Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in *Saccharomyces cerevisiae*

Amit S Joshi¹, Morgan N Thompson¹ ², Naomi Fei¹, Maik Hüttemann ³, and Miriam L Greenberg¹

¹Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202
³Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201
²Current address: Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

To whom correspondence should be addressed: Miriam L Greenberg, 5047 Gullen Mall Biological Sciences Bldg., Detroit, Michigan 48202. Telephone: 313-577-5202; Fax: 313-577-6891; Email: mlgreen@sun.science.wayne.edu

Key words: Cardiolipin, Phosphatidylethanolamine, Mitochondrial fusion, Barth syndrome, Membrane lipids, Yeast, Mitochondria

Background: Cells lacking both cardiolipin and mitochondrial phosphatidylethanolamine are inviable, suggesting that these lipids have overlapping functions.

Results: The loss of both lipids leads to decreased mitochondrial fusion and fragmented mitochondria.

Conclusion: One overlapping function of these lipids is in mitochondrial fusion.

Significance: Decreased mitochondrial fusion may partly explain the variation in clinical presentation observed in Barth syndrome.

ABSTRACT: The two non-bilayer forming mitochondrial phospholipids cardiolipin (CL) and phosphatidylethanolamine (PE) play crucial roles in maintaining mitochondrial morphology. We have previously shown that CL and PE have overlapping functions and the loss of both is synthetically lethal. Because the lack of CL does not lead to defects in the mitochondrial network in *Saccharomyces cerevisiae*, we hypothesized that PE may compensate for CL in the maintenance of mitochondrial morphology. To test this hypothesis, we constructed a conditional mutant *crd1Δpsd1Δ* containing null alleles of *CRD1* (CL synthase) and *PSD1* (mitochondrial phosphatidylserine decarboxylase), in which the wild type *CRD1* gene is expressed on a plasmid under control of the TET*OFF* promoter. In the presence of tetracycline, the mutant exhibited highly fragmented mitochondria, loss of mtDNA, and reduced membrane potential, characteristic of fusion mutants. Deletion of *DNM1*, required for mitochondrial fission, restored the tubular mitochondrial morphology. Loss of CL and mitochondrial PE led to reduced levels of small and large isoforms of the fusion protein Mgm1p, possibly accounting for the fusion defect. Taken together, these data demonstrate for the first time in *vivo* that CL and mitochondrial PE are required to maintain tubular mitochondrial morphology and have overlapping functions in mitochondrial fusion.

INTRODUCTION: Mitochondria exist as dynamic, double membrane-bound organelles. Mitochondrial membranes are enriched in phospholipids and proteins that are required for mitochondrial biogenesis and for maintenance of mitochondrial morphology and the tubular network (1). CL and PE are non-bilayer forming phospholipids in the mitochondrial membranes (2,3) that play an essential role in mitochondrial function. While cells lacking CL or mitochondrial PE are viable, the loss of both phospholipids is lethal, suggesting that these lipids have overlapping functions that are essential (4). Several recent studies have implicated the involvement of CL and mitochondrial PE in the maintenance of mitochondrial morphology (5-7). CL and PE are fusogenic phospholipids that form hexagonal phases in the presence of divalent cations, which
Mitochondrial phospholipids and fusion

functions in mitochondrial fusion phospholipids CL and PE have overlapping hypotheses that the mitochondrial assemble in a CL-dependent manner (20). We was shown activity of the s-Mgm1p (19,20). Moreover, it demonstrated that CL stimulates the GTPase activity of s-Mgm1p (19,20). Moreover, it was shown in vitro that s-Mgm1p and l-Mgm1p assemble in a CL-dependent manner (20). We hypothesized that the mitochondrial phospholipids CL and PE have overlapping functions in mitochondrial fusion in vivo. Consistent with this hypothesis, we demonstrate that cells lacking both CL and mitochondrial PE have reduced levels of both Mgm1p isoforms and exhibit excessive fragmentation of mitochondria and defects in mitochondrial fusion.

EXPERIMENTAL PROCEDURES:

Yeast strains, plasmids and growth media: The S. cerevisiae strains used in this study, listed in Table 1, are isogenic to BY4741 and BY4742. The single mutants were obtained from the MATα yeast deletion collection obtained from Dr. John Lopes. Double and triple mutants used in this study were obtained by tetrad dissection. Synthetic complete media contained standard concentration of amino acids, all the essential components of DIFCO vitamin-free yeast nitrogen base, 0.2% ammonium sulphate and glucose (2%). Synthetic drop-out media contained all of the aforementioned ingredients except the amino acid used as a selectable marker. Complex media contained yeast extract (1%), peptone (2%), with glucose (2%) (YPD) or galactose (2%) (YP-galactose) as carbon source. All the plasmids were amplified and extracted using standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol (21). The v5 epitope-tagged CRD1 gene was cloned into the pCM189 plasmid (ATCC), in which, the TET OFF promoter regulates the expression of cloned gene, using the BamHI and NotI restriction sites. The existing URA3 marker of the plasmid was replaced by HIS3 using EcoRV and Clal restriction sites. Bacterial transformations were performed using dam E.coli to avoid Dam methylase sensitivity to the Clal restriction enzyme.

Fluorescence microscopy: Fluorescence microscopy was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3 digitally charge-coupled device camera operated by QCapture2 software. All pictures were taken at 1,000 X. To stain mitochondrial DNA, yeast cells were cultured to the mid-log phase, fixed in 70% ethanol at room temperature for 30 min, washed two times with distilled water, and stained with 1 µg/ml DAPI (Sigma) for 5 min. Mitochondria were visualized by transforming the cells with either plasmid pYX142 or pYX122 expressing GFP fused to the mitochondrial presequence, pre Su9 (22) (provided by Dr. Benedikt Westermann) or pYX142-mtRFP expressing mitochondria targeted RFP (provided by Dr. Janet Shaw). Cells were harvested in the appropriate medium and viewed under fluorescence microscopy.

Electron microscopy: Cells were grown in 100 ml YPD to an A550 of 0.5. After harvesting, cells were prepared for EM using the osmium thiocarbohydrazide osmium fixation method (23).

In vivo fusion assay: The mitochondrial in vivo fusion assay was performed as described (13,24). MATα cells of WT, crd1Δ, and psd1Δ, were transformed with pYX122-mtGFP and MATα cells were transformed with pYX122-mtRFP. MATα cells of the conditional mutant crd1Δpsd1Δ were transformed with pYX142-mtRFP, and MATα cells with pYX142-mtGFP. MATα cells of the conditional mutant crd1Δpsd1Δfis1Δ were transformed with pYX142-mtGFP, and MATα with pYX142-mtRFP. MATα cells of crd1Δpsd1Δfis1Δ were transformed with pYX142-mtGFP and MATα with pYX142-mtRFP. Cells were grown in 5 ml selective media to an A550 of 0.5. After centrifugation, cells of opposite mating type were mixed and spotted on an YPD plate. After 3.5 hours of incubation at 30 C, cells were
observed for mitochondrial fusion. The images were merged and analyzed using Image J software.

**Extraction, separation and analysis of yeast total phospholipids:** Yeast cells were grown in the presence of $^{32}$P, (10 µCi/ml) in the indicated growth conditions. Total phospholipids were extracted and analyzed by TLC as described (25). The developed chromatograms were analyzed by phosphorimaging and the phospholipids were quantified using Image Quant software.

**Flow cytometry:** Mitochondrial membrane potential was measured using whole cells as described (26). Cells were grown in YP-galactose media to the mid-logarithmic phase. Actively growing cells ($5 \times 10^4$ cells) were incubated at 30°C with the dye tetramethyl rhodamine methyl ester (TMRM) (50nM) for 30 mins. To induce a decrease in membrane potential, control cells were treated with sodium azide (20 mM). Fluorescence was measured using a flow cytometer. The results were analyzed using WinMDI2.9 software.

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

**RESULTS:**

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

Previous studies have shown that loss of CL (crd1Δ) is lethal in combination with loss of mitochondrial PE (psd1Δ), but not cytosolic PE (psd2Δ) (4). To gain insight into the overlapping roles of these mitochondrial lipids, we constructed a conditional mutant, crd1Δpsd1Δ, in which CRD1 is expressed from a plasmid under the control of the TET$^{OFF}$ promoter. This mutant lacks mitochondrial PE and CL in the presence of tetracycline, but contains CL in the absence of tetracycline. We used this conditional mutant as a tool to identify functions of these phospholipids in mitochondrial morphology and mitochondrial fusion. The conditional double mutant grew normally on YPD. The addition of tetracycline (200 µg/ml), which shut off CRD1 expression, inhibited growth of the double mutant but did not affect growth of WT, crd1Δ, or psd1Δ cells (Fig. 1A). To determine if tetracycline did indeed regulate CRD1 expression, we measured the levels of CL in crd1Δpsd1Δ cells. In psd1Δ, CL was synthesized, although levels were reduced compared to those of WT, consistent with previous studies (4). In crd1Δpsd1Δ grown in the absence of tetracycline, CL levels were 40% of those of psd1Δ, indicating that CL levels from plasmid CRD1 are less than CL levels obtained from genomic CRD1. In the presence of tetracycline, CL was greatly diminished to only 14% of the levels in psd1Δ, indicating that expression from the TET$^{OFF}$ promoter was greatly (but not completely) repressed. Tetracycline itself did not affect CL levels in cells lacking the plasmid, which were similar in psd1Δ cells grown in the presence and absence of the drug (Fig. 1B).

To determine if CL and mitochondrial PE play a role in the maintenance of mitochondrial morphology, we compared the mitochondrial network in WT, crd1Δ, psd1Δ, and crd1Δpsd1Δ cells transformed with plasmids expressing mitochondria targeted GFP (mtGFP) (22) (Fig 2A). At least 500 cells of each strain were observed for each biological replicate (Fig 2B). Cells exhibited a normal tubular mitochondrial network in crd1Δ, consistent with earlier findings (27), indicating that the lack of CL by itself does not affect the mitochondrial network. The lack of mitochondrial PE had a small but significant effect on the mitochondrial network, as ~23% of psd1Δ cells exhibited fragmented mitochondria. Unlike the WT-like tubular mitochondrial network, the majority of psd1Δ cells had short tubular mitochondria consistent with a mitochondrial morphology defect in these cells. The morphology of crd1Δpsd1Δ cells grown in the absence of tetracycline was similar to that of psd1Δ cells. However, the addition of tetracycline severely affected the mitochondrial network, leading to excessive mitochondrial fragmentation similar to that observed in fusion mutants (Fig. 2A, 2B). These findings suggested that loss of both CL and mitochondrial PE leads to a defect in mitochondrial fusion. Tetracycline
Mitochondrial phospholipids and fusion

by itself did not affect the mitochondrial network in WT, crd1Δ and psd1Δ cells (data not shown). To determine if the increase in mitochondrial fragmentation correlated with the loss of CL, a time course experiment was performed in which crd1Δpsd1Δ cells were grown in YPD containing 32P, in the presence or absence of tetracycline. Total phospholipids and mitochondrial morphology were examined at 5, 8 and 11 hrs. Total CL decreased by ~11%, ~31%, and ~55% while the percentage of mitochondrial fragmentation increased during this time to ~20%, ~45% and ~96% at 5, 8 and 11 hrs, respectively (Fig. 2C, 2D). These findings indicate that the increase in mitochondrial fragmentation corresponded with a decrease in CL in the crd1Δpsd1Δ cells.

Electron microscopic examination of the mutants revealed that crd1Δ mitochondria were somewhat smaller than those of WT, but relatively unremarkable (data not shown). Mitochondria in psd1Δ cells and in crd1Δpsd1Δ cells grown in the absence of tetracycline also appeared smaller than WT. This phenotype was more significant in crd1Δpsd1Δ cells grown in the presence of tetracycline. Thus, the loss of both CL and mitochondrial PE led to highly fragmented mitochondria, consistent with defective fusion (Fig. 2F).

To determine the role of CL and mitochondrial PE in mitochondrial fusion, we performed an in vivo fusion assay (13,24) as described in “Experimental Procedures.” In this assay, we examined mitochondrial fusion events in zygotes acquired by mating haploids of opposite mating types of WT, crd1Δ, psd1Δ, and crd1Δpsd1Δ cells transformed with either mtGFP or mitochondria tagged RFP (mtRFP). As expected, crd1Δ cells exhibited complete mixing of mitochondrial content, indicating that the lack of CL alone does not affect mitochondrial fusion (Fig. 3A). Fusion occurred but was decreased in psd1Δ cells, suggesting that the lack of PE causes somewhat reduced fusion even when CL is present. As expected, the fusion phenotype of crd1Δpsd1Δ cells grown in the absence of tetracycline was similar to that of psd1Δ cells. However, in the presence of tetracycline, a complete block of mitochondrial fusion was observed in all the crd1Δpsd1Δ zygotes examined, consistent with the defective mitochondrial network observed in the absence of both CL and mitochondrial PE (Fig. 3A). These results indicate that when both CL and mitochondrial PE are deficient, mitochondrial fusion does not occur.

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

Several studies have reported that cells defective in mitochondrial fusion lose mitochondrial DNA (mtDNA) (10,18,24,28,29). Therefore, we hypothesized that crd1Δpsd1Δ cells would exhibit mtDNA loss. To address this possibility, WT, crd1Δ, psd1Δ, and crd1Δpsd1Δ cells were grown with or without tetracycline to the mid-logarithmic growth phase at 30°C. Cells were observed under the fluorescence microscope after DAPI staining for the presence of mtDNA (Fig 4A). As expected, the majority of crd1Δpsd1Δ cells (~80%) grown in the absence of tetracycline at the permissive temperature of 30°C retained the mtDNA. This was consistent with our previous study showing that crd1Δ cells retained mtDNA at 30°C but exhibited mtDNA loss only at elevated temperatures (30). However, in the presence of tetracycline, only ~20% of crd1Δpsd1Δ cells had mtDNA (Fig. 4B).

Mitochondrial fusion as determined by in vitro assay involves distinct steps of outer and inner membrane fusion (31). In addition to functional protein complexes, fusion of the outer membrane requires low GTP levels and a proton gradient, while inner membrane fusion requires large amounts of GTP and an inner membrane potential. It is therefore possible that a decreased membrane potential led to the fusion defect in crd1Δpsd1Δ cells. To test this possibility, we used a flow cytometry assay to measure mitochondrial membrane potential (ΔΨm) in intact WT, crd1Δ, psd1Δ, and crd1Δpsd1Δ cells grown with or without tetracycline (26) in YP-galactose rather than YP-glucose to ensure actively respiring mitochondria. Cells were grown at 30°C to the mid-logarithmic growth phase and then incubated with the voltage-dependent probe tetramethylrhodamine methyl ester (TMRM) (50 nM) for 30 minutes. The accumulation of TMRM in mitochondria is driven by the ΔΨm, which is determined by the difference in yellow fluorescence and forward scatter in the form of fluorescence peaks (26). Values were calculated relative to the control, i.e., crd1Δpsd1Δ cells grown in the absence of tetracycline. As seen in Fig. 4C, crd1Δpsd1Δ cells in the presence of tetracycline exhibited a
decrease in membrane potential similar to that observed in these cells in the presence of sodium azide, a cytochrome c oxidase inhibitor that reduces the $\Delta \Psi m$ (26). These observations were consistent with a reduced membrane potential in cells lacking both CL and mitochondrial PE. It was recently demonstrated that mitochondrial fusion in mammalian cells requires high $\Delta \Psi m$ levels and is prevented by depolarization (32). Thus, the observed decrease of $\Delta \Psi m$ could be one explanation for the fusion defects in the crd1Δpsd1Δ mutant cells.

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

We wished to determine if the mitochondrial fragmentation observed in crd1Δpsd1Δ cells could be explained by increased fission rather than decreased fusion. Fusion and fission regulate mitochondrial morphology in an antagonistic manner (33). Previous studies have shown that three major proteins regulate mitochondrial fission, Dnm1p (34-36), Fis1p (36), and Mdv1p (37,38). Abolishing mitochondrial fission by deletion of any of these genes leads to net-like mitochondria. In contrast, eliminating fusion by deletion of MGM1, FZO1 or UGO1 leads to fragmentation, which can be restored to normal tubular morphology by deletion of the fission gene DNM1 (13,35). If mitochondrial fragmentation in crd1Δpsd1Δ cells results from a defect in fusion and not increased fission, then disruption of mitochondrial fission would restore mitochondria to the normal tubular morphology. Therefore, we examined if the fragmented mitochondrial morphology of crd1Δpsd1Δ cells could be rescued to normal tubular mitochondrial morphology by deletion of the fission gene DNM1. To do so, we constructed a crd1Δpsd1Δdnm1Δ conditional mutant containing the plasmid with the TETOFF regulated CRD1 expression plasmid, as well as a plasmid expressing mtGFP (Fig. 5A). In the absence of tetracycline, when CRD1 is expressed, the crd1Δpsd1Δdnm1Δ cells would be expected to exhibit net-like mitochondria characteristic of a fission defect. However, in the presence of tetracycline, the cells would be predicted to lack both fission and fusion and, hence, would exhibit WT tubular mitochondrial morphology.

As seen in Fig. 5B, ~76% of crd1Δpsd1Δdnm1Δ cells grown in the absence of tetracycline exhibited net-like mitochondria, the predicted phenotype. The remaining cells (~18%) exhibited tubular mitochondria, most likely because fusion was decreased in these cells due to the low level of expression of CRD1 (Fig 1). In the presence of tetracycline, only ~32% of cells exhibited net-like mitochondria while the majority (~45%) exhibited tubular mitochondria, as predicted. These findings suggest that both fission and fusion were defective in these cells and that the fragmented mitochondrial morphology in crd1Δpsd1Δ cells was rescued by deletion of the fission gene DNM1 (Fig. 5A, 5B). Tetracycline itself did not affect the mitochondrial morphology in dnm1Δ (data not shown). Interestingly, a significant number of crd1Δpsd1Δdnm1Δ cells (~22%) grown in the presence of tetracycline, had fragmented mitochondria, as the network exhibited the appearance of a string of beads (data not shown). This morphology suggested the presence of a persistent fusion defect in the absence of CL and mitochondrial PE.

To further investigate the block in fusion, we performed an in vivo mitochondrial fusion experiment by mating crd1Δpsd1Δdnm1Δ cells of opposite mating types, in which one mating type contained mtGFP and the other mating type contained mtRFP. We observed decreased fusion in cells grown without tetracycline, and a complete block in mitochondrial fusion in cells grown with tetracycline (Fig. 5C). Cells grown without tetracycline that exhibited net-like structures had no defect in mitochondrial fusion (Fig. 5C). Cells grown in the presence of tetracycline displayed a complete block of mitochondrial fusion. Similar observations were made in the conditional mutant crd1Δpsd1Δfis1Δ (Fig. S1). These experiments suggest that crd1Δpsd1Δdnm1Δ cells exhibited a fusion defect due to loss of CRD1 and PSD1. Taken together, these studies indicate that mitochondrial fragmentation observed in crd1Δpsd1Δ cells is a result of defective fusion and not due to increased fission.

To determine if deletion of the fission gene FIS1 or DNM1 could rescue the lethality of the double mutant, we crossed crd1Δdnm1Δ and crd1Δfis1Δ with psd1Δ and carried out meiotic tetrad analysis to identify viable triple mutants. However, triple mutants were not detected in 72 tetrads of the diploid crd1Δfis1ΔPSD1/CRD1FIS1psd1Δ or 75 tetrads of the diploid...
Therefore, while CL and mitochondrial PE have overlapping functions in mitochondrial fusion, rescue of the fusion defect could not rescue the synthetic lethality.

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

The current study suggests that one common function of CL and PE is mitochondrial fusion. It has been reported that the lack of CL destabilizes the anchoring, assembly, and GTPase activity of fusion protein Mgm1p in vitro (19,20,39). To test if mitochondrial PE compensates for the loss of CL and stabilizes the fusion proteins in vivo, we determined the steady state levels of fusion proteins Fzo1p, Ugo1p, l-Mgm1p and s-Mgm1p in WT, crd1Δ, psd1Δ, and crd1Δpsd1Δ cells. The crd1Δpsd1Δ cells exhibited significantly diminished levels of l-Mgm1p and s-Mgm1p (Fig 3B, 3C). Fzo1p levels were slightly decreased and Ugo1p was not affected (Fig. 3B, 3C). To determine if the loss of Mgm1p isoforms correlated with the loss CL in crd1Δpsd1Δ, cells were grown in the presence or absence of tetracycline, proteins were extracted from cells harvested at 5, 8 and 11 hrs, and the levels of Mgm1p isoforms were determined by Western blot (Fig 2E). The isoform levels were severely diminished at 11 hrs, which correlated with increased mitochondrial fragmentation as seen in Fig 2C. These data indicate that the defect in mitochondrial fusion in crd1Δpsd1Δ can be attributed at least in part to the reduced levels of s-Mgm1p and l-Mgm1p.

**DISCUSSION:**

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

Our previous studies have shown a synthetic lethal interaction between crd1Δ and psd1Δ mutant cells, suggesting essential overlapping roles of CL and mitochondrial PE (4). PE synthesized by the non-mitochondrial pathway (Psd2p catalyzed PE synthesis in Golgi/vacuole) (40-42) did not rescue this lethality. Externally synthesized PE is inefficiently transported to the inner mitochondrial membrane, as reduced levels of PE were observed in the inner mitochondrial membrane of the psd1Δ mutant cells (43). Taken together, these studies suggested that PE synthesized in the mitochondrial inner membrane has functions that cannot be compensated by externally synthesized PE. In the current study, we demonstrate that the loss of mitochondrial phospholipids CL and PE leads to mitochondrial fragmentation (Fig. 2A, 2B, and 2F) and defective mitochondrial fusion (Fig. 3A). Although mitochondrial fusion is an overlapping function of CL and PE, the lack of mitochondrial fusion is probably not the cause of lethality observed in crd1Δpsd1Δ cells, as lethality was not rescued by deletion of the fission gene FIS1 or DNM1. Mitochondria are required not only for cellular bioenergetics, but also for the synthesis of essential metabolites. In addition, our previous studies have shown that CL is required for non-mitochondrial functions, including vacuolar function, the high osmolarity glycerol (44) pathway, and cell wall synthesis (45-47). Thus, it is possible that lethality in cells lacking CL and PE could be caused by deficiencies in both mitochondrial and non-mitochondrial functions. The identification of suppressors of crd1Δpsd1Δ synthetic lethality will very likely identify the essential cellular functions shared by these phospholipids. These studies are currently in progress.

How do CL and mitochondrial PE affect mitochondrial fusion? Non-bilayer lipids are known to affect the function and stability of many mitochondrial membrane proteins (48). Recent studies have proposed that scaffolding proteins such as prohibitin recruit membrane proteins to CL and PE rich regions, forming protein rich lipid domains (49). The lack of CL and mitochondrial PE might influence the distribution of these domains, which in turn would affect several mitochondrial processes, including mitochondrial fusion. While early
Mitochondrial phospholipids and fusion

studies suggested that the non-bilayer forming phospholipids CL and PE play an important role in mitochondrial fusion, very little was known about the mechanism by which this could occur (8,50,51). In this study, we show that the lack of CL and mitochondrial PE leads to reduced steady state levels of both large and small isoforms of Mgm1p (Fig. 2E, 3B), which are required for fusion. Recent studies have shown that l-Mgm1p acts as an anchor in the inner membrane (17). Both CL and PE are synthesized and predominantly localized in the inner mitochondrial membrane, and the loss of both CL and mitochondrial PE might affect the stability of this isoform, leading to its degradation. The formation of s-Mgm1p requires functional mitochondrial protein import machinery, membrane potential and adequate ATP levels, all of which are defective in cells lacking CL (52-56). This is a first report describing overlapping roles of CL and mitochondrial PE in fusion in vivo, and suggests a mechanistic role for these phospholipids in regulating mitochondrial structure and function.

How is the role of CL and PE in mitochondrial fusion relevant to human disease? The role of mitochondrial phospholipids in fusion is relevant to studies that implicate function of mitochondrial fusion in cardiac function (57). Fragmented mitochondria are associated with the loss of Opa1 (the human homolog of Mgm1p) in mitochondrial myopathies involving cardiac and skeletal muscle (53) and in ischemic cardiomyopathy (58). Overexpression of the fusion proteins Fzo1/2 (human homolog of Fzo1p) prevents cardiac cell death from ischemia (59). Elucidating the role of CL and PE in mitochondrial fusion may also shed light on defects observed in lymphoblast mitochondria from patients with Barth syndrome (BTHS), a severe genetic disorder characterized by dilated cardiomyopathy and skeletal myopathy (60,61). BTHS is caused by mutation in the CL remodeling enzyme tafazzin, resulting in decreased CL and altered fatty acid composition of major mitochondrial phospholipids, including CL and PE (62). Defects in mitochondrial fusion may account for the observed morphological variation in BTHS mitochondria, including enlarged size, fragmentation, adhesion of opposing membranes and deformed intercristae space observed in BTHS lymphoblasts as well as in cardiac and skeletal muscle mitochondria of the mouse model of BTHS (63,64). Identifying the role of CL and PE in mitochondrial fusion may thus explain, in part, the wide variation in the clinical presentation observed in BTHS.

References:

1. Gohil, V. M., and Greenberg, M. L. (2009) Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand. *J Cell Biol* **184**, 469-472
2. Gonzalvez, F., and Gottlieb, E. (2007) Cardiolipin: setting the beat of apoptosis. *Apoptosis* **12**, 877-885
3. Ardail, D., Privat, J. P., Egret-Charlier, M., Levrat, C., Lerme, F., and Louisot, P. (1990) Triggering of mannosyltransferase activity in inner mitochondrial membranes by dolichyl-monophosphate incorporation mediated through phospholipids or fatty acids. *J Biol Chem* **265**, 18797-18802
4. Gohil, V. M., Thompson, M. N., and Greenberg, M. L. (2005) Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine and cardiolipin biosynthetic pathways in Saccharomyces cerevisiae. *J Biol Chem* **280**, 35410-35416
5. Tamura, Y., Endo, T., Iijima, M., and Sesaki, H. (2009) Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. *J Cell Biol* **185**, 1029-1045
6. Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., Brugger, B., Westermann, B., and Langer, T. (2009) The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. *J Cell Biol* **184**, 583-596
Mitochondrial phospholipids and fusion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements: We are grateful to Jodi Nunnari and Suzanne Hoppins for discussions and useful suggestions in this study. EM images were taken at The Integrated Imaging Center (The Johns Hopkins University). We thank Vishal Gohil, Vinay Patil, and Shuliang Chen for valuable suggestions, Icksoo Lee for help with the FACS experiment and Cunqi Ye for assistance with protein work.
FOOTNOTES

*This work was supported by grants from The National Institutes of Health (R21 HL 084218) and The Barth Syndrome Foundation to MLG, and by Wayne State University Graduate Enhancement Research Fellowship and Graduate Enhancement Research Funds to ASJ.

1To whom correspondence should be addressed: Miriam L Greenberg, 5047 Gullen Mall Biological Sciences Bldg., Detroit, Michigan 48202. Telephone: 313-577-5202; Fax: 313-577-6891; Email: mlgreen@sun.science.wayne.edu

§ The abbreviations used are: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; PDME, phosphatidyldimethylethanolamine; WT, wild type.

FIGURE LEGENDS

FIGURE 1: Tetracycline dependent growth of the conditional mutant crd1Δpsd1Δ. (A) Tenfold serial dilutions of cell suspensions were spotted on YPD plates supplemented with 200 µg/ml tetracycline (Tet) where indicated and incubated at 30 °C. (B) Cells were grown in YPD for 12 hrs in the presence or absence of tetracycline (Tet). Steady state labeling, phospholipid extraction, 1-D TLC, phosphorimaging and quantification were carried out as described in “Experimental Procedures.” CL levels are quantified as percent of total phospholipids. Mean values ± standard deviation of two independent experiments are shown.

FIGURE 2: Mitochondrial fragmentation observed in crd1Δpsd1Δ cells. (A) Mitochondria were visualized using mtGFP. Cells were grown at 30 °C to log phase in synthetic leucine deficient medium with or without 200 µg/ml tetracycline (Tet) and examined by fluorescence microscopy. Bars, 1µm. (B) Quantitation of cells containing fragmented mitochondria. Values are mean ± SE (n = 3). At least 500 cells were visualized in each experiment. (C-F) The crd1Δpsd1Δ mutant cells were grown at 30 °C in the presence or absence of 200 µg/ml tetracycline (Tet) and harvested at the indicated times. (C) CL levels were analyzed by 1-D TLC as described in “Experimental procedures” and relative levels of CL are indicated. Values are mean ± SE (n = 3). (D) Cells containing fragmented and tubular mitochondrial morphology were quantified. Values are mean ± SE (n = 3). (E) Total cell proteins were extracted and analyzed by SDS-PAGE followed by Western blot. (F) Aliquots of crd1Δpsd1Δ cells were fixed as described in “Experimental procedures” and thin sections were examined by electron microscopy. Labels m and v indicate mitochondria and vacuole (white area), respectively. Bars, 500nm.

FIGURE 3: crd1Δpsd1Δ cells exhibit defective mitochondrial fusion, loss of mitochondrial DNA and reduced membrane potential. (A) Cells of opposite mating types were transformed with either mtGFP or mtRFP. Mitochondrial fusion was examined by observing merged images of mtGFP and mtRFP in WT (a-d), crd1Δ (e-h), psd1Δ (i-l) and crd1Δpsd1Δ cells grown without (m-p) or with (q-t) tetracycline (Tet). Bars, 1µm. (B) Total cellular proteins were analyzed by SDS-PAGE followed by Western blot. Steady state levels of Mgm1p, Fzo1p and Ugo1p were measured. α-tubulin was used as a loading control. (C) Quantitation of fusion proteins. Values are mean ± SE (n = 3).

FIGURE 4: crd1Δpsd1Δ cells exhibit loss of mitochondrial DNA and reduced membrane potential. (A) Cells were grown in YP-gal to log phase at 30 °C with or without 200 µg/ml tetracycline (Tet) and stained with DAPI. Bars, 1µm. (B) Total cellular proteins were analyzed by SDS-PAGE followed by Western blot. Dissipation of the mitochondrial membrane potential demonstrated as TMRM fluorescence (%) in crd1Δpsd1Δ cells grown to log phase in YP-gal with or without tetracycline (Tet) and stained with TMRM. Cells were also treated with sodium azide as control.

FIGURE 5: crd1Δpsd1Δdnm1Δ cells are defective in mitochondrial fusion. (A) Mitochondria were visualized in the crd1Δpsd1Δdnm1Δ mutant using mtGFP. Cells were grown at 30°C to log
phase in synthetic deficient glucose medium with 200 μg/ml tetracycline (Tet) where indicated and examined by fluorescence microscopy. Bars, 1μm. (B) Cells containing tubular, fragmented and net-like mitochondria were quantified. Values are mean ± SE (n = 3). At least 100 cells were visualized in each experiment. (C) crd1Δpsd1Δdnm1Δ cells of opposite mating types were transformed with either mtGFP or mtRFP. Mitochondrial fusion was examined by observing merged images of mtGFP and mtRFP in zygotes of crd1Δpsd1Δdnm1Δ grown without (a-d) or with (e-h) tetracycline (Tet). Bars, 1μm.
**Table 1: Strains used in this study.**

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

| Strain             | YPD          | YPD + Tet   |
|--------------------|--------------|-------------|
| WT                 | ![YPD WT](image) | ![YPD + Tet WT](image) |
| crd1Δ              | ![YPD crd1Δ](image) | ![YPD + Tet crd1Δ](image) |
| psd1Δ              | ![YPD psd1Δ](image) | ![YPD + Tet psd1Δ](image) |
| crd1Δpsd1Δ         | ![YPD crd1Δpsd1Δ](image) | ![YPD + Tet crd1Δpsd1Δ](image) |

**B**

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

Fig. 1
Fig. 2

A

WT  crd1Δ  psd1Δ  -Tet  +Tet

crd1Δpsd1Δ

B

% Fragmented mitochondria

WT  crd1Δ  psd1Δ  -Tet  +Tet

crd1Δpsd1Δ
**Fig. 2**

**C**

|       | 5 hrs | 8 hrs | 11 hrs |
|-------|-------|-------|--------|
| - Tet |       |       |        |
| + Tet |       |       |        |

relative CL levels (%)

crd1Δpsd1Δ

**D**

|       | 5 hrs | 8 hrs | 11 hrs |
|-------|-------|-------|--------|
| - Tet |       |       |        |
| + Tet |       |       |        |

% Cells

- Tubular
- Fragmented

**E**

|       | 5 hrs | 8 hrs | 11 hrs |
|-------|-------|-------|--------|
| - Tet |       |       |        |
| + Tet |       |       |        |

α-Tubulin

l-Mgm1
s-Mgm1

**F**

- Tet
+ Tet

m
V

Downloaded from http://www.jbc.org/ by guest on March 17, 2020
Fig: 3

A

| Strain | crd1Δ | psd1Δ |
|--------|-------|-------|
| WT | ![a](image) | ![i](image) | ![m](image) | ![q](image) |
| crd1Δ | ![e](image) | ![j](image) | ![n](image) | ![r](image) |
| psd1Δ | ![i](image) | ![k](image) | ![o](image) | ![s](image) |
| Merge | ![l](image) | ![p](image) | ![t](image) |  |

B

| Strain | crd1Δ | psd1Δ |
|--------|-------|-------|
| WT | ![a](image) | ![l-Mgm1](image) | ![s-Mgm1](image) |
| crd1Δ | ![e](image) | ![Mgm1p](image) |  |
| psd1Δ | ![i](image) | ![Ugo1p](image) |  |
| Fzo1p | ![k](image) | ![Fzo1p](image) |  |
| α-Tubulin | ![l](image) | ![Tubulin](image) |  |

C

| Strains | Mgm1p-large | Mgm1p-small | Ugo1p | Fzo1p |
|---------|-------------|-------------|-------|-------|
| BY4742 (WT) | 1 | 1 | 1 | 1 |
| crd1Δ | 0.658 ± 0.08 | 1.115 ± 0.358 | 0.834 ± 0.039 | 0.783 ± 0.06 |
| psd1Δ | 0.639 ± 0.045 | 1.155 ± 0.231 | 0.918 ± 0.083 | 0.859 ± 0.024 |
| crd1Δpsd1Δ (- Tet) | 0.329 ± 0.06 | 0.822 ± 0.324 | 0.921 ± 0.043 | 0.513 ± 0.134 |
| crd1Δpsd1Δ (+ Tet) | 0.138 ± 0.08 | 0.344 ± 0.037 | 0.986 ± 0.182 | 0.511 ± 0.138 |
**Fig: 4**

A. 

- Tet  
+ Tet

B. 

- Tet  
+ Tet

C. 

- Tet  
+ Tet  
Na-Azide

---

**crd1Δpsd1Δ**
A

\( crd1\Delta psd1\Delta dnm1\Delta \)

-B

+Tet

\( crd1\Delta psd1\Delta dnm1\Delta \)

B

![](chart.png)

C

mtGFP

mtRFP

Merge

\( crd1\Delta psd1\Delta dnm1\Delta \)

-Tet

+Tet

Fig: 5
Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae
Amit S. Joshi, Morgan N. Thompson, Naomi Fei, Maik Hutteman and Miriam L. Greenberg

J. Biol. Chem. published online March 20, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.330167

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/03/20/M111.330167.DC1