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

The anionic phospholipid cardiolipin and its precursor phosphatidylglycerol are synthesized and localized in the mitochondrial inner membrane of eukaryotes. They are required for structural integrity and optimal activities of a large number of mitochondrial proteins and complexes. Previous studies showed that loss of anionic phospholipids leads to cell inviability in the absence of mitochondrial DNA. However, the mechanism linking loss of anionic phospholipids to petite lethality was unclear. To elucidate the mechanism, we constructed a crd1Δrho° mutant, which is viable and mimics phenotypes of pgs1Δ in the petite background. We found that loss of cardiolipin in rho° cells leads to elevated expression of Swe1p, a morphogenesis checkpoint protein. Moreover, the retrograde pathway is activated in crd1Δrho° cells, most likely due to the exacerbation of mitochondrial dysfunction. Interestingly, the expression of SWE1 is dependent on retrograde regulation as elevated expression of SWE1 is suppressed by deletion of RTG2 or RTG3. Taken together, these findings indicate that activation of the retrograde pathway leads to up-regulation of SWE1 in crd1Δrho° cells. These results suggest that anionic phospholipids are required for processes that are essential for normal cell division in rho° cells.

The phospholipid cardiolipin (CL) is ubiquitous in eukaryotes. It is predominantly present in the mitochondrial inner membrane, where it interacts with a large number of mitochondrial proteins (1, 2). The interactions of CL with mitochondrial membrane proteins are required for optimal protein function as dissociation of CL from these proteins results in inactivation of the complexes or decreases in protein activities (3–9).

In the yeast Saccharomyces cerevisiae, the absence of CL leads to reduced mitochondrial membrane potential, perturbation of coupling, instability of electron transport chain supercomplexes, and impaired protein import (10–15). The identification of the genes encoding the CL biosynthetic enzymes phosphatidylglycerolphosphate synthase (PGS1) (16, 17) and CL synthase (CRD1) (18–20) in yeast has enabled analyses of the functional role of CL in vivo. Mutants in CRD1, which are blocked in the final step of CL synthesis, can synthesize the precursor lipid phosphatidylglycerol (PG), the levels of which are significantly increased in the crd1Δ mutant (18, 19). Analysis of the crd1Δ mutant indicates that CL is not essential for yeast viability at optimal growth temperatures. In contrast, the pgs1Δ mutant cannot synthesize PG and CL (16). It appears that PG can substitute for CL for some mitochondrial and cellular functions as the pgs1Δ mutant exhibits a more severe growth defect than that of the crd1Δ mutant. Previous studies showed that the pgs1Δ mutant fails to maintain mtDNA without 1 M sorbitol and exhibits defective cell wall biogenesis and defective growth at 37 °C, even on non-fermentable carbon sources (21, 22). These defects suggest that CL biosynthesis plays an important role in numerous cellular processes apart from mitochondrial bioenergetics.

S. cerevisiae is a petite positive yeast that can grow without mtDNA (23). However, mutations in PGS1 lead to the petite lethal phenotype, as evidenced by the finding that the pgs1Δ mutant cannot survive ethidium bromide mutagenesis (17, 24, 25). The pgs1Δ mutant grown on glucose exhibits a spontaneous loss of mtDNA at 30 °C, which may thus contribute to the deleterious phenotypes of the mutant (21). Interestingly, disruption of CRD1 does not lead to loss of mtDNA or to petite lethality, most likely because the PG that accumulates in crd1Δ substitutes for some essential functions of CL.

Loss of mtDNA and mitochondrial dysfunction lead to a variety of cellular responses (26–28). One of the most dramatic of these is induction of mitochondrial retrograde regulation, a pathway of communication from mitochondria to the nucleus (29, 30). In this pathway, mitochondrial dysfunction is signaled to the nucleus, which responds with a wide range of changes in nuclear gene expression that may compensate for the mitochondrial defects (31). The key signaling proteins in the retrograde pathway are Rtg2p and the transcription factors Rtg1p and Rtg3p (32, 33). Rtg2p senses mitochondrial dysfunction...
and transmits this signal to the heterodimer Rtg1p/Rtg3p, which translocates from the cytoplasm to the nucleus and activates transcription of target genes, such as CIT2 and DLD3 (34, 35). The promoter regions of CIT2 and DLD3 contain an inverted repeat of an R box (GTCAC) that provides the binding sites for the Rtg1p/Rtg3p heterodimer (34, 36). The expression of CIT2 and DLD3 is dramatically increased in a strain- and carbon source-dependent manner when the retrograde response is activated (34, 35).

In addition to the mitochondrial retrograde response, loss of mitochondrial DNA in yeast is also associated with cell division defects. In a wide scale screen for mutants that produce rho+ petite cells (lacking mtDNA), Newlon et al. (37, 38) found that mutations in CDC8 and CDC21, which encode thymidylate kinase and thymidylate synthase, respectively, arrested mtDNA replication and caused a high frequency of petite formation. In contrast, a cdc28 mutant exhibited increased mitochondrial genome stability and decreased rates of spontaneous and ethidium bromide-induced petite formation (39, 40). How these genes affect mitochondrial genome stability is unknown. During cell division, mtDNA replication and mitochondrial inheritance must be precisely controlled so that mitochondria are properly transmitted to daughter cells. In this light, it would be expected that some mutations affecting mitochondria might result in a cdc (cell division cycle) mutant phenotype. Previous studies have shown that ERV1, a flavin-linked sulfhydryl oxidase of the mitochondrial intermembrane space, is essential for cell viability and for the biogenesis of functional mitochondria (41). Elimination of ERV1 leads to loss of mtDNA and cell division delay with a cdc phenotype (42). Interestingly, some genes for maintenance of mitochondrial inheritance and morphology, including MMM1, MDM1, and OLE1, showed a significant association with cell division as mutants in these genes exhibit defects in mitochondrial distribution and morphology and are unable to complete cell division (43–45).

Because mitochondrial dysfunction and loss of mtDNA are associated with cdc defects, we hypothesized that the deleterious phenotypes seen in CL mutants could be attributed to perturbation of cell division. Although the pgs1Δ mutant is petite lethal unless supplemented with sorbitol, a crd1ΔrhoΔ mutant is viable and could be used to address the hypothesis. In this study, we demonstrated that loss of mtDNA in *crd1Δ* leads to cell division delay triggered by elevated expression of Swe1p, a morphogenesis checkpoint protein. The induction of Swe1p was dependent on the retrograde pathway, which was activated in the *crd1ΔrhoΔ* mutant. This is the first demonstration that mitochondrial dysfunction leads to cell division defects that are mediated by retrograde regulation.

### Experimental Procedures

**Yeast Strains, Growth Media, and Growth Conditions**—The *S. cerevisiae* strains and plasmids used in this work are listed in Table 1. Synthetic complete medium contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), uracil (20 mg/liter), yeast nitrogen base without amino acids (Difco), and glucose (2%). Synthetic drop-out medium contained all of the above ingredients except the amino acid used as a selectable marker. Complex medium (YPD) contained yeast extract (1%), peptone (2%), and glucose (2%). YPD medium was supplemented with 1 M sorbitol. Solid medium contained agar (2%) in addition to the above mentioned ingredients.

To construct deletion mutants, the entire open reading frame of the target gene was replaced by *KanMX4* using PCR-mediated homologous recombination in the wild type strain. The *KanMX4* cassette was amplified from pUG6 using primers consisting of 50 nucleotides identical to the target gene flanking regions at the 5′ end and 21 nucleotides for the amplification of the *KanMX4* gene at the 3′ end. The PCR product was transformed into wild type cells, and transformants were selected on YPD medium containing G418 (200 μg/ml). Disruption of the target gene was confirmed by the absence of the target PCR product using primers against the target gene coding sequences.

To generate rho+ derivatives, parental rho+ cells were cultured in YPD medium containing 20 μg/ml ethidium bromide to the early stationary phase. Single cells were then plated on solid YPD and replicated to YPGE. Colonies that were inviable on YPGE and replicated to YPGE were isolated for use in this study.
with DAPI. Loss of mtDNA was confirmed by failure to complement rho− tester strains for growth on YPGE medium.

**Plasmid Construction**—To construct a SWE1-overexpressing plasmid, a 2533-bp sequence containing the entire open reading frame of SWE1 was amplified from yeast genomic DNA using SacI-tagged primer 18SWE1-f (5′-GACAGAGCTCAGATGAGTTTTGAGGAGG-3′) and BamHI-tagged primer 18SWE1-r (5′-TATTTGGGATCCATAACATGCGGCCCATAG-3′). The PCR products were ligated to pYPGK18 cut with SacI and BamHI, downstream of the PGK1 promoter.

**Phospholipid** Determination—Yeast cells were grown in the presence of [32P]Pi (10 μCi/ml) at 30 °C to the early stationary phase. Cells were then washed and digested by Zymolyase to yield spheroplasts. Total lipids were extracted from spheroplasts with chloroform:methanol (2:1) (v/v). The extracted lipids were applied to a boric acid-treated TLC plate, which was developed in the one-dimension solvent system chloroform/triethylamine/ethanol/water (30:35:35:7). Developed chromatograms were quantified by phosphorimaging (46).

**Fluorescence and Microscopic Analysis**—All microscopy was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3 digital CCD camera operated by QCapture2 software. Pictures in the same pattern were taken at the same magnification (×1000). To stain nuclear and 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.

**Visualization of Chitin Distribution** was performed by Oregon Green 488-tagged wheat germ agglutinin (WGA) staining. Yeast cells grown to the early stationary phase and washed once with PEM buffer containing 0.1 M PIPES, 5 mM EGTA, 5 mM MgCl2 (pH 6.9). To 100 μl of cell suspension, 1 μl of WGA-Oregon Green 488 (Molecular Probes) was added, and cells were incubated at room temperature for 5 min (47).

**Visualization of the Actin Cytoskeleton** was visualized using rhodamine-phalloidin (Molecular Probes), which binds specifically to polymerized actin. Yeast cells grown to the mid-log phase were harvested by centrifugation at 2000 × g and fixed in the medium with 4% formaldehyde for 10 min. The cells were resuspended in phosphate-buffered saline containing 4% formaldehyde and incubated for 60 min. Rhodamine-phalloidin (2 μl) was added to a 100-μl cell suspension according to the manufacturer’s instructions followed by incubation in the dark for 60 min. Stained cells were washed three times with phosphate-buffered saline before visualization with fluorescence microscopy (48).

**To stain mitochondria, cells grown to the mid-log phase were incubated with DiOC6(3) (Molecular Probes), a membrane potential-dependent probe, or MitoTracker Green FM (Molecular Probes), a membrane potential-independent probe, in 10 mM HEPES buffer containing 5% glucose at room temperature for 15 min. Mitochondria were observed and photographed following the procedures of the manufacturer. In living cells, mitochondria were visualized using a fusion protein consisting of the mitochondrial signal sequence of Cox4p fused to Discosoma red fluorescent protein (COX4-DsRed), which was expressed by a centromere-based plasmid (49).

To view septin localization, CDC3-GFP, CDC11-GFP, or CDC12-GFP was expressed from centromeric plasmid. Yeast cells containing these septin-GFP constructs were grown to the mid-log phase, and live cells were examined directly under the fluorescence microscope using Olympus BX41 NIB filter.

**Real-time PCR**—Yeast cultures (10 ml) were grown to the early stationary phase, cells were harvested, and total RNA was isolated using the RNeasy mini kit (Qiagen). The RNA samples were treated with DNase from a DNA-free kit (Ambion) to remove contaminating genomic DNA. cDNAs were synthesized with a Reverse-iT first strand synthesis kit (ABgene) according to the manufacturer’s protocol. Real-time PCR reactions were performed in a 50-μl volume using ABsolute quantitative PCR SYBR Green mix (ABgene) in a 96-well plate. Duplicates for each sample were included for each reaction. The real-time PCR primers used in this work are listed in Table 2. ACT1 was used as the internal control, and the RNA level of the gene of interest was normalized to ACT1 levels. PCR reactions were initiated at 95 °C for 10 min for denaturation followed by 40 cycles consisting of 30 s at 95 °C and 60 s at 57 °C.

**Western Blot Analysis of Swe1p**—Yeast cells grown to the mid-log phase were harvested and subjected to mechanical breakage with glass beads at 4 °C. After separation from glass beads and cell debris by centrifugation, protein extracts were boiled immediately with protein gel sample buffer. Total proteins (50 μg of protein) were separated on 8% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated in 5% nonfat milk with anti-Swe1p rabbit IgG (y-311, Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Swe1p was visualized using an ECL chemiluminescence detection system (GE Healthcare) with α-tubulin as the loading control.

**Flow Cytometry**—Yeast cells were cultured in YPD medium at 30 °C and harvested at the indicated growth phase. After cells were fixed with 75% ethanol and digested with RNase A at room temperature for 4 h, cellular DNA was stained with 50 μg/ml propidium iodide at 4 °C for 12 h. Stained cells were sonicated briefly before proceeding with flow cytometry. Cellular DNA quantification was performed using a FACSArray (BD Biosciences) 96-well plate flow cytometer.

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**TABLE 2**

The real-time PCR primers used in this study

| Gene | Primers | Sequence (5′ to 3′) | Product length |
|------|---------|---------------------|---------------|
| ACT1 | Forward  | CCGTCCGTTCCTTCCACTATCGC | 218 |
|      | Reverse  | CAGATCTGAGGTTCCCGAAGCG | |
| SWE1 | Forward  | GGTTATGATTACACGTGGACACC | 309 |
|      | Reverse  | CTGCGATTACAGCCTCCTTGGC | |
| CIT2 | Forward  | CAGCTTTGATACACCAATCTAGGTT | 309 |
|      | Reverse  | CAGCCGGGCTGGTTTGAATCTTT | |
| DLD3 | Forward  | ACAACCGCTGGTTTAACTCTTC | 258 |
|      | Reverse  | CAAACCGCCTGCTTGTAACTTCC | |
RESULTS

Loss of mtDNA in crd1Δ Results in Slow Growth and Decreased PG Accumulation—As discussed above, pgs1Δ exhibits the petite lethal phenotype in the absence of sorbitol (21). To determine whether crd1Δ exhibits petite lethality, we generated a crd1Δrho° mutant. The crd1Δrho° cells were viable on YPD medium (Fig. 1A) but exhibited a very long lag in growth and decreased cell density in the stationary phase (Fig. 1B). One possible explanation for the severe growth defect in the crd1Δrho° cells is that the loss of mtDNA led to a decrease in synthesis of PG. Because the increase in PG in the crd1Δ mutant may partially compensate for lost CL, the loss of mtDNA may diminish this excess PG to a level that can no longer compensate. This would be consistent with previous studies showing that PG and CL synthesis are decreased in the absence of a mitochondrial genome (50–54). Consistent with this, we found that loss of mtDNA led to a 42% decrease in CL levels in WT cells (Fig. 1C). In the crd1Δ mutant, which lacks CL but accumulates PG, the loss of mtDNA led to a 48% decrease in PG levels (Fig. 1C). Therefore, the decreased PG levels may contribute to the severe growth defect of crd1Δrho° cells.

The crd1Δrho° Mutant Shows Cell Division Defects—Previous studies demonstrated that the pgs1Δ mutant exhibits defects in cell wall biosynthesis and maintenance of cell integrity (21). Consequently, it is hypersensitive to cell wall-perturbing agents such as caffeine and calcofluor white and exhibits an enlarged cell morphology. Although PG accumulation is decreased in the crd1Δrho° mutant (Fig. 1C), crd1Δrho° cells were not sensitive to caffeine and calcofluor white (data not shown), suggesting that they contain sufficient PG to maintain cell wall integrity. However, crd1Δrho° cells did have an enlarged cell morphology phenotype, and 13.4% (±0.6% S.D.) of cells exhibited elongated buds in the mid-log phase, suggestive of a cell division defect (Fig. 2A). In the presence of a plasmid-borne copy of the CRD1 gene, wild type cell morphology was completely restored. This suggested that CL is essential for normal cell division in the absence of mtDNA. Consistent with this, DAPI staining of nuclear DNA revealed incoordination between nuclear division and cell division in crd1Δrho° cells. As seen in Fig. 2B, in contrast to rho° cells in the CRD1 background (WT rho°), crd1Δrho° cells exhibited multinucleate mother cells and anucleate daughter cells. Mother and daughter cells were characterized by failure to separate and were linked by long necks. DNA content analysis by flow cytometry also revealed a dramatic delay in cell division. Although the crd1Δ mutant showed a relatively normal distribution of cells in G1 and G2/M, the crd1Δrho° mutant accumulated cells with a G2/M DNA content and had essentially no cells in G1 (Fig. 2C). Interestingly, the mutant also accumulated cells with a DNA content greater than G2/M DNA content (Fig. 2C, indicated by 4C), suggesting additional DNA synthesis prior to cell division. As described further below, all of these cell division defects could be rescued by mutations in the cell cycle checkpoint gene, SWE1.

In addition to the incoordination between nuclear and cell division, chitin deposition was altered in crd1Δrho° cells (Fig. 2D). Chitin ring formation was absent, and bud scars were hardly distinguishable from the rest of the cell wall, both in morphologically abnormal cells and in cells with normal morphology, indicating that chitin deposition in the bud scars did
not occur in these cells. Chitin plays an important role in cell division, and synthesis of chitin is regulated during the cell cycle. Chitin is distributed asymmetrically in the cell wall, largely at the presumptive bud site before bud emergence (55–57), and forms the primary septum at cytokinesis (58). The dynamic synthesis and restricted localization of chitin underscores its importance in cell division. Therefore, loss of normal chitin deposition was consistent with a cell division defect in crd1Δrho° cells.

During cell division, the formation of chitin rings is dependent on the presence of septins (59, 60). Yeast cells have five septins that are involved in vegetative growth, Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1 (61, 62). Early in the cell cycle, septins polymerize at the presumptive bud site, forming a ring that

FIGURE 2. Cell division defects in crd1Δrho° cells. A, WT, rho°, crd1Δ, and crd1Δrho° cells were cultured in YPD medium. The crd1Δrho° cells transformed with pYPGK18 or pYPGK18CRD1 were cultured in synthetic drop-out medium (Leu°). Cell morphology was examined and photographed microscopically after growth to the mid-log phase at 30 °C. The scale bar represents 3 μm. B, cells were grown to the mid-log phase in YPD at 30 °C. The nucleus was visualized by staining with DAPI as described under “Experimental Procedures.” The scale bar represents 2 μm. C, cells were grown to mid-log phase in YPD medium at 30 °C and processed for flow cytometry as described under “Experimental Procedures” (1C, G1 DNA content; 2C, G2/M DNA content; 4C, greater than G2/M DNA content). D, cells were grown to the early stationary phase in YPD at 30 °C. Chitin deposition was visualized by staining with WGA-Oregon Green 488 as described under “Experimental Procedures.” The scale bar represents 2 μm. E, cells transformed with the GFP-tagged Cdc3p were grown to the mid-log phase in synthetic drop-out medium at 30 °C, and living cells were examined directly under the fluorescence microscope. The scale bar represents 2 μm. All images in the same pattern were taken at the same magnification (×1000).
Up-regulation of Swe1p in crd1Δrho°

A

![Graph showing SWE1 mRNA levels in different conditions.](image)

B

![Image showing Swe1p and α-Tubulin expression.](image)

**FIGURE 3. Swe1p is up-regulated in crd1Δrho° cells.** A, total RNA was extracted from cells grown to the mid-log phase in YPD at 30 °C. SWE1 mRNA levels were determined by real-time PCR as described under “Experimental Procedures.” Expression was normalized to the RNA levels of the internal control ACT1. Error bars represent the range of the three independent experiments. B, total protein was extracted from cells grown to the mid-log phase in YPD at 30 °C. Swe1p was identified by Western blot. Wild type cells transformed with pYPGK18SWE1 were used as a positive control, swe1Δ was used as a negative control, and α-tubulin was used as a loading control.

expands into an hourglass structure and acts as a scaffold to localize the chitin synthase III complex to the mother side of the neck, where it deposits chitin in the cell wall (58, 59, 62). Because chitin localization is dependent on the septins, the observation of defective chitin deposition in crd1Δrho° cells suggested that these cells have defects in septin localization. To test this possibility, strains containing Cdc3p-GFP, Cdc12-GFP, and Cdc11-GFP were used for visualization of septin rings. As seen in Fig. 2E and supplemental Fig. S9A, WT, WT rho°, crd1Δ, and crd1Δrho° cells with normal morphology had normally localized septin rings, whereas crd1Δrho° cells with abnormal morphology exhibited misplaced septin rings localized around the neck close to the side of the daughter cells, indicating that the misplaced septin ring may be a consequence of abnormal cell morphology. This result suggested that the chitin deposition is independent of septin in crd1Δrho° cells as crd1Δrho° cells did not exhibit ring-form chitin deposition.

**Cell Division Defects in the crd1Δrho° Mutant Result from Elevated Expression of Swe1p—**Coordination of cell cycle events during yeast cell division is maintained by the morphogenesis checkpoint control that monitors errors in critical cell cycle processes and acts to delay cell division until cells recover from errors (63–66). Cell division delay is mediated by Swe1p, which phosphorylates and inhibits the cyclin-dependent kinase Cdc28p (67, 68). Previous studies have shown that conditional overexpression of SWE1 can cause cell division delay and the formation of elongated buds, similar to the crd1Δrho° phenotype (67, 69). To explore a possible role for SWE1 in the crd1Δrho° phenotype, we examined the mRNA level of SWE1 in crd1Δrho° cells. The SWE1 mRNA was increased 6.8-fold in the mutant when compared with the WT (Fig. 3A). Consistent with the increased RNA levels, Swe1p was significantly accumulated in crd1Δrho° cells (Fig. 3B). These results raised the possibility that Swe1p up-regulation may contribute to the crd1Δrho° phenotype. Consistent with this possibility, we showed that constitutive overexpression of SWE1 in WT cells led to an elongated cell morphology and to the presence of binucleated cells, similar to the crd1Δrho° mutant (supplemental Fig. S1A). Cells constitutively overexpressing SWE1 also had a very similar DNA content profile to crd1Δrho° cells, with a loss of G1 cells, and an accumulation of cells with postreplicative (G2/M DNA content) and greater than G2/M DNA content (4C) DNA content (supplemental Fig. S1B).

To determine whether Swe1p expression is required for the cell division defects of crd1Δrho° cells, we deleted SWE1 in the crd1Δrho° mutant. The phenotypes of elongated budding cells, aberrant chitin deposition, misplaced septin, and postreplicative DNA content were rescued in the crd1Δswe1Δrho° mutant (Fig. 4 and supplemental Figs. S3 and S9B). However, disruption of SWE1 in crd1Δrho° was unable to alleviate the slow growth defects. The crd1Δswe1Δrho° mutant maintained a prolonged lag phase similar to that seen in crd1Δrho° cells (supplemental Fig. S4). A few crd1Δswe1Δrho° cells exhibited enlarged cell morphology with diffuse DNA fragmentation (Fig. 4A). To determine whether crd1Δswe1Δrho° cells are arrested in the cell cycle after DNA replication in lag phase, DNA content was monitored in the first 10 h after cells were inoculated in fresh medium. Cytometric analysis showed that crd1Δswe1Δrho° cells did not exhibit postreplicative DNA accumulation in lag phase (supplemental Fig. S5), indicating that the slow growth defect of crd1Δswe1Δrho° is not due to cell division delay after DNA replication. Taken together, these findings suggested that the crd1Δrho° mutant undergoes cell division defects controlled by Swe1p.

Stabilization and accumulation of Swe1p are triggered by actin depolarization (70, 71). We observed that actin polarization, as detected by phalloidin staining, was maintained in crd1Δrho° cells (supplemental Fig. S6). Similar to the WT, the actin cortical patches clustered at the bud tip. Because budding cells were elongated, the actin cable extended through the long neck to the bud tip. These results suggested that in crd1Δrho° cells, increased expression of the SWE1 gene leads to an accumulation of Swe1p (Fig. 3).
sion delay in crd1Δrho° cells. To address this possibility, we observed mitochondrial morphology in dividing cells stained with DiOC6(3), which stains mitochondria in a potential-dependent manner, MitoTracker Green FM, which is potential-independent, and a plasmid-borne Cox4-DsRed fusion protein. As shown in Fig. 5, mitochondria in WT and crd1Δ cells exhibited an extended tubular morphology. The WT rho° cells displayed a reduced mitochondrial network. In contrast, normal tubule-like mitochondria were completely absent from crd1Δrho° cells, which contained large mitochondrial patches in mother and budding daughter cells. These results indicated that loss of both mtDNA and CL perturbed mitochondrial integrity.

As discussed above, the retrograde pathway is activated in yeast cells in response to mitochondrial dysfunction, resulting in increased expression of genes in anapleurotic pathways (29, 30). The degree to which the retrograde response is activated is dependent on the genetic background of cells and the carbon source used for growth (26, 27, 35, 78). For example, rho° cells in the PSY142 strain background exhibit a robust retrograde response in which CIT2 expression is up-regulated as much as 10-fold (26). In contrast, the expression of CIT2 is only slightly increased in rho° cells derived from the W303-1B strain background (27).

In the FGY3 strain background used in this study, neither crd1Δ nor rho° cells exhibited altered expression of CIT2 and DLD3 in glucose. Strikingly, however, the crd1Δrho° mutant exhibited a dramatic 10-fold increase in CIT2 and DLD3 expression (Fig. 6, A and B). To determine whether the increase in CIT2 and DLD3 resulted from activation of the retrograde pathway, RTG2 and RTG3 were deleted in crd1Δrho° cells. As expected, deletion of either RTG2 or RTG3 eliminated the increased expression of CIT2 and DLD3 (Fig. 6, C and D). Surprisingly, deletion of RTG2 or RTG3 from crd1Δrho° also reduced expression of SWE1 and Swe1p to wild type levels (Figs. 3B and 6E). These findings suggested that the mitochondrial retrograde pathway is involved in SWE1 expression. Moreover, the elongated budding cell morphology, defective chitin deposition, misplaced septin localization, and postreplicative DNA content were completely suppressed in crd1Δrtg2Δrho° and crd1Δrtg3Δrho° cells (supplemental Figs. S7–S10).
FIGURE 5. The crd1Δrho° mutant exhibited severe defects in mitochondrial integrity. Cells grown to the mid-log phase were incubated with DiOC6(3) or MitoTracker Green FM and examined under the fluorescence microscope. The scale bar represents 2 μm.

In conclusion, our results suggest a model in which loss of mtDNA in CL-deficient cells leads to severe mitochondrial dysfunction. This triggers activation of the retrograde pathway and up-regulation of SWE1, resulting in cell division delay.

**DISCUSSION**

The current study, which addressed the hypothesis that loss of CL from cells lacking mtDNA leads to cell division defects, has resulted in several novel findings. (i) The loss of CL from rho° cells led to cell division delay. (ii) Cell division delay was triggered by up-regulation of Swe1p. (iii) The induction of SWE1 stemmed from activation of the retrograde pathway as genetic elimination of the pathway decreased SWE1 expression. These findings indicate for the first time that mitochondrial anionic phospholipids are required for normal cell division in rho° cells and that cell division is controlled by the retrograde pathway in crd1Δrho° cells.

The finding of cell division defects in crd1Δrho° cells strongly supports the hypothesis that anionic phospholipids play an important role in maintenance of normal cell division in the absence of mtDNA. Unlike pgs1Δ, the crd1Δ mutant is not petite lethal and does not exhibit sensitivity to cell wall perturbing agents, suggesting that the phospholipid PG satisfies the anionic phospholipid requirement for cell wall biogenesis and cell viability (22). However, PG levels are significantly decreased in crd1Δrho° cells when compared with those of crd1Δ, and this deficiency may exacerbate mitochondrial dysfunction. Consistent with this, the normal tubular network of mitochondria is absent from crd1Δrho° cells (Fig. 5). Furthermore, crd1Δrho° cells fail to maintain mitochondrial integrity and contain fragmented mitochondrial patches in mother and budding daughter cells (Fig. 5). Restoration of CL by a plasmid borne CRD1 gene reverses the enlarged cell size and elongated budding cell phenotypes (Fig. 2A), indicating that the dysfunction is due to loss of CL from rho° cells. Other mutations that impair mitochondrial function and morphology have been linked to cell division defects, such as mmm1, mdm10, and mmm12 (43, 79, 80). Interestingly, deletion of any of these genes is synthetically lethal with crd1 (81). However, the mechanisms linking mitochondria to cell division have not been previously identified.

The presence of postreplicative DNA accumulation, multinucleate mother cells, and elongated anucleate budding daughter cells revealed the incoordination of nuclear division and cell division in crd1Δrho° cells (Fig. 2B). It appears that excess apical budding growth continues both in the presence and in the absence of nuclear division. This may result from elevated SWE1 expression because overexpression of SWE1 in WT cells can also cause the binucleated phenotype and cell division delay (supplemental Fig. S1). Swe1p negatively regulates the mitotic Clb-Cdc28p complexes by phosphorylating residue Tyr-19. As a consequence, Cdc28p cannot induce the switch from polar to isotropic bud growth, resulting in the formation of elongated buds (82, 83). Consistent with a previous study (67), we found that overexpression of SWE1 in WT cells resulted in the absence of a G1 DNA content peak and accumulation of G2/M and greater than G2/M DNA content (supplemental Fig. S1B). The increase in SWE1 expression and accumulation of Swe1p (Fig. 3) could thus easily account for the postreplicative DNA accumulation, multinucleate mother cells, elongated anucleate budding, misplaced septin, and defective chitin deposition in crd1Δrho° cells (Fig. 2, D and E). These findings are also consistent with reports that overexpression of SWE1 leads to elongated budding cells (67) and that Swe1p strongly influences septin localization by inhibiting Clb-Cdc28p (69). Defective chitin deposition may also be attributed to Swe1p-mediated inhibition of Cdc28p as the cdc28 mutant showed aberrant distribution of chitin in the cell wall (84).

Although disruption of SWE1 completely suppressed the postreplicative DNA accumulation, elongated budding cell phenotype, and restored localization of septin and chitin in crd1Δrho° cells (Fig. 4, A–C), it did not rescue the slow growth defect (supplemental Fig. S4). Although yeast can survive without mtDNA, which encodes respiratory chain components for oxidative phosphorylation, the tricarboxylic acid cycle and the biosynthesis of cellular metabolites, including some amino acids and lipids, are essential (72). These essential reactions require the import of nuclear-encoded proteins and the incorporation of lipids synthesized in other organelles (85). CL is localized in the mitochondrial inner membrane and affects the stability of various inner membrane protein complexes, including respiratory chain complexes and metabolite carriers (1). Moreover, CL is required for mitochondrial inner membrane fusion that facilitates content exchange and mtDNA maintenance (86). Surprisingly, we have recently shown that CL is involved in outer membrane protein
biogenesis as the loss of CL impairs the assembly pathways of several mitochondrial outer membrane proteins (87). In addition, disruption of CRD1 is synthetically lethal with endoplasmic reticulum-mitochondria encounter structure (ERMES) components, which are indispensable for efficient interorganelle phospholipid exchange and mitochondrial morphology and inheritance (43, 79–81). As shown in Fig. 5, loss of mtDNA in \textit{crd1}/H9004 led to severe mitochondrial morphology defects, which may affect the essential cellular functions of mitochondria, such as protein import, lipid exchange, and biosynthesis of metabolites. Therefore, severe mitochondrial defects are the main cause of growth defects in \textit{crd1}/H9004 \textit{rho}°. Disruption of SWE1 in \textit{crd1}/H9004 \textit{rho}° cells did not rescue mitochondrial morphology defects (supplemental Fig. S11), suggesting that the slow growth defect in \textit{swe1crd1}/H9004 \textit{rho}° cells is due to exacerbated mitochondrial dysfunction. Critical to understanding this mechanism is the induction of the retrograde response in \textit{crd1}/H9004 \textit{rho}° cells. Neither WT \textit{rho}° nor \textit{crd1} cells exhibited an increased retrograde response in the strain background used in this study (FGY3). In fact, the retrograde regulation appears to be triggered by altered mitochondrial metabolism, as opposed to an altered mitochondrial genome (26, 88). Deletion of RTG2 or RTG3 eliminated the elevated expression of SWE1, elongated budding cell morphology, misplaced septin ring, defective chitin deposition, and postreplicative DNA content in \textit{crd1}/H9004 \textit{rho}° cells (Fig. 3 and supplemental Figs. S7–S10). One possible explanation for elevated SWE1 expression is that it is a target of the effectors of the retrograde pathway, Rtg1p/Rtg3p, that bind as a heterodimer to the R box (GTCAC) region in promoters of RTG target genes, including CIT2, DLD3, CIT1,

\textbf{FIGURE 6.} Deletion of RTG2 or RTG3 suppressed elevated expression of SWE1 in \textit{crd1}/H9004 \textit{rho}° cells. Total RNA was extracted from cells grown to the mid-log phase in YPD at 30 °C, and mRNA levels of CIT2, DLD3, and SWE1 were determined by real-time PCR as described under “Experimental Procedures.” Expression was normalized to the mRNA levels of the internal control ACT1. Error bars represent the range of the three independent experiments.
Up-regulation of Swe1p in crd1Δrho°

Figure 7. Model for retrograde pathway-mediated cell division delay in crd1Δrho° cells. The loss of mtDNA in crd1Δ cells leads to exacerbation of mitochondrial dysfunction, which is sensed by Rtg2p. The Rtg2p transmits this signal to the heterodimer Rtg1p/Rtg3p, resulting in translocation of Rtg1p/Rtg3p from the cytoplasm to the nucleus where it activates transcription of Swe1. The up-regulation of Swe1p serves as a compensatory mechanism to delay the cell division, preventing the generation of abnormal daughter cells.

ACO1, IDH1, and IDH2 (31, 33, 34, 36, 89). The R box is localized at −100 bp to −400 bp upstream from the open reading frame and is necessary for maximal gene expression. Inspection of the 5′-flanking region of the Swe1 gene revealed the presence of an R box localized at −356 bp, which could serve as a putative Rtg1p/Rtg3p binding site. Further work will be required to address this question.

Collectively, our results suggest that in the absence of both mtDNA and CL, cell division is compromised due to exacerbation of mitochondrial dysfunction, which triggers activation of the retrograde pathway. Swe1p is up-regulated by mitochondrial retrograde response and delays the cell division, possibly as a mechanism to avoid the generation of abnormal daughter cells. A model depicting this regulation is shown in Fig. 7.

The pgs1Δ mutant, lacking both PG and CL, loses mtDNA spontaneously in the absence of 1 M sorbitol and exhibits more severe growth defects than crd1Δrho° cells (Fig. 1A). It is tempting to speculate that the petite lethal phenotype of pgs1Δ is attributed to cell division defects due to the absence of both mtDNA and anionic phospholipids. Similar to crd1Δrho° cells, pgs1Δ cells display defective deposition of chitin (21), which indicates a possible cell division defect. The yeast cell wall consists of three major structural polysaccharides, mannans, glucans, and chitin (90, 91). Mannans and glucans are synthesized continuously during the cell cycle and are distributed uniformly in the cell wall, whereas chitin displays periodic synthesis and restricted localization (90). The importance of chitin distribution in cell division was underscored by the observation that some cell cycle mutations cause uniform distribution of chitin, such as cdc3 and cdc28 (84). In addition to the potential cell division defect due to the absence of both mtDNA and anionic phospholipids, the pgs1Δ cells may also be subject to defective cell division stemming from perturbation of cell wall biogenesis (21). Fks1p, a catalytic subunit of 1,3-β-glucan synthase, was barely detectable in pgs1Δ cells, which contained markedly reduced 1,3-β-glucan (22). As the cell wall morphology checkpoint monitors cell wall biosynthesis during the cell division, the fks1Δ mutant, which has a significant defect in glucan synthesis, exhibits G2 arrest (92). Therefore, the defective cell wall might contribute to a cell division delay in pgs1Δ.

In summary, we have shown that CL is required for normal cell division in the absence of mtDNA. These studies show for the first time that mitochondrial dysfunction leads to cell division defects that are mediated by retrograde regulation. Our findings provide novel insights into the essential cellular functions of CL and shed light on the mechanism underlying petite lethality of pgs1Δ.

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