be defective in crd1Δ. Further studies are necessary to address this possibility.

4.2.1 The FAB pathway

As shown in Fig. 4.5, the first step of the FAB pathway is the synthesis of PI3P from PI, catalyzed by phosphatidylinositol (PI) 3-kinase, Vps34p (Auger et al., 1989; Slessareva et al., 2006). Fab1p, which localizes to the vacuole membrane, catalyzes the second step, converting vacuolar PI3P to PI(3,5)P2 (Dove et al., 2002; Gary et al., 1998; Yamamoto et al., 1995). The reverse reaction to dephosphorylate PI(3,5)P2 to PI3P is catalyzed by PI(3,5)P2 phosphatase, Fig4p (Rudge et al., 2004), which is also needed for maximum function of Fab1p (Duex et al., 2006). Fab1p can be activated by Vac7p and Vac14p (Bonangelino et al., 1997; Duex et al., 2006; Gary et al., 2002), the latter of which is physically associated with Fig4p (Duex et al., 2006). Atg18p negatively regulates Vac7p (Efe et al., 2005). This regulatory pathway controls levels of PI(3,5)P2, which affect vacuole size and acidification. It is known that hyperosmotic stress leads to increased PI(3,5)P2, resulting in shrunken and fragmented vacuoles (Bonangelino et al., 2002; Dove et al., 1997), while loss of PI(3,5)P2 by deletion of
Fig. 4.5 The FAB pathway and its regulation. Revised from Efe et al., 2005.
4.2.2 Is the FAB pathway perturbed in *crd1Δ*?

To determine if there is perturbation of the FAB pathway in *crd1Δ*, I assessed genetic interaction between *crd1Δ* and *fig4Δ*, because Fig4p is essential for both synthesis and turn over of PI(3,5)P₂. If the FAB pathway in *crd1Δ* is perturbed, no matter whether there is altered synthesis of PI(3,5)P₂, or altered turn-over of PI(3,5)P₂, I should see genetic interaction between *FIG4* and *CRD1*. The ability of single cells to form colonies was observed, using our lab generated *crd1Δfig4Δ*, *fig4Δ*, *crd1Δ* and WT strains in the FGY background. As shown in Fig. 4.6, deletion of *FIG4* partially rescued the *crd1Δ* growth defect at 37.5°C, suggesting that the FAB pathway may be perturbed in *crd1Δ*. Levels of PI(3,5)P₂ may be decreased, and deletion of *FIG4* may rescue the growth defects of *crd1Δ* by blocking the dephosphorylation of PI(3,5)P₂.

4.2.3 PI3P and PI(3,5)P₂ are localized normally on the vacuole membrane of *crd1Δ*.

The findings described in section 4.2.2 provided a clue that the growth and vacuole defects in *crd1Δ* may be due to perturbation of the FAB pathway. Thus, it was necessary to determine if PI3P and PI(3,5)P₂ on the vacuole membrane of *crd1Δ* are decreased.

To directly observe if there is loss of PI3P or PI(3,5)P₂ on the vacuole membrane, both *crd1Δ* and WT cells were transformed with the pRS415-GFP-FYVE and pRS415-GFP-ATG18 plasmids (kindly provided by Rania Deranieh), while *fab1Δ* containing the
Fig. 4.6 Deletion of *FIG4* partially rescues *crd1Δ* temperature sensitivity. Cells were pre-cultured in liquid YPD at 30°C to the mid-log phase. Approximately 200 cells of each strain were plated on YPD plates and incubated at 37.5°C for 3 days.
pRS415-GFP-ATG18 plasmid (kindly provided by Rania Deranieh) was used as a negative control. The pRS415-GFP-FYVE plasmid expresses a protein in which GFP is fused to the FYVE zinc finger domain. Binding of the FYVE domain of this probe to PI3P enables the monitoring of PI3P intracellular localization. The pRS415-GFP-ATG18 plasmid expresses a PI(3,5)P2 specific fluorescent lipid-associated reporter (FLARE), on which GFP is fused to the PI(3,5)P2 binding domain of Atg18p. At elevated temperature, PI3P and PI(3,5)P2 vacuolar localization in crd1Δ was similar to that of WT (Fig 4.7). In contrast, there is a loss of PI(3,5)P2 on the vacuole membrane in fab1Δ (Fig 4.8), suggesting that loss of vacuolar PI(3,5)P2 may be the cause of the vacuole defects in fab1Δ but not crd1Δ. However, normal PI3P and PI(3,5)P2 vacuole localization does not exclude the possibility that PI(3,5)P2 levels are slightly decreased in crd1Δ, which would not be discernible by comparison of fluorescence intensity. Analysis of PI3P and PI(3,5)P2 levels by HPLC would be required to conclusively show altered FAB pathway function.

4.2.4 Are protein levels of the FAB pathway regulators altered in crd1Δ?

Although overexpression of FAB1 did not increase PI(3,5)P2 levels (Gary et al., 1998), overexpression of FAB1 suppressed the vacuole defects in vac14Δ (Dove et al., 2002). This finding suggests that, other than regulating synthesis of PI(3,5)P2, Fab1p also directly controls vacuole size and acidification by a mechanism still unknown. Thus, the protein levels of the FAB pathway regulators are also important for vacuole morphology and acidification. Although Chen showed that the mRNA levels of FAB1,
Fig. 4.7 *crd1Δ* exhibits normal PI3P localization at the vacuolar membrane. Cells were pre-cultured at 30 °C to the early log phase and then transferred to 37°C at a starting A550 of 0.5 for 8h. PI3P localization was then observed using fluorescence microscopy.
Fig. 4.8 Normal vacuolar localization of PI(3,5)P₂ in crd1Δ. Cells were pre-cultured at 30°C to the early log phase and then transferred to 37°C at a starting A₅₅₀ of 0.5 for 8h. Cells were then observed using fluorescence microscopy to determine PI(3,5)P₂ localization.
VAC7, VAC14, VPS15, VPS34, FIG4, and ATG18 were not affected in crd1Δ cells, the protein levels were not examined (Chen et al., 2008b).

The optimal way to address this question is to detect the protein levels of these regulators by Western blot. However, primary antibodies against these proteins are not commercially available. Thus, I employed an indirect approach to address this question. If overexpression of FAB1 or VAC7 suppresses the vacuole defects in crd1Δ, it suggests that Fab1p or Vac7p protein levels may be decreased in the mutant, which may account for the vacuole defects. crd1Δ in the BY4742 background was transformed with vectors expressing either FAB1 or VAC7 (or empty pYPGK18 vector as a negative control), plated on leu- plates, and incubated at 30°C or 38°C. The ability of single cells to form colonies was observed. Overexpression of FAB1 and VAC7 in crd1Δ did not rescue the growth defects of crd1Δ (Fig 4.9), indicating that Fab1p and Vac7p protein levels may be unaltered in crd1Δ. However, it is possible that there is targeted degradation of the FAB1 and VAC7 mRNA in crd1Δ, resulting in not only degradation of endogenous FAB1 and VAC7 mRNA, but also FAB1 and VAC7 mRNA that is expressed from the overexpression vector.

To definitively ascertain if protein levels of FAB pathway regulators are altered in CL deficient cells, Western blot detections of these proteins are required. Because the primary antibodies against these proteins are not commercially available, an alternative approach is to tag each FAB pathway regulator with the hemagglutinin (HA) tag to enable the detection of these proteins by Western blot.
Fig. 4.9 Overexpression of FAB1 and VAC7 does not rescue crd1Δ temperature sensitivity. Cells were pre-cultured in liquid SC leu· at 30°C to the mid-log phase. Approximately 200 cells of each strain were plated on SC leu· plates and incubated at 38°C for 3 days.
4.3 Are the vacuole defects in \textit{crd1\textDelta} caused by perturbation of the Ena1 Na$^+$-ATPase exporter?

Vacuole size and acidification are regulated by PI(3,5)P$_2$, as well as ion pumps and channels presented in the vacuole membrane. These include the H$^+$-Ca$^{2+}$ exchanger Vcx1p, the Ca$^{2+}$ pump Pmc1p, the Ca$^{2+}$ channel Yvc1p, the H$^+$-Na$^+$ exchanger Nhx1p, and the voltage-sensitive Cl$^-$ channel Gef1p (Bonilla and Cunningham, 2002; Ke et al., 2013; Li and Kane, 2009). Deletion of \textit{NHX1}, but not the genes encoding the other pumps or channels, suppresses vacuole defects and temperature sensitivity of \textit{crd1\textDelta} (Chen et al., 2008b). There is defective vacuolar V-ATPase activity and reduced proton transport in \textit{crd1\textDelta}, causing decreased intravacuolar H$^+$ levels (Chen et al., 2008b). Deletion of \textit{NHX1} blocks the vacuolar efflux of H$^+$ and influx of Na$^+$, which may counteract the decreased vacuolar H$^+$ level. However, the mechanism whereby deletion of \textit{NHX1} rescues the vacuole morphology remains to be elucidated.

Because deletion of \textit{NHX1} blocks the influx of Na$^+$ into the vacuole, it is reasonable to speculate that there may be excessive Na$^+$ in the vacuole of \textit{crd1\textDelta}. Na$^+$ is sequestered in the vacuole when intracellular Na$^+$ levels are increased (Li et al., 2012). If there is excessive intra-vacuolar Na$^+$ in \textit{crd1\textDelta}, it may indicate increased intracellular Na$^+$ in this strain.

The amount of intracellular Na$^+$ in \textit{Saccharomyces cerevisiae} is tightly regulated by activity of the ion pumps and channels on the cell membrane. The cell membrane influx of Na$^+$ is mainly induced by the Na$^+$-K$^+$ transporter Trk system and the non-
specific cation channel Nsc1. Conversely, the efflux of Na⁺ is induced by the Na⁺/K⁺-ATPase Ena1p and the Na⁺, K⁺/H⁺ antiporter Nha1p (Ke et al., 2013). Although Ena1p and Nha1p are both involved in cell Na⁺ efflux, Nha1p plays a larger role in K⁺ extrusion and cell survival under acidic conditions, while Ena1p is more involved in Na⁺ extrusion and cell survival under alkaline conditions (Jung et al., 2012). In WT cells, heat shock stimulates activity of the Na⁺-K⁺ pump (the Trk system) without affecting intracellular Na⁺ levels (Boonstra et al., 1984), suggesting that the efflux of Na⁺ may be upregulated to counteract stimulation of the Na⁺-K⁺ pump at elevated temperature. Thus, Na⁺ efflux is essential for cell survival under heat stress.

4.3.1 Deletion of ENA1 does not exacerbate crd1Δ temperature sensitivity

I hypothesized that Na⁺ efflux is defective in crd1Δ at 39°C, resulting in increased intracellular Na⁺. To alleviate toxicity caused by increased intracellular Na⁺, vacuolar Na⁺ influx is increased, resulting in increased osmotic pressure and vacuole enlargement. To test my hypothesis, I investigated if there is genetic interaction between CRD1 and ENA1, which encodes the major Na⁺ efflux transporter. Synthetic lethality would suggest that Ena1p is defective in crd1Δ. A crd1Δena1Δ double mutant was constructed by tetrad dissection and examined for temperature sensitivity. Deletion of ENA1 did not exacerbate crd1Δ temperature sensitivity (Fig. 4.10), suggesting that Ena1p may not be defective in crd1Δ.

4.3.2 Upregulation of ENA1 expression may be partially impaired in crd1Δ
Fig. 4.10 Deletion of *ENA1* does not affect *crd1Δ* temperature sensitivity. Cells were pre-cultured in liquid YPD at 30°C to the mid-log phase. Aliquots were adjusted to 2x10⁸ cells/ml and then diluted in a 10X serial dilution. Cells were spotted on YPD plates with the most diluted spot containing 2000 cells, and the plates were incubated at 30°C or 39°C for 2 days.
Ena1p is regulated at the transcriptional level (Ke et al., 2013; Márquez JA and R., 1996; Platara et al., 2006). To determine if there is defective Na⁺ efflux in crd1Δ at 39°C, it is necessary to determine if ENA1 gene expression is decreased in crd1Δ compared to WT at 39°C. This would suggest that Na⁺ efflux will be decreased, leading to increased intracellular Na⁺. WT and crd1Δ cells were incubated at either 30°C or 39°C for 2 hours. ENA1 expression in these strains was determined by RT-PCR. At 39°C, ENA1 expression was upregulated in both WT and crd1Δ. However, upregulation of ENA1 expression in crd1Δ was less than in WT (Fig. 4.11), suggesting that upregulation of ENA1 expression may be partially impaired in crd1Δ at elevated temperature. Impaired ENA1 upregulation may lead to inadequate Na⁺ efflux and accumulation of intracellular Na⁺, leading to vacuole enlargement.

4.3.3 Overexpression of ENA1 does not rescue crd1Δ temperature sensitivity

As seen in Fig.4.11, there may be impaired upregulation of ENA1 in crd1Δ at 39°C. I then determined if overexpression of ENA1 rescues crd1Δ. crd1Δ was transformed with a vector (pYPGK18) overexpressing ENA1, spotted on the leu- plates and incubated at 30°C or 39°C. Overexpression of ENA1 did not affect crd1Δ temperature sensitivity (Fig. 4.12), indicating that the growth and vacuole defects in crd1Δ at elevated temperature are probably not due to impaired upregulation of ENA1 in crd1Δ.

The findings in Section 4.3.2 and 4.3.3 are controversial. To conclusively address if there is increased intracellular Na⁺, the total Na⁺ level in crd1Δ at 39°C should be
Fig. 4.11 Inadequate *ENA1* upregulation in *crd1Δ* compared to WT, at elevated temperature. WT and *crd1Δ* cells were cultured in YPD medium to the mid-log phase at 30°C and then incubated at either 30°C or 39°C for 2 hours. *ENA1* expression was determined by RT-PCR.
Fig. 4.12 Overexpression of *ENA1* does not rescue *crd1Δ* temperature sensitivity. Cells were precultured in liquid SC leu at 30°C to the mid-log phase. Aliquots were adjusted to 2x10⁸ cells/ml and then diluted in a 10X serial dilution. Cells were spotted on YPD plates with the most diluted spot containing 2000 cells, and the plates were incubated at 30°C or 39°C for 2 days.
directly measured by isotachophoresis (Nakamura et al., 1993), an analytical chemistry technique used for quantification of ionic analytes.

5. Summary

In this chapter, I suggest future experiments to elucidate how CL regulates mitophagy and MAPK pathways. In addition, experiments to investigate the role of CL in vacuole homeostasis were described, and future directions related to this work have been proposed. Much about CL remains to be elucidated. I encourage my junior labmates to continue to explore the mystery of how CL contributes to human life and health.
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11.


ABSTRACT

CARDIOLIPIN REGULATES MITOPHAGY THROUGH THE PKC PATHWAY

by

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Cardiolipin (CL), the signature phospholipid of mitochondrial membranes, is important for cardiovascular health. Perturbation of CL metabolism is implicated in cardiovascular disease (CVD). The link between CL and CVD may be explained by the physiological roles of CL in pathways that are cardioprotective, such as autophagy/mitophagy and the mitogen-activated protein kinase (MAPK) pathways. My dissertation work focuses on elucidating how CL influences mitophagy and MAPK pathways.

\( \text{crd1}^\Delta \) was synthetically lethal/sick with the general autophagy mutants \( \text{atg8}^\Delta \), \( \text{atg18}^\Delta \) and mitophagy mutant \( \text{atg32}^\Delta \), suggesting that autophagy/mitophagy may be deficient in cells lacking CL. Microscopic examination of mitophagy revealed decreased translocation of GFP-tagged mitochondrial proteins into the vacuole of \( \text{crd1}^\Delta \) cells. This was confirmed by a decreased level of free GFP generated by
cleavage of GFP-tagged mitochondrial protein after delivery into the vacuole by
mitophagy. These findings indicated that mitophagy is decreased in CL-deficient cells.
Expression of ATG8 was increased in crd1Δ cells at 37˚C, suggesting that nonselective
autophagy was upregulated to compensate for decreased mitophagy.
The PKC and HOG MAPK pathways are known to be required for mitophagy.
crd1Δ growth defects are exacerbated by deletion of HOG pathway genes SHO1,
SSK1, STE50 and HOG1, and rescued by stimulating the HOG pathway and
upregulating the PKC pathway. These findings suggested the possibility that MAPK
pathways are defective in crd1Δ cells. Phosphorylation of Slt2p and Hog1p in response
to stimulants was decreased in crd1Δ, consistent with defective activation of these
MAPK pathways. Interestingly, upregulating PKC by transforming the cell with a vector
expressing a constitutively activated Pkc1p rescued defective mitophagy in crd1Δ.

These results suggest that the mechanism underlying defective mitophagy
caused by loss of CL is a defective PKC pathway.
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