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# Cardiolipin and Mitochondrial Phosphatidylethanolamine Have Overlapping Functions in Mitochondrial Fusion in *Saccharomyces cerevisiae**[S]

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**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.

**The two non-bilayer forming mitochondrial phospholipids cardiolipin (CL) and phosphatidylethanolamine (PE) play crucial roles in maintaining mitochondrial morphology. We have shown previously 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 tubular morphology and fusion. 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 mitochondrial DNA, and reduced membrane potential, characteristic of fusion mutants. Deletion of *DNM1*, required for mitochondrial fission, restored the tubular mitochondrial morphology. Loss of CL and mitochondrial PE led to reduced levels of small and large isoforms of the fusion protein Mgm1p, possibly accounting for the fusion defect. Taken together, these data demonstrate 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.**

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[3] and PE are non-bilayer forming phospholipids in the mitochondrial membranes (2, 3) that play an essential role in mitochondrial function. Although cells lacking CL or mitochondrial PE are viable, the loss of both phospholipids is lethal, suggesting that these lipids have overlapping functions that are essential (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 confer negative curvature to the mitochondrial membrane (8, 9). In the current study, we investigated the role of CL and PE in mitochondrial fusion.

Highly conserved protein machinery strictly regulates the process of mitochondrial fusion, and recent studies suggest that phospholipids also play a vital role in this process. Mitochondrial fusion in the yeast *Saccharomyces cerevisiae* primarily requires three proteins. These include the outer membrane GTPase, Fzo1p (Mfn1 and Mfn2 in mammals) (10, 11), the inner membrane GTPase, Mgm1p (Opa1 in mammals) (12, 13), and the outer membrane protein Ugo1p, which links the two GTPases to form a functional complex (14–16). In *S. cerevisiae*, Mgm1p exists as long (l-Mgm1p) and short isoforms (s-Mgm1p), both of which are required for mitochondrial fusion (17, 18). *In vitro* studies demonstrated that CL stimulates the GTPase activity of the 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

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[3] The abbreviations used are: CL, cardiolipin; PE, phosphatidylethanolamine; PDME, phosphatidyldimethylethanolamine; TMRM, tetramethyl rhodamine methyl ester; mtGFP, mitochondria-targeted GFP; RFP, red fluorescent protein.

**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* | Ref. 4 |
| *crd1Δ* | *MATa, his 301, leu 200, met 1500, ura 300, crd1Δ::KanMX4* | Invitrogen |
| *psd1Δ* | *MATa, his 301, leu 200, met 1500, ura 300, psd1Δ::KanMX4* | Invitrogen |
| *psd1Δ* | *MATα, his 301, leu 200, lys 200, ura 300, psd1Δ::KanMX4* | This study |
| *dnm1Δ* | *MATa, his 301, leu 200, met 1500, ura 300, dnm1Δ::KanMX4* | Invitrogen |
| *fis1Δ* | *MATa, his 301, leu 200, met 1500, ura 300, fis1Δ::KanMX4* | Invitrogen |
| *crd1Δpsd1Δ* | *MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, pCM189-CRD1* | This study |
| *crd1Δpsd1Δ* | *MATa, his 301, leu 200, lys 200, met 1500, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, pCM189-CRD1* | This study |
| *crd1Δpsd1Δfis1Δ* | *MATa, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, fis1Δ::KanMX4, pCM189-CRD1* | This study |
| *crd1Δpsd1Δfis1Δ* | *MATα, his 301, leu 200, lys 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, fis1Δ::KanMX4, pCM189-CRD1* | This study |
| *crd1Δpsd1Δdnm1Δ* | *MATa, his 301, leu 200, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, dnm1Δ::KanMX4, pCM189-CRD1* | This study |
| *crd1Δpsd1Δdnm1Δ* | *MATα, his 301, leu 200, lys 200, met 1500, ura 300, crd1Δ::URA3, psd1Δ::KanMX4, dnm1Δ::KanMX4, pCM189-CRD1* | This study |

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 *MATa* yeast deletion collection obtained from Dr. John Lopes. Double and triple mutants used in this study were obtained by tetrad dissection. Synthetic complete media contained standard concentration of amino acids, all the essential components of Difco vitamin-free yeast nitrogen base, 0.2% ammonium sulfate, and glucose (2%). Synthetic drop-out media contained all of the aforementioned ingredients except the amino acid was used as a selectable marker. Complex media contained yeast extract (1%), peptone (2%), with glucose (2%) (YPD) or galactose (2%) (YP-galactose) as a carbon source. All of 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 ClaI restriction sites. Bacterial transformations were performed using $dam^-$ *E. coli* to avoid Dam methylase sensitivity to the ClaI restriction enzyme.

*Fluorescence Microscopy*—Fluorescence microscopy was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3 digitally charge-coupled device camera operated by QCapture2 software. All pictures were taken at 1000×. 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 of YPD to an $A_{550}$ 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 *MATa* cells were transformed with pYX142-mtRFP. *MATa* cells of the conditional mutant *crd1Δpsd1Δ* were transformed with pYX142-mtRFP, and *MATα* cells were transformed with pYX142-mtGFP. *MATa* cells of the conditional mutant *crd1Δpsd1Δfis1Δ* were transformed with pYX142-mtGFP, and *MATα* with pYX142-mtRFP. *MATα* cells of *crd1psd1dnm1Δ* were transformed with pYX142-mtGFP and *MATa* with pYX142-mtRFP. Cells were grown in 5 ml of selective media to an $A_{550}$ of 0.5. After centrifugation, cells of opposite mating type were mixed and spotted on an YPD plate. After 3.5 h of incubation at 30° C, cells were observed for mitochondrial fusion. The images were merged and analyzed using Image J software.

*Extraction, Separation, and Analysis of Yeast Total Phospholipids*—Yeast cells were grown in the presence of $^{32}P_i$ (10 μCi/ml) in the indicated growth conditions. Total phospholipids were extracted and analyzed by TLC as described (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) (50 nM) for 30 min. 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 Mitochondrial Network and Mitochondrial Fusion Is Defective in Absence of CL and Mitochondrial PE*—Previous studies have shown that loss of CL (*crd1Δ*) is lethal in

| Strains | % CL |
|---|---|
| BY4742 (WT) | 100 |
| *crd1*Δ | Not detectable |
| *crd1*Δ*psd1*Δ (- Tet) | 18.5 ± 1.62 |
| *crd1*Δ*psd1*Δ (+ Tet) | 7.1 ± 0.14 |
| *psd1*Δ (- Tet) | 51.2 ± 1.13 |
| *psd1*Δ (+ Tet) | 46.6 ± 4.80 |

FIGURE 1. **Tetracycline-dependent growth of the conditional mutant *crd1*Δ*psd1*Δ.** *A*, 10-fold serial dilutions of cell suspensions were spotted on YPD plates supplemented with 200 μg/ml tetracycline (*Tet*) where indicated and incubated at 30° C. *B*, Cells were grown in YPD for 12 h in the presence or absence of tetracycline. Steady state labeling, phospholipid extraction, one-dimensional TLC, phosphorimaging, and quantification were carried out as described under "Experimental Procedures." CL levels are quantified as percent of total phospholipids. Mean values ± S.D. of two independent experiments are shown. *PC,* phosphatidylcholine; *PI,* phosphatidylinositol; *PS,* phosphatidylserine; *PG,* phosphatidylglycerol; *PA,* phosphatidic acid.

combination with loss of mitochondrial PE (*psd1*Δ), but not cytosolic PE (*psd2*Δ) (4). To gain insight into the overlapping roles of these mitochondrial lipids, we constructed a conditional mutant, *crd1*Δ*psd1*Δ, in which *CRD1* is expressed from a plasmid under the control of the $TET_{OFF}$ promoter. This mutant lacks mitochondrial PE and CL in the presence of tetracycline but contains CL in the absence of tetracycline. We used this conditional mutant as a tool to identify functions of these phospholipids in mitochondrial morphology and mitochondrial fusion. The conditional double mutant grew normally on YPD. The addition of tetracycline (200 μg/ml), which shut off *CRD1* expression, inhibited growth of the double mutant but did not affect growth of WT, *crd1*Δ, or *psd1*Δ cells (Fig. 1*A*). To determine whether tetracycline did indeed regulate *CRD1* expression, we measured the levels of CL in *crd1*Δ*psd1*Δ cells. In *psd1*Δ, CL was synthesized, although levels were reduced compared with those of WT, consistent with previous studies (4). In *crd1*Δ*psd1*Δ grown in the absence of tetracycline, CL levels were 40% of those of *psd1*Δ, indicating that CL levels from plasmid *CRD1* are less than CL levels obtained from genomic *CRD1*. In the presence of tetracycline, CL was greatly diminished to only 14% of the levels in *psd1*Δ, indicating that expression from the $TET_{OFF}$ promoter was greatly (but not completely) repressed. Tetracycline itself did not affect CL levels in cells lacking the plasmid, which were similar in *psd1*Δ cells grown in the presence and absence of the drug (Fig. 1*B*).

To determine whether CL and mitochondrial PE play a role in the maintenance of mitochondrial morphology, we compared the mitochondrial network in WT, *crd1*Δ, *psd1*Δ, and *crd1*Δ*psd1*Δ cells transformed with plasmids expressing mitochondria targeted GFP (mtGFP) (Fig. 2*A*) (22). At least 500 cells of each strain were observed for each biological replicate (Fig. 2*B*). Cells exhibited a normal tubular mitochondrial network in *crd1*Δ, 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 *psd1*Δ cells exhibited fragmented mitochondria. Unlike the WT-like tubular mitochondrial network, the majority of *psd1*Δ cells had short tubular mitochondria consistent with a mitochondrial morphology defect in these cells. The morphology of *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline was similar to that of *psd1*Δ cells. However, the addition of tetracycline severely affected the mitochondrial network, leading to excessive mitochondrial fragmentation similar to that observed in fusion mutants (Fig. 2, *A* and *B*). These findings suggested that loss of both CL and mitochondrial PE leads to a defect in mitochondrial fusion. Tetracycline by itself did not affect the mitochondrial network in WT, *crd1*Δ, and *psd1*Δ cells (data not shown). To determine whether the increase in mitochondrial fragmentation correlated with the loss of CL, a time course experiment was performed in which *crd1*Δ*psd1*Δ cells were grown in YPD containing $^{32}P_i$, in the presence or absence of tetracycline. Total phospholipids and mitochondrial morphology were examined at 5, 8, and 11 h. Total CL decreased by ~11, ~31, and ~55%, whereas the percentage of mitochondrial fragmentation increased during this time to ~20, ~45, and ~96% at 5, 8, and 11 h, respectively (Fig. 2, *C* and *D*). These findings indicate that the increase in mitochondrial fragmentation corresponded with a decrease in CL in the *crd1*Δ*psd1*Δ cells.

Electron microscopic examination of the mutants revealed that *crd1*Δ mitochondria were somewhat smaller than those of WT but relatively unremarkable (data not shown). Mitochondria in *psd1*Δ cells and in *crd1*Δ*psd1*Δ cells grown in the absence of tetracycline also appeared smaller than WT. This phenotype was more significant in *crd1*Δ*psd1*Δ cells grown in the presence of tetracycline. Thus, the loss of both CL and mitochondrial PE led to highly fragmented mitochondria, consistent with defective fusion (Fig. 2*F*).

To determine the role of CL and mitochondrial PE in mitochondrial fusion, we performed an *in vivo* fusion assay (13, 24) as described under "Experimental Procedures." In this assay, we examined mitochondrial fusion events in zygotes acquired by mating haploids of opposite mating types of WT, *crd1*Δ, *psd1*Δ,

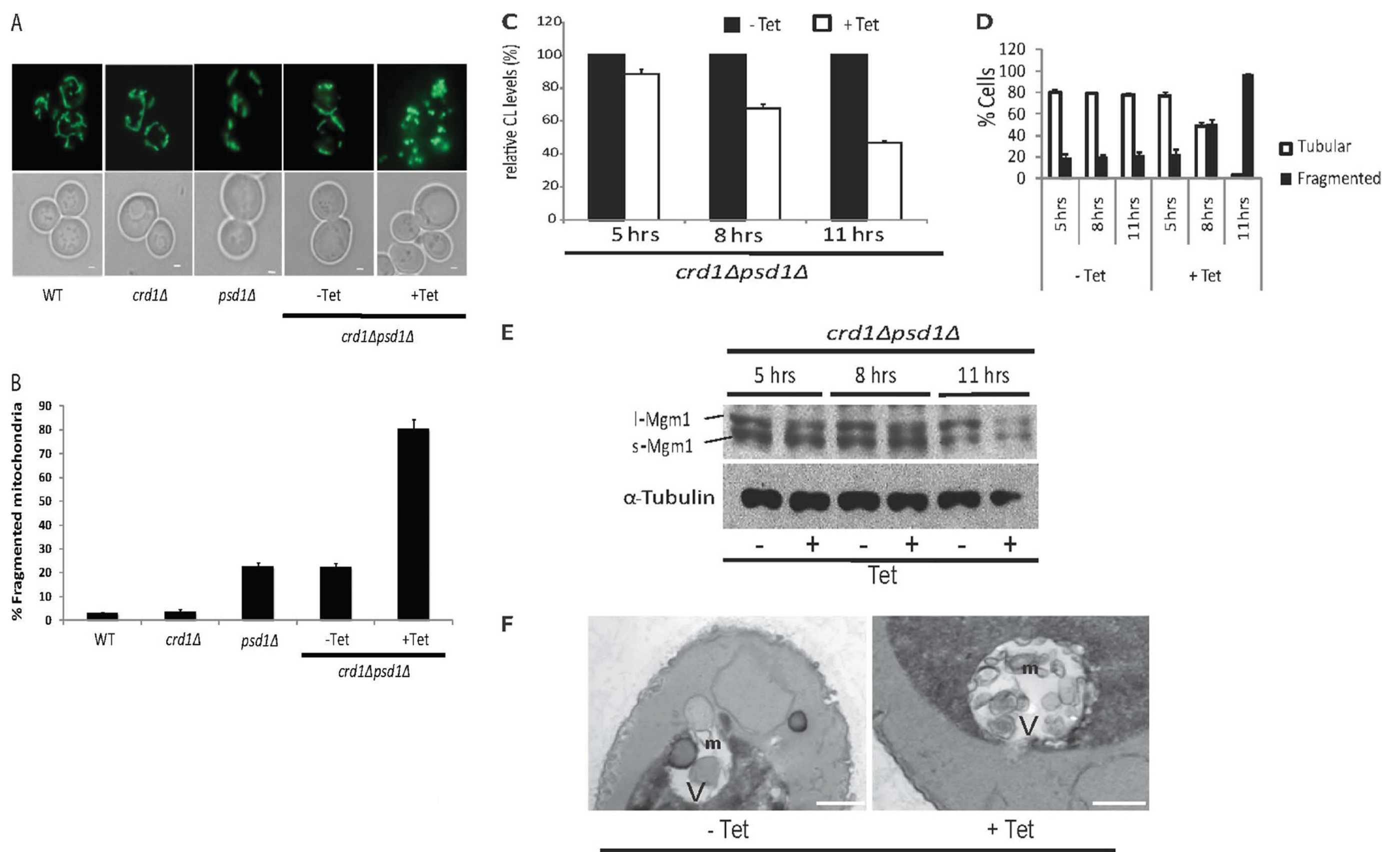


FIGURE 2. **Mitochondrial fragmentation observed in *crd1Δpsd1Δ* cells.** *A*, mitochondria were visualized using mtGFP. Cells were grown at 30° C to log phase in synthetic leucine deficient medium with or without 200 $\mu$g/ml tetracycline (*Tet*) and examined by fluorescence microscopy. *Bars*, 1 $\mu$m. *B*, quantitation of cells containing fragmented mitochondria. Values are mean ± S.E. ($n = 3$). At least 500 cells were visualized in each experiment. *C–F*, the *crd1Δpsd1Δ* mutant cells were grown at 30° C in the presence or absence of 200 $\mu$g/ml tetracycline and harvested at the indicated times. *C*, CL levels were analyzed by one-dimensional TLC as described under "Experimental Procedures," and relative levels of CL are indicated. Values are mean ± S.E. ($n = 3$). *D*, cells containing fragmented and tubular mitochondrial morphology were quantified. Values are mean ± S.E. ($n = 3$). *E*, total cell proteins were extracted and analyzed by SDS-PAGE followed by Western blot. *F*, aliquots of *crd1Δpsd1Δ* cells were fixed as described under "Experimental Procedures," and thin sections were examined by electron microscopy. Labels *m* and *V* indicate mitochondria and vacuole (*white area*), respectively. *Bars*, 500 nm.

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. 3*A*). 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. 3*A*). 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. 4*A*). As expected, the majority of *crd1Δpsd1Δ* cells (~80%) grown in the absence of tetracycline at the permissive temperature of 30° C retained 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. 4*B*).

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, whereas 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 ($\Delta\Psi_m$) 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 TMRM (50 nM) for 30 min. The accumulation of TMRM in mitochon-

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 |

FIGURE 3. ***crd1Δpsd1Δ* cells exhibit defective mitochondrial fusion.** *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 panels*), *crd1Δ* (*e–h panels*), *psd1Δ* (*i–l panels*), and *crd1Δpsd1Δ* cells grown without (*m–p panels*) or with (*q–t panels*) tetracycline (*Tet*). *Bars*, 1 μm. *B*, total cellular proteins were analyzed by SDS-PAGE followed by Western blot. Steady state levels of Mgm1p, Fzo1p, and Ugo1p were measured. α-Tubulin was used as a loading control. *C*, quantitation of fusion proteins. Values are mean ± S.E. ($n = 3$).

dria is driven by the $\Delta\Psi_m$, 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. 4*C*, *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 whether the mitochondrial fragmentation observed in crd1Δ psd1Δ 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 fis-

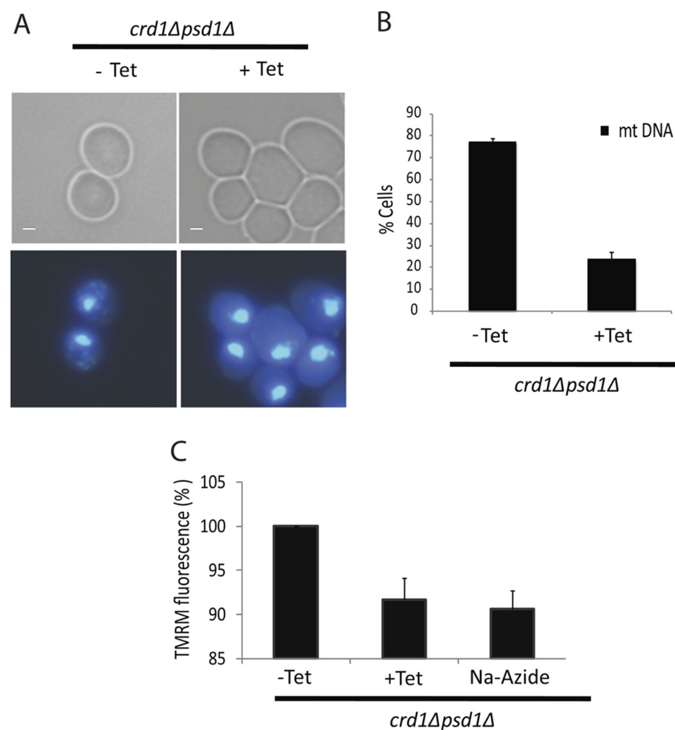


FIGURE 4. ***crd1Δpsd1Δ* cells exhibit loss of mitochondrial DNA and reduced membrane potential.** *A*, cells were grown in YP-gal to log phase at 30° C with or without 200 μg/ml tetracycline (*Tet*) and stained with DAPI. *Bars*, 1 μm. *B*, quantitation of cells containing mtDNA. Values are mean ± S.E. ($n$ = 3). At least 500 cells were visualized in each experiment. *C*, dissipation of the mitochondrial membrane potential demonstrated as TMRM fluorescence (%) in *crd1Δpsd1Δ* cells grown to log phase in YP-gal with or without tetracycline and stained with TMRM. Cells were also treated with sodium azide as a control.

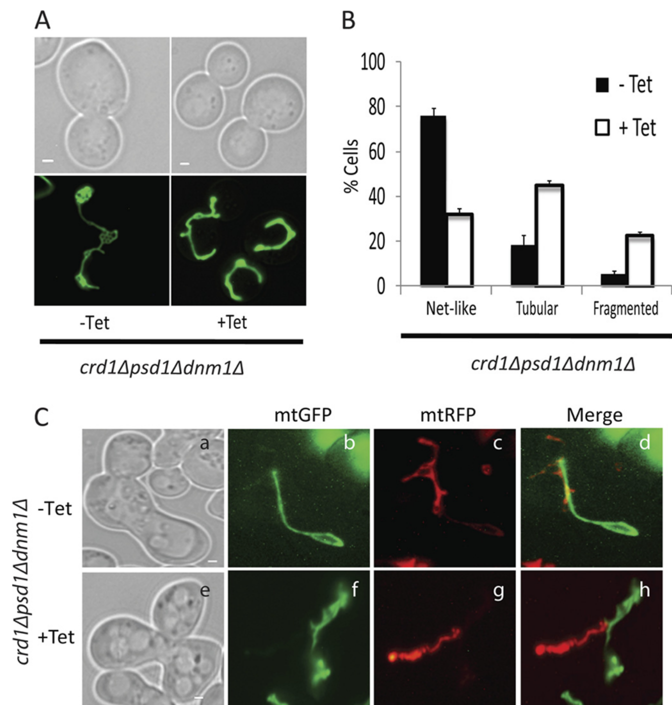


FIGURE 5. ***crd1Δpsd1Δdnm1Δ* cells are defective in mitochondrial fusion.** *A*, mitochondria were visualized in the *crd1Δpsd1Δdnm1Δ* mutant using mtGFP. Cells were grown at 30° C to log phase in synthetic deficient glucose medium with 200 μg/ml tetracycline (*Tet*) where indicated and examined by fluorescence microscopy. *Bars*, 1 μm. *B*, cells containing tubular, fragmented, and net-like mitochondria were quantified. Values are mean ± S.E. ($n$ = 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 panels*) or with (*e–h panels*) tetracycline. *Bars*, 1 μm.

sion would restore mitochondria to the normal tubular morphology. Therefore, we examined whether 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 *crd1Δ psd1Δdnm1Δ* conditional mutant containing the plasmid with the $TET_{OFF}$-regulated *CRD1* expression plasmid, as well as a plasmid expressing mtGFP (Fig. 5*A*). 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. 5*B*, ~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, whereas 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. 5, *A* and *B*). 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 *crd1Δpsd1Δ dnm1Δ* 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. 5*C*). Cells grown without tetracycline that exhibited net-like structures had no defect in mitochondrial fusion (Fig. 5*C*). Cells grown in the presence of tetracycline displayed a complete block of mitochondrial fusion. Similar observations were made in the conditional mutant *crd1Δpsd1Δfis1Δ* (supplemental 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 whether 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 *crd1Δdnm1ΔPSD1/CRD1DNM1psd1Δ*. Therefore, although 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 whether 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. 3, *B* and *C*). Fzo1p levels were slightly decreased and Ugo1p was not affected (Fig. 3, *B* and *C*). To determine whether 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 h, and the levels of Mgm1p isoforms were determined by Western blot (Fig. 2*E*). The isoform levels were severely diminished at 11 h, which correlated with increased mitochondrial fragmentation as seen in Fig. 2*C*. 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 1-Mgm1p.

## DISCUSSION

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

Our previous studies have shown a synthetic lethal interaction between *crd1Δ* and *psd1Δ* mutant cells, suggesting essential overlapping roles of CL and mitochondrial PE (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. 2, *A*, *B*, and *F*) and defective mitochondrial fusion (Fig. 3*A*). 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. Although early studies suggested that the non-bilayer forming phospholipids CL and PE play an important role in mitochondrial fusion, very little was known about the mechanism by which this could occur (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 (Figs. 2*E* and 3*B*), 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 Mfn1/2 (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.

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