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Endoplasmic reticulum stress affects the transport of phosphatidylethanolamine from mitochondria to the endoplasmic reticulum in *S. cerevisiae*

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Abstract

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are two of the most abundant phospholipids in cells. Although both lipids can be synthesized in the endoplasmic reticulum (ER), in *S. cerevisiae* PE can also be produced in mitochondria and endosomes; this PE can be transported back to the ER where it is converted to PC. In this study we found that dithiothreitol (DTT), which induces ER stress, decreases PE export from mitochondria to the ER. This results in decreased levels of total cellular PC and mitochondrial PC. These decreases were not caused by changes in levels of PC synthesizing or degrading enzymes. PE export from mitochondria to the ER during ER stress was further reduced in cells lacking Mdm10p, a component of an ER-mitochondrial tethering complex that may facilitated lipid exchange between these compartments. We also found that reducing mitochondrial PC levels induces mitophagy. In conclusion, we show that ER stress affected PE export from mitochondria to ER and the Mdm10p is important for this process.

Keywords

ER stress; Mitochondria; Phosphatidylcholine; ERMES; Lipid transport

1. Introduction

The endoplasmic reticulum (ER) plays crucial roles in cellular metabolism including protein folding, redox homeostasis and lipid biosynthesis [1]. Many environmental and physiological insults disturb ER homeostasis and activate the collection of intracellular signalling pathways, called the unfolded protein response [2]. A number of studies suggest

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that the unfolded protein response plays an important role in maintaining lipid homeostasis [3].

Phospholipids are a class of lipids that are major constituents of cell membranes [4]. In the absence of exogenous ethanolamine or choline, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are mainly synthesized through the CDP-DAG pathway (Fig. 1) [4]. In the CDP-DAG pathway, phosphatidylserine (PS) is synthesized in the ER [5] and is transported to mitochondria and endosome, where PS decarboxylases (Psd1p in mitochondria or Psd2p in endosome) convert the PS to PE [6–7]. Then, the PE is exported from mitochondria or endosome back to the ER where PC synthesis occurs. In the ER, the enzyme Cho2p converts the PE into phosphatidylmonomethylethanolamine (PMME), which is further methylated to form PC by Opi3p (Fig. 1) [8]. Alternatively, PE and PC are also synthesized in the ER through the Kennedy pathway (Fig. 1), which requires exogenous ethanolamine and choline [9]. PC is important for cellular [10–13] and mitochondrial functions [14–15]. Thus export of PE from mitochondria or endosomes to the ER for the synthesis of PC is vital [4]. Yeast strains that are defective in PC biosynthesis have mitochondrial defects and generate respiratory-deficient (“petite”) cells with a high frequency [13,16]. Defective mitochondria in these cells can be eliminated by mitochondrial autophagy (mitophagy), which delivers damaged mitochondria to the vacuole (lysosome) for degradation [17].

Phospholipids can be exchanged between organelles at zones of close contact between organelles called as membrane contact sites [18–20]. The ER-mitochondria encounter structure (ERMES) is a tether that connects the ER and mitochondria [21]. The number and size of ERMES complexes are regulated by the conserved GTPase protein, Gem1p [22]. The ERMES complex consists of four proteins, Mmm1p, Mdm10p, Mdm12p and Mdm34p, and may facilitate lipid exchange between the ER and mitochondria [21]. However, the mechanism of the phospholipid exchange between the ER and the mitochondria is yet to be elucidated. In this study we found that ER stress decreases levels of cellular PC by affecting the export of PE from the mitochondria to the ER. In addition, we found that PE export from mitochondria to ER during ER stress was further reduced in cells lacking Mdm10p, suggesting that ERMES may play a critical role in mitochondria to ER PE transport during ER stress. We also show that depletion of PC in mitochondria under ER stress caused mitochondrial dysfunctions that induced mitophagy.

2. Materials and methods

2.1. Yeast strains, media and growth conditions

Yeast strains used in this study are listed in Table 1. A PCR-based procedure was used to generate and verify gene deletions [23]. Yeast were grown in synthetic complete (SC) media containing 0.67% yeast nitrogen base, either 2% dextrose (SC-D) or 2% lactate (SC-L), and appropriate amino acids. For ER stress, the cells were grown in 3 ml of SC-D medium for 8 to 10 h, diluted to A_{600} value of 0.006 and grown at 30 °C with 0.5 mM or 1 mM dithiothreitol (DTT) or without DTT until a final A_{600} value of 1.0 was achieved. For the DTT-sensitive strains, cells were grown up to an A_{600} value of 0.3 and then exposed to ER stress for 3 or 4 generations.

2.2. Growth characteristics of yeast cells on solid and liquid media

The relative growth rate of yeast strains on solid media was determined by spot tests. Cells were grown overnight at 30 °C in 3 ml of SC-D medium. The cultures were diluted to an A_{600} value of 0.02 and incubated at 30 °C until the mid-logarithmic growth phase. Then, the cells were harvested, washed and re-suspended with sterile water to A_{600} value of 1.0, 5-fold serial dilutions in water were prepared and 5 μ l of each dilution was spotted on either SC-D or SC-L plates. Growth was assessed after the plates had been incubated for two or four days at 30 °C. To determine the growth rate of strains in liquid media, cells were grown in 3 ml of SC-D medium to saturation overnight, diluted to A_{600} value of 0.001 in the same medium either with (0.5 to 1.5 mM DTT) or without DTT and A_{600} values of the cultures were determined over time up to 48 h.

2.3. Phospholipid analysis of whole cells

Cells were grown to mid-logarithmic growth phase at 30 °C in SC-D medium either with 0.5 or 1 mM DTT or without DTT, harvested by centrifugation and washed twice with sterile water. The lipids were extracted from the cell homogenate using the procedure of Bligh and Dyer [24]. The phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silica gel plates using chloroform/methanol/ammonium hydroxide (65/35/5 v/v), followed by chloroform/acetic acid/methanol/water (75/25/5/2.2 v/v). The phospholipids were visualized with iodine vapour, scraped off from the plate and quantified using the method of Rouser et al. [25]. The protein concentration was determined by the method of Bradford [26] using BSA as a standard.

2.4. Pulse-chase labelling of phospholipids with [3 H]serine

To reveal the impact of ER stress on PE and PC synthesis, wild type (WT) cells ($A_{600} \sim 0.3$) were labelled with [3 H]serine (10 μ Ci/ml) for 45 min in SC-D. The cells were then washed and resuspended in fresh SC-D either, with 0.5 or 1 mM DTT or without DTT and harvested at the indicated times. Lipids were extracted as described in the previous section and phospholipids were separated by TLC [27] using the solvent system chloroform:methanol:2-propanol:0.25% KCl:triethylamine in the ratio 30:9:25:6:18 (v/v). The radioactivity associated with individual phospholipids was measured using a liquid scintillation counter (PerkinElmer Life Sciences), using 5 ml of toluene based scintillation mixture.

2.5. Isolation and purification of mitochondria

The isolation and purification of the mitochondria was performed as described previously [19,28]. Briefly, crude mitochondria were prepared, suspended in Buffer-A (0.6 M sorbitol, 20 mM K^+ MES and 1 mM PMSF) and layered on a density gradient composed of 20–50% sucrose in Buffer-A. The gradient was centrifuged at 100,000 X g for 1 h at 4 °C. The mitochondria were harvested from the gradient, diluted four-fold in Buffer-A, and centrifuged at 12,000 $\times$ g for 10 min at 4 °C. The resulting mitochondrial pellet was re-suspended in Buffer-A and used for lipid analysis. The mitochondrial protein concentration was determined by the method of Bradford [26].

2.6. *In vivo* [^3H]serine and [^{14}C]choline labelling and phospholipid analysis

The yeast strains were grown in SC-D medium until the mid-log phase. The cultures were washed twice with sterile cold water and re-suspended in 2 ml of SC-D medium lacking serine (A_{600} of 0.2) either, with 0.5 or 1 mM DTT or without DTT. The reaction was initiated by adding 10 $\mu\text{Ci/ml}$ of [^3H]serine, and the cultures were grown for 3 h at 30 °C with vigorous shaking. The yeast cells were harvested and lipids were extracted and separated as described previously [24,25,27]. The separated phospholipids were scraped and the radioactivity was quantified using a liquid scintillation counter. To assess PC degradation, another set of cells were labelled with 5 $\mu\text{Ci/ml}$ [^{14}C]choline and the radioactivity of metabolites containing choline was quantified in aqueous and non-aqueous phase using a liquid scintillation counter.

2.7. Phospholipid N-methyltransferases assay

The yeast cells were grown overnight and microsomes were pre-pared by the method of Zinser et al. [11]. Phospholipid methylation assays were performed by the method of Gaynor and Carman [29]. Briefly, the PEMT assay mixture contains 10 μg microsomal protein, 50 mM Tris buffer (pH 9.0), 0.2 mM PE and 0.5 mM S-adenosyl-L-[methyl- ^3H]-methionine (10,000 cpm/nmol) and was incubated for 10 min at 30 °C. The PLMT assay mixture, contains 0.2 mM PMME, 0.5 mM S-adenosyl-L-[methyl- ^3H]-methionine (10,000 cpm/nmol), 50 mM Tris buffer (pH 7.5), 10 mM MgCl_2 , 10 μg microsomal protein and was incubated for 10 min at 30 °C. The reaction was stopped by the addition of 0.5 ml of 0.1 M HCl, and lipids were extracted with chloroform and methanol (2:1). The chloroform soluble metabolites were analyzed by TLC [27]. PMME, PDME, and PC were identified with authentic standards and the associated radioactivity was measured by using liquid scintillation counter (PerkinElmer LifeSciences).

2.8. Mitophagy analysis

For the mitophagy assay, cells expressing a GFP-tagged OM45 were initially grown in YPL medium (up to 10 h) and then transferred to SC-D medium and grown until logarithmic growth phase (A_{600} of 0.8) with or without 0.5 or 1 mM DTT or 50 μM 2-hydroxyethylhydrazine (2-HEH). For macro autophagy analysis, cells expressing GFP-Atg8 were grown in SC-D medium (up to 12 h), diluted and grown until logarithmic growth phase (A_{600} of 1 to 1.5) with or without DTT/2-HEH. The stability of OM45-GFP and GFP-Atg8 was analyzed by Western blotting [30].

2.9. Western blotting

Yeast cell-free lysate were prepared by breaking the cells with glass beads in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail. Total proteins (50 μg) were separated by SDS-PAGE using 8 or 10% gels and transferred to nitrocellulose membranes at 120 V for 2 h. The resulting membrane was incubated with primary antibody for 2 h at room temperature, followed by incubation with an HRP-conjugated secondary antibody (1:5000) for 1 h. The anti-Kar2 antiserum was used to measure the Kar2p levels (1:5000). Anti-Pgk1 was used to identify Pgk1p (1:5000). Anti-GFP (1:5000) was used to detect GFP-Atg8 and OM45-GFP. The

membrane was washed with PBS containing 0.1% Tween-20, and the proteins were detected using a chemiluminescent substrate (ECL) system (Thermo scientific). The intensity of Kar2p was normalized to the Pgk1p expression levels, and the relative intensity data were calculated using the ImageJ analysis software [31].

2.10. Statistical analyses

Data were analyzed using the SPSS software package v10.0 (the Statistical Package for the Social Science for Windows 10.0). All the values reported in this paper represent the mean of three independent experiments. Statistical analyses were performed using Student's t-tests (two-tailed, unpaired) and differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Effect of ER stress on yeast cell growth

DTT is known to induce ER stress [32]. To elucidate the effect of ER stress on the phospholipid metabolism, WT cells were grown in media with increasing concentrations of DTT (0.5, 1.0 and 1.5 mM). The cells showed a significant growth impairment even at 0.5 mM DTT (Fig. 2A) but were able to tolerate a maximum of 1.0 mM DTT (Fig. 2A); a severe growth defect was found when cells were cultured in media with 1.5 mM DTT. Therefore, 0.5 and 1 mM DTT concentrations were used in subsequent experiments. To measure the extent of ER stress, we determined levels of Kar2p, an ER chaperone that is induced during ER stress [33]. Treating cells with 0.5 and 1 mM DTT significantly increased Kar2p expression levels by 2 and 3.3-fold respectively (Fig. 2B), indicating that these treatments induce ER stress.

3.2. ER stress affected the steady-state PC levels in cells

We found that ER stress significantly increased PE levels and decreased PC levels in WT cells (Fig. 3A). The phosphatidylinositol (PI) level was also significantly increased with 1 mM DTT. No significant changes were observed in amount of PS and cardiolipin (CL) (Fig. 3A) in the whole cells during ER stress. Next, we determined how mitochondrial phospholipid levels changed during ER stress and found that levels of mitochondrial PC were significantly decreased while PE simultaneously increased (Fig. 3B). To determine whether the PC depletion during ER stress affected the mitochondrial function, we grew the cells in respiratory growth conditions. As expected, the WT cells exposed to ER stress were unable to grow on respiratory conditions (data not shown). This suggests either that ER stress or PC depletion in mitochondria prevents mitochondrial function.

3.3. PC decrease during ER stress was not due to increased turnover by the phospholipases

The decrease in PC during ER stress could be caused by a decrease in the rate of PC synthesis or an increase in PC degradation. To measure the stability of PC over time, we labelled cellular phospholipids and determined their stability over time. WT cells were labelled with [^3H]serine for 45 min in a medium without ethanolamine or choline (most PC is generated by the CDP-DAG pathway in this growth condition, Fig. 1). The cells were then

washed, resuspended in fresh media, and the amount of PS, PE, and PC determined over 4 h. Cells were grown in media that lacked DTT or contained 0.5 or 1.0 mM DTT (Fig. 4A to C). Panel A shows the synthesis pattern of PE and PC from PS in the WT control cells. At the 0 h, 60% of the radioactivity was associated with PS, whereas PE and PC accounted for 25% and 10%, respectively. In the WT control cells, after 4 h, the PS decreased to 10%, and the PE increased to approximately 40%, whereas the labelled PC levels reached to 50% (Fig. 4A). Cells exposed to 0.5 or 1 mM DTT for 2 to 4 h, reduced the PC levels to approximately 20% and increased PE up to 50%. These findings indicate that DTT-induced ER stress could be due to either a defect in PC synthesis or increased PC degradation.

We determined whether PC degradation was occurring in two ways. First, we measured the levels of lyso PC (LPC), a product of PC degradation, during ER stress by labelling the cells with [14 C]choline and found that ER stress did not increase the levels of LPC (Fig. 4D). We also measured the choline containing metabolites (glycerophosphocholine, phosphocholine, and choline) in the aqueous phase, and the amount of radioactivity was significantly decreased with 1 mM DTT (Fig. 4E). These experiments collectively suggested that, the decrease in PC level under ER stress was not due to increased turnover by phospholipases and therefore might be due to a defect in PC synthesis.

3.4. ER stress did not alter the activity of the PC synthesizing enzymes

Next, we wanted to know whether the enzymatic activity of, the methyltransferases that convert PE to PC, Cho2p and the Opi3p, was reduced by ER stress. The addition of 0.5 and 1 mM DTT to WT or *psd1* strains did not alter the activity of Cho2p or the Opi3p (Table 2). We also measured the PC level in both the *cho2* and *opi3* deletion cells during ER stress, and found 10 to 15% of PC decrease (data not shown). The supplementation of choline increased the cellular PC level under ER stress implying that the enzymes involved in PC synthesis through the Kennedy pathway were not affected (data not shown). Previous studies have indicated that yeast cells lacking protein UnProceSsed1 (Ups1p) accumulate PE in mitochondria [34]. We tested the levels of PE in cells lacking either Ups1p or either of the related proteins Ups2p and Ups3p during ER stress and found that Ups proteins were not responsible for PE accumulation in the mitochondria (data not shown). Collectively, these findings suggest that treating cells with DTT does not significantly alter the ability of cells to synthesize PC.

3.5. ER stress reduces PE export from mitochondria to the ER

Our findings suggest that reduced levels of PC during ER stress was neither due to increased degradation (Fig. 4 D,E) nor due to a defect in PC synthesis (Table 2). Since PC is made from PE that is produced in mitochondria and endosomes, we wondered PE export from these organelles is reduced by ER stress. To determine the rate of PE to PC conversion, we labelled cells with [3 H]serine, which is used to produce PS that is subsequently converted to PE and then PC [8] (Fig. 1). In WT cells, we observed a decrease in PC level (Fig. 5A), which might be due to a defect in the transport of PE from either mitochondria or endosomes to the ER. To distinguish between these possibilities we labelled cells lacking Psd1p, which is in mitochondria, or Psd2p, which is in endosomes [6–7]. Cells lacking Psd1p (*psd1*) did not show any significant difference in the level of PS, PE and PC during

ER stress (Fig. 5B), suggesting that PE from the endosome to the ER is not affected by ER stress. In contrast, *psd2* cells showed a defect in the conversion of PE to PC under ER stress (Fig. 5C). These results suggest that, ER stress might affect the transport of PE from the mitochondria to the ER and result in a reduced level of PC.

3.6. PC depletion in mitochondria induces mitophagy

We found that ER stress decreases the PC levels and renders cells unable to grow in the respiratory conditions, which requires functional mitochondria. To determine the effect of mitochondrial PC depletion on mitochondrial function, we used 2-hydroxyethylhydrazine (2-HEH), an ethanolamine analogue that is converted to phosphatidylethylhydrazine and inhibits the PE methyltransferases Cho2p and Opi3p [35]. The 2-HEH treated cells showed a significant growth defect under respiratory conditions (Fig. 6A) and decreased mitochondrial PC by ~35% (Fig. 6B). These defects were corrected by adding choline to the growth medium, which allows cells to produce PC by the Kennedy pathway (Fig. 6A and data not shown). These results suggested that, 2-HEH induced growth defect was due to a depletion of PC that results in mitochondrial dysfunction.

Previous reports from our laboratory and others have suggested that ER stress induces autophagy in yeast cells [36] and that defects in the PC synthesis increased mitophagy [15]. Here we also observed a reduction in mitochondrial PC levels that caused mitochondrial dysfunction (Fig. 6A). Thus, we studied the levels of mitophagy by using a well-established OM45-GFP processing assay, in which the appearance of free GFP is used to indicate that mitochondria have been degraded by autophagy [30]. We used *atg1* cells, which cannot induce autophagy, as a negative control. Both ER stress and 2-HEH induced mitophagy in WT cells (Fig. 7). The addition of choline to the growth medium increased cellular PC levels during ER stress (data not shown) but mitophagy and autophagy induction were not rescued (Fig. 7). The addition of DTT and 2-HEH increased the free GFP signal from OM45-GFP in WT cells. As expected, the *atg1* strain expressing OM45-GFP did not generate any processed GFP during ER stress (Fig. 7).

3.7. PE export from mitochondria to ER under ER stress is reduced in cells lacking Mdm10p

The [³H]serine labelling study in *psd2* cells (Fig. 5C) suggested that reduced PC levels during ER stress might be due to a defect in PE transport from mitochondria to the ER. The ERMES complex connects the ER and mitochondria for lipid transport [21], and may be required for PE export from mitochondria during ER stress. We determined the steady-state PC and PE levels during ER stress in *mmm1*, *mdm10*, *mdm12*, *mdm34* and *gem1* strains lacking Psd2p (Fig. 8A). PC levels during ER stress were decreased in all the ERMES mutant strains including *psd2 gem1* strain and were most decreased in *psd2 mdm10* cells (Fig. 8B). This finding suggests that Mdm10p is particularly important for PE export from mitochondrial to ER during ER stress. Steady-state PC levels were also remarkably decreased in *psd2 mdm10* cells during ER stress (Fig. 8B). We also determined the phospholipid composition of an ER-enriched fraction of membranes derived from WT cells during ER stress (Fig. 8C). The PC level in the ER-enriched membranes was not altered during ER stress in WT or *psd2* cells. Interestingly, the levels of PC in ER-

enriched membranes were significantly decreased in membranes from a *psd2 mdm10* strain compared to *psd2* cells. Moreover, this effect was amplified by ER stress. The PE levels in ER-enriched membranes were also significantly more decreased in membranes from *psd2 mdm10* cells than from *psd2* cells (both with or without DTT) (Fig. 8C). Based on these results, we propose that a defect in PE export from mitochondria to ER results in decreased cellular levels of PC during ER stress.

3.8. PC transport from ER to mitochondria is not altered by ER stress

During ER stress, the export of PE from the mitochondria to the ER was affected (Figs. 5 and 8B). We wanted to analyze the impact of ER stress on the transport of PC from the ER to the mitochondria. Hence, we studied the transport of PC from ER to mitochondria by labelling WT and *cho2 opi3* cells with [¹⁴C]choline for 2 h under ER stress. In *cho2 opi3* cells PC is synthesized only by the Kennedy pathway at ER using exogenous choline (Fig. 1). Thus, if the ER stress affected the PC transport from ER to mitochondria, *cho2 opi3* cells might have a decreased PC level in mitochondria. However, we found that the addition of 0.5 mM DTT to WT or *cho2 opi3* cells did not decrease the levels of mitochondrial PC as compared to untreated control cells (Fig. 9A). We also measured steady state mitochondrial PE and PC levels in *psd2 mdm10* and *cho2 opi3* cells with 0.5 mM or without DTT. This analysis revealed that *psd2 mdm10* cells have a significantly reduced amount of PC when grown with DTT, consistent with the idea that they have a defect in PE export from mitochondria to ER where PE is converted to PC (Fig. 9B). Collectively, these results suggest that ER stress specifically affects PE export from mitochondria to ER but does not alter PC transport from ER to mitochondria.

4. Discussion

In this study we demonstrate that ER stress decreases cellular PC levels by reducing PE export from mitochondria to ER. We show that *psd2 mdm10* cells exhibit severely reduced mitochondrial PE export during ER stress. In addition, we show that ER stress decreases mitochondrial PC level but the transport of PC from ER to mitochondria is unaffected. Decreasing PC levels in mitochondria induced mitochondrial dysfunction and mitophagy. Taken together, our data indicate that ER stress affects PE export from mitochondria to ER and that this export is facilitated by Mdm10p.

The cellular functions of PC and PE are well known [4] but only limited studies address their importance in stress conditions. Here we found that ER stress decreases both cellular and mitochondrial PC levels. In yeast, PC is degraded by different phospholipases to maintain the PC homeostasis [37]. Thus, the observed PC decrease could be due to an increased PC degradation under ER stress. Indeed, we found that ER stress reduced levels of LPC and other choline containing metabolites suggesting that phospholipases were not responsible for the decrease. Therefore, reduced PC could be due to decreased activity of PC synthesizing enzymes (Cho2p and Opi3p). Surprisingly, we do not see any decrease in the activity of Cho2p and Opi3p under ER stress. These findings indicate that ER stress does not affect the activity of PC synthesis or degradation enzymes. Instead, our findings suggest

that mitochondrial and cellular PC levels decrease due to defect in export of PE from mitochondria or endosome to ER.

We used either *psd1* or *psd2* cells to study PE export from mitochondria or endosome to ER during ER stress. In *psd1* cells, PE is synthesized in endosome [6] whereas in *psd2* cells PE is produced in mitochondria [7]. PE is then exported to ER for PC synthesis [8]. Interestingly, we observed an increased PE and decreased PC levels under ER stress only in *psd2* cells; *psd1* cells did not show any defect under ER stress. Therefore, PC reduction during ER stress is due to a defect in PE export from mitochondria to ER where PE is converted to PC.

Previous studies have indicated that yeast strains lacking any one of the members of ERMES complex exhibit a defect in mitochondrial morphology and phospholipid transport [21]. However, a subsequent study suggested that ERMES is not directly involved in PS transport to mitochondria [38]. As shown in Fig. 8A–C, *psd2 mdm10* cells exhibited severe defects in PE export from mitochondria to ER. These findings suggest that the ERMES complex, particularly Mdm10p, is required for PE export from mitochondria to ER during ER stress. This result is consistent with the findings of Kornmann et al., [21] where they demonstrate that yeast cells lacking ERMES proteins exhibit a poor PS to PC conversion. Our results suggest that under ER stress inducing conditions, Mdm10p is required for export of PE from mitochondria to ER.

We show that in ER stress inducing conditions cells exhibit decreased levels of mitochondrial PC. Consequently, cells are unable to grow in respiratory conditions that require mitochondrial function. To check whether the respiratory growth defect is due to PC depletion and not because of ER stress, we used ethanolamine analogue 2-HEH, which specifically inhibits PC synthesizing enzymes [34]. We found that cells treated with 2-HEH also exhibit a respiratory growth defect, which was rescued by choline supplementation indicating the growth defect in respiratory conditions was due to PC deficiency.

Previous studies have reported that a defect in PC synthesis induces mitophagy [21], probably because PC plays an important role in mitochondrial membrane biogenesis [20]. Our results suggest that PC depletion caused by ER stress or 2-HEH treatment induces mitophagy (Fig. 7) and that depletion of mitochondrial PC alone could induce mitophagy. In conclusion, our findings reveal the importance of Mdm10p for export of PE from mitochondria to ER for PC synthesis during ER stress.

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Fig. 1.

Schematic representation of phospholipid biosynthesis in yeast cells. The pathway depicts the biosynthesis of phospholipids, including the steps focused on this study. The steps involved in the CDP-DAG pathway are shown in pale orange and the Kennedy pathway is shown in pale blue. The enzymes involved in the steps are also indicated. Abbreviations: G-3-P, glyceraldehyde-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; CDP-DAG, cytidinediphosphatediacylglycerol; PS, phosphatidylserine; PE, phosphatidyl ethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PGP, phosphatidyl glycerophosphate; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyl dimethylethanolamine; PI, phosphatidylinositol; P, phosphate; Cho, choline; CL, cardiolipin; Etn, ethanolamine; Ins, inositol; Gat1/2p, glycerol-3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase1/2; Slc1p, 1-acyl-sn-glycerol-3-phosphate acyltransferase; Cds1p, CDP-diacylglycerol synthase1; Tam41p, Mitochondrial phosphatidatecytidyltransferase (CDP-DAG synthase); Cho1p, phosphatidylserine synthase1; Psd1/2p, phosphatidylserine decarboxylase1/2; Cho2p, phosphatidylethanolaminemethyltransferase; Opi3p, phosphatidyl monomethylethanolaminemethyltransferase; Pis1p, phosphatidylinositol synthase1; Pgs1p, phosphatidylglycerolphosphate synthase1; Gep4p, phosphatidyl glycerophosphatase; Crd1p, cardiolipin synthase; Eki1p/Cki1p, ethanolamine/choline kinase1; Ect1p, ethanolamine-phosphate cytidyltransferase1; Pct1p, choline phosphate cytidyltransferase1; Cpt1p, choline phosphotransferase1; Ept1p, ethanolamine phosphotransferase1.

Fig. 2. Effect of ER stress on yeast cell growth. (A) WT cells were grown in SC-dextrose (SC-D) medium in the presence or absence of 0.5, 1, or 1.5 mM DTT. Growth was measured at the indicated times. (B) Western blotting analysis of Kar2p in WT cells grown either with or without DTT. The lanes were loaded with 50 μ g of total protein and Kar2p levels were represented relative to Pgk1p levels. Kar2p, karyogamy2; Pgk1p, phosphoglycerate kinase1. These values are the mean $\pm$ SD of three independent experiments. The control cells were compared with the other groups with a statistical significance threshold of * $p < 0.05$.

Fig. 3. Phospholipid pattern of wild type cells during ER stress. (A) Phospholipid composition of the cell-free homogenate and (B) mitochondria from the WT strain. The cells were grown in the absence or presence of 0.5 or 1 mM DTT. The mitochondria isolation, lipid extraction, and analysis were performed as described in the methods. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin. Others include PA, phosphatidic acid, LPL, lyso phospholipids; PDME, phosphatidyl dimethylethanolamine. The results are presented as nmol of phospholipid

phosphorous/mg cell free total protein or mitochondrial protein and values are the mean $\pm$ SD of three independent experiments. Control cells were compared with other groups with a statistical significance threshold of * $p < 0.05$. NS, not significant.

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Fig. 4.

Effect of ER stress on phosphatidylcholine levels. (A) The WT strains were pulse labelled with [^3H]serine for 45 min, washed, and grown in SC-dextrose medium for 4 h with no DTT, (B) 0.5 mM DTT or (C) 1 mM DTT. The mean deviation in the experiment was $\leq 10\%$, and so the error bars were omitted for better clarity in panels A, B and C. (D) WT cells were grown with [^{14}C]choline and the amount of lyso-PC was measured with or without DTT treatment. (E) The radioactivity associated with the aqueous phase was quantified. The data represent the mean $\pm$ SD of three independent experiments. For panels D and E, control cells were compared with other groups with a statistical significance threshold of * $p < 0.05$. NS, not significant. For other abbreviations, see Fig. 1.

Fig. 5.

In vivo amino glycerophospholipid synthesis and transport during ER stress. (A) *In vivo* PS synthesis (labelling was initiated with [3 H]serine) and its conversion into PE and PC were measured. The PS, PE and PC formation under ER stress was measured in the WT, (B) *psd1* and (C) *psd2* strains. The radioactivity associated in amino glycerophospholipids was quantified as described in the methods section. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine. The data represent the mean $\pm$ SD of

three independent experiments. The respective genotype control cells were compared with DTT treated cells with a statistical significance threshold of * $p < 0.05$, NS, not significant.

Fig. 6. Growth and phospholipid analysis of wild-type cells after 2-hydroxyethylhydrazine (2-HEH) treatment. (A) WT yeast strains were serially diluted and spotted on SC-dextrose or SC-lactate plates, with or without 50 μ M 2-HEH in the presence or absence of choline (Cho) and incubated at 30 °C for 48 h. (B) Phospholipid composition of the mitochondria from the WT cells grown with or without 2-HEH. Mitochondria isolation and phospholipid analysis were performed as described in the methods. The phospholipid contents were represented as nmol of phospholipid phosphorous/mg of mitochondrial protein. The data represent the mean $\pm$ SD of three independent experiments. The control cells were compared with their 2-HEH treated groups with a statistical significance of * $p < 0.05$, NS, not significant. For abbreviations see Fig. 1.

Fig. 7.

Phosphatidylcholine depletion induces mitophagy in yeast cells. WT and *atg1* strains were exposed to ER stress and mitophagy was monitored by OM45-GFP processing. The WT and *atg1* strains were grown in the absence or presence of 0.5 or 1 mM DTT and with or without 4 mM choline. Mitophagy was also monitored in cells treated with 50 μ M 2-hydroxyethylhydrazine (2-HEH). Macroautophagy was studied in WT and *atg1* strains using GFP-Atg8 processing. Phosphoglycerate kinase1 (Pgk1) was used as a loading control. An asterisk (*) symbol indicates a nonspecific band.

Fig. 8.

Defect in ER-mitochondria tethering (ERMES) complex decreased the levels of phosphatidylcholine during ER stress in yeast cells. (A) PS synthesis and its conversion to PE and PC were studied in the *psd2*, *psd2 mmm1*, *psd2 mdm10*, *psd2 mdm12*, *psd2 mdm34* and *psd2 gem1* strains. The cells were labelled with [³H]serine for 2 h and the radioactivity associated with PC in the *psd2* control strains was considered as 100%. The strains were exposed to 0.5 mM DTT and the formation of PC in ERMES mutant strains with *psd2* background was compared with *psd2* control cells. (B) The phospholipids in

cell-free homogenates of *psd2* and *psd2 mdm10* strains were quantified in the presence and absence of 0.5 mM DTT. (C) The ER phospholipid composition of the ER in WT, *psd2* and *psd2 mdm10* strains grown under ER stress is shown. PC, phosphatidylcholine; PE, phosphatidylethanolamine; others include PS, phosphatidylserine; PI, phosphatidylinositol; lyso phospholipids and phosphatidylmethylethanolamine. The data represent the mean $\pm$ SD of three independent experiments. The WT control cells were compared with other groups with a statistical significance of * $p < 0.05$, NS, not significant.

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Fig. 9. Phosphatidylcholine transport from ER to mitochondria was not affected under ER stress. (A) The *in vivo* transport of PC from ER to mitochondria was studied in WT, *cho2 opi3* cells under ER stress using [14 C]choline for 2 h. (B) Steady state mitochondrial PE and PC levels were measured in *cho2 opi3* and *psd2 mdm10* cells as described in the methods section. PE, phosphatidylethanolamine (PE) and PC, phosphatidylcholine. The data represent the mean $\pm$ SD of three independent experiments. In A, WT control cells were compared with all other groups. In B, control cells of the respective strain was compared

with 0.5 mM DTT treated cells with a statistical significance threshold of * $p < 0.05$, NS, not significant.

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Table 1

Yeast strains used in this study

Strain	Genotype	Reference
Wild type (WT)	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0	[36]
psd1	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd1::KAN ^R	"
psd2	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3	This study
cho2	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 cho2::KAN ^R	"
opi3	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 opi3::KAN ^R	"
atg1	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 atg1::KAN ^R	"
cho2 opi3	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 cho2::KAN ^R ; opi3::URA3	"
psd2 mmm1	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; mmm1::KAN ^R	"
psd2 mdm10	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; mdm10::KAN ^R	"
psd2 mdm12	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; mdm12::KAN ^R	"
psd2 mdm34	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; mdm34::KAN ^R	"
psd2 gem1	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; gem1::KAN ^R	"
psd2 ups1	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; ups1::KAN ^R	"
psd2 ups2	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; ups2::KAN ^R	"
psd2 ups3	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 psd2::URA3; ups3::KAN ^R	"
WT + OM45-GFP	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 OM45-GFP::hphMX4	[39]
atg1 + OM45-GFP	BY4741 MATa his3 1 leu2 0 met15 0 ura3 0 atg1::KAN ^R OM45-GFP::hphMX4	[39]

Table 2

Phospholipid N-Methyltransferase catalytic activity in WT and *psd1* strains under ER stress. The enzyme activity was measured and expressed as pmole methyl incorporated/ min/mg protein. PMME, phosphatidyl monomethylethanolamine; PDME, phosphatidyl dimethylethanolamine; PC, phosphatidylcholine. The data represent the mean $\pm$ S.D. of three independent experiments. WT control cells were compared with other groups with a statistical significance of * $p < 0.05$, NS-not significant.

Strain	Product	
	PMME	PDME + PC
WT	pmol methyl incorporated/min/mg protein	
Control	1.60 $\pm$ 0.05	6.9 $\pm$ 0.31
0.5 mM DTT	1.58 $\pm$ 0.02 ^{NS}	7.0 $\pm$ 0.46 ^{NS}
1.0 mM DTT	1.56 $\pm$ 0.03 ^{NS}	7.0 $\pm$ 0.49 ^{NS}
<i>psd1</i>		
Control	1.64 $\pm$ 0.06 ^{NS}	6.7 $\pm$ 0.39 ^{NS}
0.5 mM DTT	1.58 $\pm$ 0.07 ^{NS}	6.8 $\pm$ 0.36 ^{NS}
1.0 mM DTT	1.54 $\pm$ 0.08 ^{NS}	6.7 $\pm$ 0.39 ^{NS}