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ORIGINAL PAPER

Endoplasmic reticulum stress and calcium imbalance are involved in cadmium-induced lipid aberrancy in *Saccharomyces cerevisiae*

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Abstract The endoplasmic reticulum is the key organelle which controls protein folding, lipid biogenesis, and calcium (Ca^{2+}) homeostasis. Cd exposure in *Saccharomyces cerevisiae* activated the unfolded protein response and was confirmed by the increased Kar2p expression. Cd exposure in wild-type (WT) cells increased PC levels and the PC biosynthetic genes. Deletion of the two phospholipid methyltransferases *CHO2* and *OPI3* modulated PC, TAG levels and the lipid droplets with cadmium exposure. Interestingly, we noticed an increase in the calcium levels upon Cd exposure in the mutant cells. This study concluded that Cd interrupted calcium homeostasis-induced lipid dysregulation leading to ER stress.

Keywords Cadmium · Phosphatidylcholine · Lipid droplets · Calcium and ER stress

Introduction

The endoplasmic reticulum (ER) plays a crucial role in protein synthesis, folding, glycosylation, secretion, and lipid synthesis (Görlach et al. 2006). ER stress, induced by dithiothreitol (DTT) and tunicamycin, led to lipid overload in *Saccharomyces cerevisiae* and is linked to calcium homeostasis (Gardarin et al. 2010). The toxicants or chemicals induce ER stress to alter the ER calcium, ER lipid composition, reactive oxygen species (ROS), and misfolded/unfolded proteins (Biogioli et al. 2008; Fu et al. 2011). Cadmium (Cd) is a toxic metal that induces ER stress, and also competes with the essential elements such as calcium, iron, zinc, and manganese, altering their intracellular ion homeostasis (Gardarin et al. 2010). Several studies indicated that exposure to Cd increased Ca^{2+} level, but the mechanism remains poorly understood (Beyersmann and Hechtenberg 1997). The Cd interacts with Ca^{2+} transport in intracellular stores, such as interference in hepatic Ca^{2+} sequestration in the microsomes (Zhang et al. 1990), or inhibition of sarcoplasmic reticulum Ca^{2+}-TPase (SERCA) (Hechtenberg and Beyersmann 1991). An alternative theory of ER stress postulates a key role for the downregulation of SERCA2 as a result of ER luminal Ca^{2+} depletion (Kharroubi et al. 2004).

In mammals, SERCA a type II P-type ATPase maintains ER calcium homeostasis (Brini and Carafoli 2009). In yeast cells, ER calcium signaling mechanisms are regulated by the Cod1/Spf1 ATPase (Cronin et al. 2002) and Pmr1 Ca^{2+}-ATPase (Antebi and Fink 1992). The Pmr1 Ca^{2+}-ATPase functions together with the calcium sequestration in the ER/Golgi complex in addition to the transportation of calcium and manganese to the secretory pathway compartments (Sorin et al. 1997; Antebi and Fink 1992). Interestingly, the ER-associated proteins are involved in maintaining ER calcium homeostasis. The molecular chaperones such as calreticulin, GRP94 or BiP, and folding enzymes (protein disulfide isomerases [PDI]) contribute to Ca^{2+} buffering in the ER lumen (Prins and Michalak 2011).

The lipotoxicity due to an enhanced ratio of phosphatidylcholine and phosphatidylethanolamine (PC/PE) in the ER, impairs SERCA2 and stimulates ER stress (Fu et al. 2011) and indicates the link between lipid and calcium. The absence

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of SERCA in *Saccharomyces cerevisiae* led to the discovery of another P-type ATPase, Cod1/Spf1p, that controls the Hmg2p degradation through calcium in the ER (Cronin et al. 2002). Together, these studies indicate an intimate relationship between the calcium and lipid homeostasis in the ER. We recently studied the accumulation of triacylglycerol (TAG) and lipid droplets (LDs) in yeast *Saccharomyces cerevisiae*, and the association of Cd stress resulted in lipid accumulation and Zn alteration (Rajakumar et al. 2016). In our lab, we also reported that Cd-induced ER stress resulted in phospholipid accumulation (Muthukumar et al. 2011). Expression of phospholipid biosynthetic enzymes get altered in response to a variety of stress conditions (Carman and Han 2009; Iwanyshyn et al. 2004) which in turn modifies the composition of phospholipids and exert tolerance against organic solvents (Ghosh et al. 2008). PC is the most abundant phospholipid in cellular membranes (Zinser et al. 1991) and crucial for the formation of the phospholipid bilayer. PC is a precursor for diverse signaling molecules (Sheikhnejad and Srivastava 1986). In ER, the two PE methyltransferases PEMT (Cho2) and PLMT (Opi3) execute a three-step methylation reaction of PE. *Cho2* catalyzes the first methylation step; however, *Opi3* is capable of promoting all three steps, although the last two steps with higher proficiency than the first one (Greenberg et al. 1983; Kodaki and Yamashita 1989; Summers et al. 1988; Gaynor et al. 1991; Preitschopf et al. 1993). The yeast cells respond to Cho2p or Opi3p deficiency by broadly varying the transcript levels (Thibault et al. 2012). The PC homeostasis is interconnected with neutral lipid metabolism. In most of the species like yeast and plants, PC is a major acyl donor for triacylglycerol (TAG) synthesis via the activation of lecithin cholesterol acyltransferase (*LRO1*) (Dahlqvist et al. 2000). The prominence of PC to health and disease was already proven by earlier studies (Farber et al. 2000; Grothe et al. 2015; Lee et al. 2014). However, the metabolism under cellular stress remains unclear. In this study, the loss of either *Cho2* or *Opi3* during cadmium exposure affected the calcium homeostasis in the ER and mitochondria.

Materials and methods

Chemical and reagents

Yeast extract, peptone, and bacteriological agar were purchased from Difco. Thin-layer silica gels 60 plates were purchased from Merck. BODIPY 493/503 was purchased from Invitrogen. Trizol, PMSF, and all other chemicals were purchased from Sigma unless specifically mentioned. The cDNA synthesizing kit was obtained from Bio-Rad. All solvents were purchased from Merck, and lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL).

Strains and growth conditions

Saccharomyces cerevisiae strains used in the study were BY4741 [MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0], *cho2*Δ, and *opi3*Δ. These strains were the kind gift from Prof. Ram Rajasekharan, CSIR-CFTRI, Mysore, INDIA. Strains were grown aerobically up to early stationary phase at 30 °C in YPD medium (1 % yeast extract, 2 % bacto-peptone, and 2 % dextrose). The yeast cells were precultured in 5 ml of YPD medium for 12 h, and then the cells were harvested and transferred to 25 ml of YPD media with or without 50-μM Cd so that the final absorption (A_{600}) of 0.1 was achieved. Following these, cells were agitated at 180 rpm at 30 °C for 12 h.

Cell viability and growth

To study the sensitivity against cadmium, wild-type and mutant strains were diluted (4-fold) and spotted on YPD plates containing 2 % agar supplemented with 0 to 100 μM of Cd and incubated for 2 days at 30 °C. Sensitivity was found to be 50 μM and so for the further experimental purposes, the cells were grown in a liquid medium with/without 50-μM Cd.

Extraction and separation of lipids

Yeast lipids were extracted using the method described by Bligh and Dyer (1959). Briefly, to the cell pellet, 2:1 ratio of chloroform and methanol were added, followed by vortexing. To this, an equal volume of acidified water (2 % phosphoric acid) was added and vigorously vortexed. The phospholipids were separated by TLC; solvents for the first dimension were chloroform/methanol/ammonia (65:35:5, *v*/v); solvents for the second dimension were chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, *v*/v). The neutral lipids were separated by using petroleum ether: diethyl ether: acetic acid (70:30:1, *v*/v) as the solvent system. Individual lipids were located by comparing the R*f* values of the unknown with the R*f* values of the standard. For the neutral lipids, the spots were visualized by post-chromatographic staining after dipping TLC plates into a solution containing 0.8-g $MnCl_2 \times 4H_2O$, 120-ml water, 120-ml methanol, and 9-ml concentrated sulphuric acid and charring at 105 °C for 30 min and quantified by densitometry scanner.

Phosphorous assay of phospholipids

Spots of phospholipid samples were scraped from TLC plate and transferred to glass tubes, and 0.65-ml perchloric acid was added and the tubes placed in a heating block until the yellow color disappears. After cooling, 3.3 ml of water, 0.5 ml of 2.5 % molybdate solution, and 0.5 ml of 10 % ascorbic acid solution were added to the tubes and mixed vigorously. The

tubes were then kept in a boiling water bath for 5 min and cooled to RT. The samples (including the standards) were read at A_{800} nm. Digestion was not needed for the standards (1 to 5 μg P/tube). The standards were diluted with water (3.3 ml) and 0.65-ml perchloric acid. The tubes containing the silica gel were centrifuged before take reading as it will interfere with the absorbance of phosphate (Siakotos et al. 1966).

Construction of protein-protein interaction network

The protein-protein interaction network of lipid proliferative pathways was constructed and analyzed using network biology approach. The protein interactions of Opi3 and Spf1 proteins were searched and retrieved from STRING database (Szklarczyk et al. 2015). The STRING database contains known and predicted protein-protein interactions derived from high throughput experiments, co-expression, co-occurrence, and gene fusion methods. The protein-protein interactions experimentally confirmed were retrieved by giving Opi3 and Spf1 proteins as seed proteins. The interactions only above the confidence score cut-off of 0.60 were chosen.

RNA isolation and real-time PCR

The wild and mutant cells were grown in YPD medium with and without Cd at 30 °C until mid-log phase. RNA was isolated using the RNA assay kit from Qiagen as described by the manufacturer instructions. After DNaseI digestion, complementary DNA (cDNA) was constructed using the high-capacity cDNA reverse transcription kit with 1× RT buffer, 1× random primer, 4-mM dNTP mix, 50-U/ml reverse transcriptase, and 1-μg total RNA. The primers were designed using the Primer Express^R Software 3.0 (Applied Biosystems), and the primer sequences are listed in Table S1. Relative mRNA levels were determined by quantitative real-time RT-PCR using SYBR Green real-time PCR master mix (Applied Biosystems) by following the manufacturer's instructions. PCR was initiated with denaturation at 94 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s, and a final extension at 72 °C for 5 min. Amplification was measured using an AB 7500 instrument (Applied Biosystems). Samples were evaluated using the ΔΔCt method described (Livak and Schmittgen 2001). The mRNA expressions were analyzed in triplicate, and the results were analyzed using relative quantification. ACT1 was used as endogenous control for normalization.

Microscopical analysis

Yeast cells were grown with or without 50-μM Cd in YPD liquid culture. For 3, 3′-dihexyloxacarbocyanine iodide (DiOC6) (Sigma) staining, cells were harvested and washed with a TE buffer. After washing, the pellet was resuspended in the TE buffer. DiOC6 was resuspended in 1-mg/ml ethanol (as DiOC6 stock solution). One microliter of DiOC6 stock solution was added to 1 ml of the yeast cell suspension. If the cell density was $A_{600} > 1.0$ at harvesting, the cell suspension was diluted with the TE buffer to an A_{600} of ~1.0. Cells stained with DiOC6 were analyzed immediately (Block-Alper et al. 2002). For Nile Red staining, yeast cells in stationary phase were washed and resuspended in phosphate-buffered saline (PBS). The cells were stained with Nile Red solution (0.0005 % in PBS, diluted from a 0.01 % stock solution) for 15 min at room temperature in the dark. The cells were then washed six times with PBS to remove the surplus dye. Cells were resuspended in 50 μl of PBS and observed with a confocal microscope directly after staining (Kimura et al. 2004). Mitochondrial damage was studied in *opi3*Δ and *cho2*Δ cells with Aconitase GFP plasmid, gifted by Prof. Markus Ralser, University of Cambridge, UK. Transformants were diluted from an overnight culture to an $A_{600} = 0.1$ in SC medium lacking uracil and were grown till mid-exponential phase. The final experiment was done by YPD media.

Image acquisition

Samples were fixed with 0.5 % (wt/vol) agarose on microscope slides. Fluorescence microscopic images were recorded on an LSM 710 Confocal microscope (Zeiss) equipped with a 100/1.40 oil objective and an AxioCam MRM camera (Zeiss) at room temperature.

Western blotting

Yeast cell lysates were prepared by breaking the cells with striled glass beads in a lysis buffer (50-mM Tris–HCl pH 7.4, 150-mM NaCl, 5-mM EDTA, and 1-mM PMSF) and the protease inhibitor cocktail. Total protein (50 μg) was subjected to SDS-PAGE. To visualize SDS-PAGE, gels were stained using a Coomassie brilliant blue staining. For immunoblot analyses, proteins were transferred to nitrocellulose membrane at 120 V for 1 h. Blots were decorated using anti-kar2p and anti-pgk1p antibodies. The resulting membrane was incubated with a 1:5000 dilution of primary antibody for 2 h, followed by a 1:2500 dilution of ALP-conjugated goat anti-rabbit IgG for 1 h. The membrane was washed again and detected with BCIP/NBT substrate system. Band intensity was quantified using the ImageJ software.

Determination of intracellular metals

Intracellular calcium and cadmium levels were determined in the wild-type and mutant strains by inductively coupled plasma atomic emission spectrometer (ICP-OES) on a Perkin Elmer 3300 (SAIF-IIT, Chennai). The cells were grown on YPD medium, and $A_{600} = 1$ of cells were collected by centrifugation, washed twice with 10-ml ice cold

1X TE buffer and then resuspended in nitric acid and heated at 200 °C for 4 h. After complete digestion, 1 ml was diluted to 10 ml in metal-free water and subjected to intracellular metal analysis (Doner and Edge 2004).

Statistics

The data analysis was done using the tools of PRISM 5.6. Each experiment was done thrice, and the data were presented as mean ± SD. Statistical analysis was carried out by analysis of variance (Oneway-ANOVA) test. The statistical probability of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ was considered to be significant.

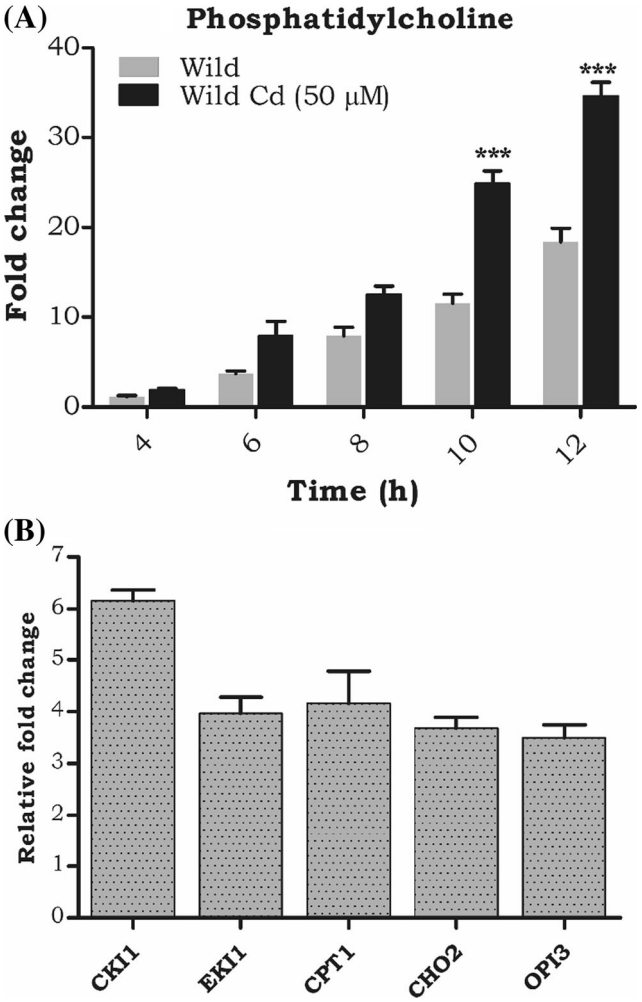


Fig. 1 Cd toxicity alters phosphatidylcholine (PC) synthesis. **a** A time-dependent study into PC accumulation during the growth phase (4–12 h), with or without 50-μM Cd. Equal amounts (A_{600} = 20OD) of cells were harvested at different time intervals. The lipids were extracted and separated on a silica TLC using solvent system **b** quantitative (qPCR) analysis of PC biosynthetic genes (CKI1, EKI1, CPT1, CHO2, and OPI3). Total RNA was isolated and converted into cDNA using 2 μg of total RNA. A1:20 dilutions of cDNA was used for the quantitative expression analysis. Actin was used as an endogenous control. Samples were analyzed in triplicate, and the expression of these genes in wild-type control cells was set as one (data not shown in the graph) and was compared with the mutants and Cd-treated cells

Results

Transcriptional alteration of PC biosynthesis during cadmium exposure

Earlier studies in our laboratory reported that cadmium exposure significantly increased the phospholipids in wild-type cells. We also analyzed the PC profiling at different time intervals (4, 6, 8, 10, and 12 h) during cadmium exposure. At 10 and 12 h, PC was increased up to 15- and 25-fold (Fig. 1A) compared to wild-type control cells. Moreover, to explore, how cadmium affects the lipid transcriptional regulation in *S. cerevisiae*, the quantitative PCR analysis of the PC biosynthetic genes CKI1, EK11, CPT1, CHO2, and OPI3 genes were quantified, and the expression of all the genes was

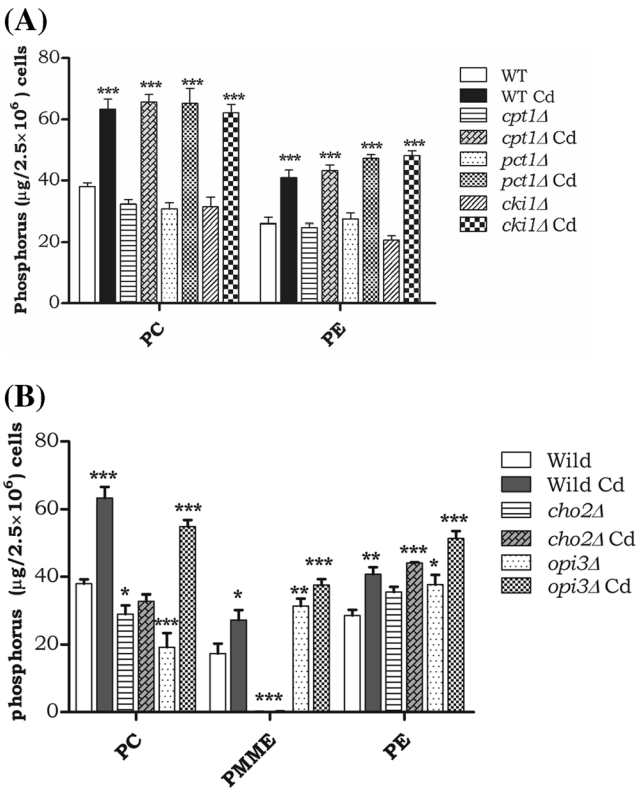


Fig. 2 Defect of PC synthesis modulates the PC production upon Cd stress. The wild-type, *cho2*Δ, and *opi3*Δ cells were grown up to mid-log phase on YPD medium; with or without 50-μM cadmium. An equal number of cells were taken, and lipids were extracted and resolved by two-dimensional silica TLC. The pattern of major membrane phospholipids PC and PE, and also quantified intermediate form of PC, like PMME. **a** The level of PC and PE in Kennedy pathway mutants (*cki1*Δ, *cpt1*Δ, and *pct1*Δ) and **b** methylation pathway mutants **(***cho2*Δ and opi3**)** phospholipid was quantified and presented as phosphorus (μg/2.5×10^6 cells). WT control cells were compared with other groups with a statistical significance of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

significantly upregulated (Fig. 1B). Further, we studied the impact of Cd on both the Kennedy and the *de novo* (methylation) CDP-DAG pathway mutants. Our results show Kennedy pathway mutants (*cki1*Δ, *cpt1*Δ, and *pct1*Δ) behaved similarly to the wild-type cells, whereas the *de novo* pathway mutants (*cho2*Δ and *opi3*Δ) were more susceptible to Cd stress (Fig. S1) and so we chose the *de novo* pathway mutants (*cho2*Δ and *opi3*Δ) for further studies.

Exposure tocadmium modulates PC levels in Kennedy and methylation pathway

Wild-type cells during 12 h of Cd exposure depicted a 50 % increase in PC, and the Kennedy pathway mutants (*cki1*Δ, *cpt1*Δ, and *pct1*Δ) also showed a palpable elevation in the accumulation of PC-like WT Cd-treated cells, as well as a similar trend, was observed in PE levels (Fig. 2A). The *cho2*Δ did not show PC alteration in the presence of Cd. But in *opi3*Δ, the PC was reduced and was quite obvious. Upon Cd exposure, the PC was significantly increased in *opi3*Δ. The intermediates of PC synthesis, phosphatidyl monomethyl ethanolamine (PMME) also accrued in *opi3*Δ. Accumulation of PE was observed in both the *cho2* and *opi3* mutant strains and was further elevated during Cd exposure (Fig. 2B). Finally, PC alteration was apparent in methylation pathway mutants *cho2*Δ and *opi3*Δ; this might have an impact in the unfolded protein response mechanism.

Defects of *cho2* and *opi3* cause TAG and LD accumulation

Both the *cho2* and *opi3* mutant strains showed a marked increase in TAG level (100 %), and Cd exposure elevated it further. The SE level was increased with Cd exposure in the wild-type and also the mutants (Fig. 3A). Consequently, we studied the LD formations and our result demonstrated increased number and size of LDs with cadmium exposure in all the strains, the especially Opi3 mutant showed significantly increase in LDs both in its numbers and size. (Fig. 3B) These results demonstrate that the methylation pathway PC synthesis are essential for both membrane and storage lipid homeostasis during Cd stress.

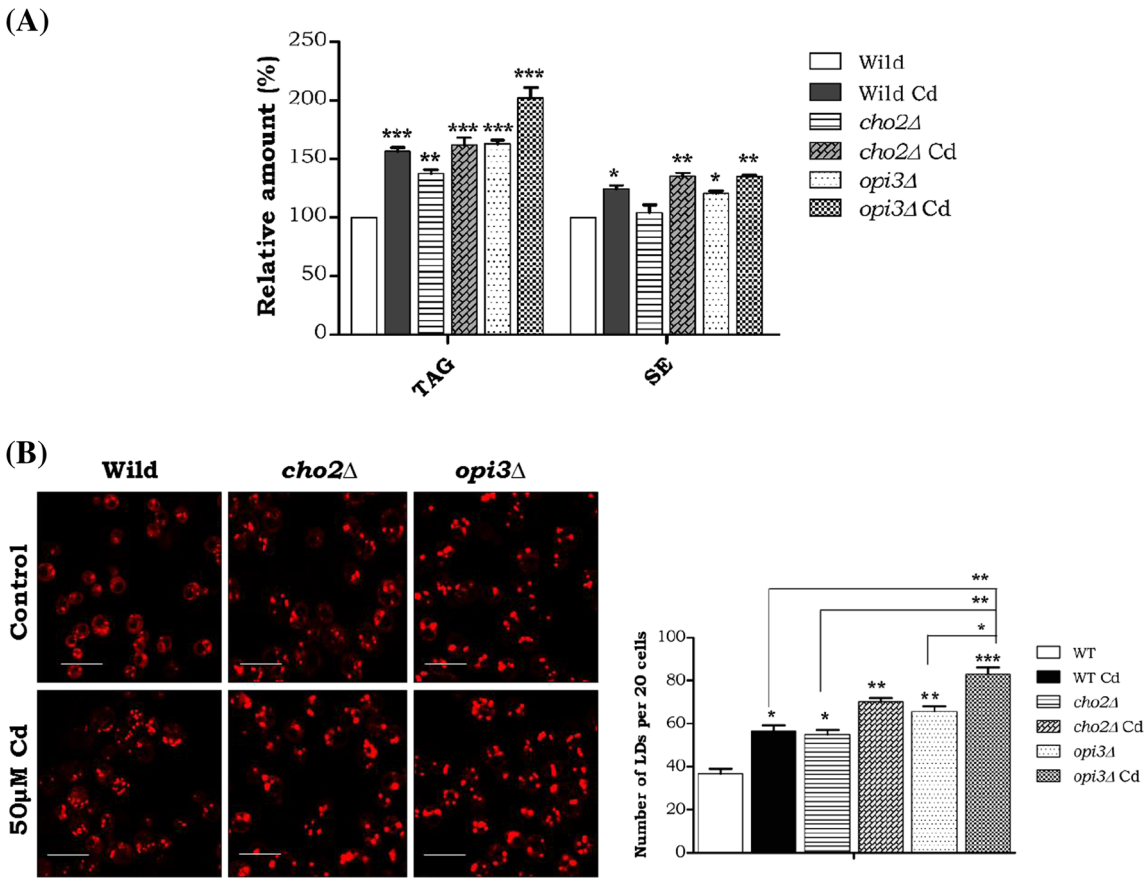


Fig. 3 Impairment of PC synthesis leads storage lipid accumulation under Cd stress. The wild-type, *cho2*Δ, and *opi3* cells were grown up to the mid-log phase on YPD medium with or without 50-mM cadmium. An equal amount of cells were taken for lipid extraction. Lipids were resolved on silica TLC and quantified. **a** *TAG* triacylglycerol, *SE* sterol esters. Samples were analyzed in triplicate and compared to the control. WT control cells were compared with other groups with a statistical significance of $*p < 0.05$, $**p < 0.01$, and $*** p < 0.001$. **b** The cells were stained with Nile red (1 μg/ml) and viewed under the fluorescent microscope. The *bar* in the figures indicates the scale of 5.0 μm

Cadmium alters intracellular calcium in the PC deletion mutants

Earlier studies in our lab reported alteration of Zn during cadmium exposure and in the current study, we focused on the cadmium interference with the biologically essential mineral such as calcium. Hence, during Cd exposure, we quantified the intracellular calcium levels in PC mutants by ICP-OES. We noticed the remarkable elevation of calcium (2-, 1.5-, and 2.5-fold) in wild-type, *cho2Δ* and *opi3Δ* Cd-treated cells (Fig. 4A). Subsequently, we analyzed transcriptional alteration of calmodulin kinase 1 (CMK1). Our results showed significant upregulation of the CMK1 in *cho2Δ* and *opi3Δ* during Cd exposure (Fig. 4C). In SGD (Saccharomyces Genome database and BIOGRIDs), the gene interaction map displays an interaction between ER calcium ATPase SPF1 and OPI3 and both are localized in ER (Fig. 4D). Also, some experimental evidence suggests the negative genetic interaction between opi3 and spf1 with a high confident score (0.66) (Table 1). We also studied the gene expression of SPF1 and PMR1 in *cho2Δ* and *opi3Δ* strains during Cd exposure. Interestingly, the SPF1 and PMR1 were downregulated in *opi3Δ* cells and upregulated in *cho2Δ* cells during Cd exposure (Fig. 4E). These results suggest that in *S. cerevisiae* during Cd exposure, the PC levels and calcium levels were altered and so had an impact on their Ca^{2+}-ATPasein ER.

Cadmium toxicity induced membrane aberrancy, ER stress, and mitochondrial damage in PC mutants

The abundance of intracellular calcium induced ER stress and may have an impact on membrane proliferation in *Saccharomyces cerevisiae*. DiOC6 the membrane specific dye quantifies the intercellular membrane content of yeast cells, and DiOC6 was used to analyze the membrane aberrancy in Cd-treated cells. Our data showed bright rings arising from the ER and nuclear membranes in the wild-type and *opi3*Δ strains upon Cd exposure. Only faint images were observed in the cadmium-unexposed cells and *cho2*Δ (−/+ cadmium) strains (Fig. 5A). We also tested ER stress by measuring Kar2p expression (ER chaperone overexpressed during UPR activation). The *cho2*Δ and *opi3*Δ cells were increased Kar2p expression that was further enhanced by Cd exposure (Fig. 5B). Furthermore, calcium alteration in ER will have an impact on the mitochondria, and

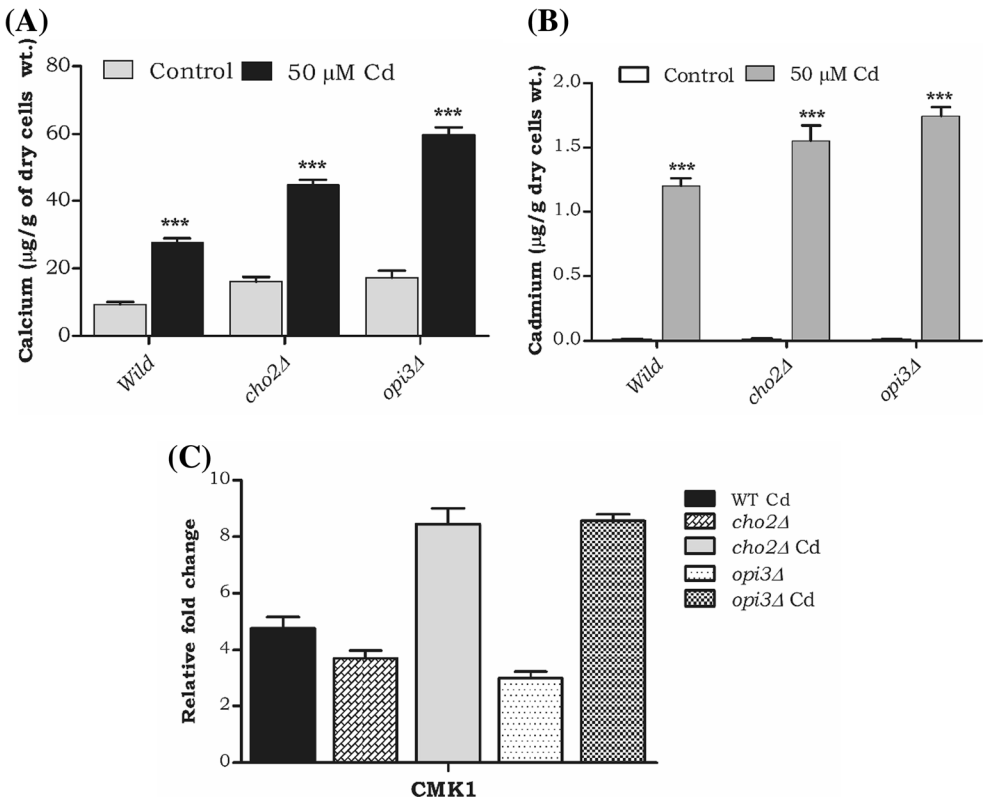


Fig. 4 A defect of methylation pathway PC leads to Ca^{2+} imbalance. **a** Analysis of intracellular calcium by ICP-MS. Wild and mutant strains were grown on nutrient rich (YPD) medium with or without Cd, after harvested and digested with Conc HNO3. *Error bars* indicate SD from two independent experiments (**p value <0.001 and*p value <0.01). **b** The level of intracellular cadmium and **c** quantitative (*qPCR*) analysis of calcium-calmodulin kinase1(*CMK1*). **d** Interaction mapping of phospholipid methyl transferase gene (OPI3) and ER calcium ATPase (SPF1) by using STRING 10.0. **e** Quantitative (qPCR) analysis of ER calcium ATPase SPF1 and PMR1. Samples were analyzed in triplicate, and the expression of these genes in wild-type control cells was set as one (data not shown in the graph) and was compared with the mutants and Cd-treated cells

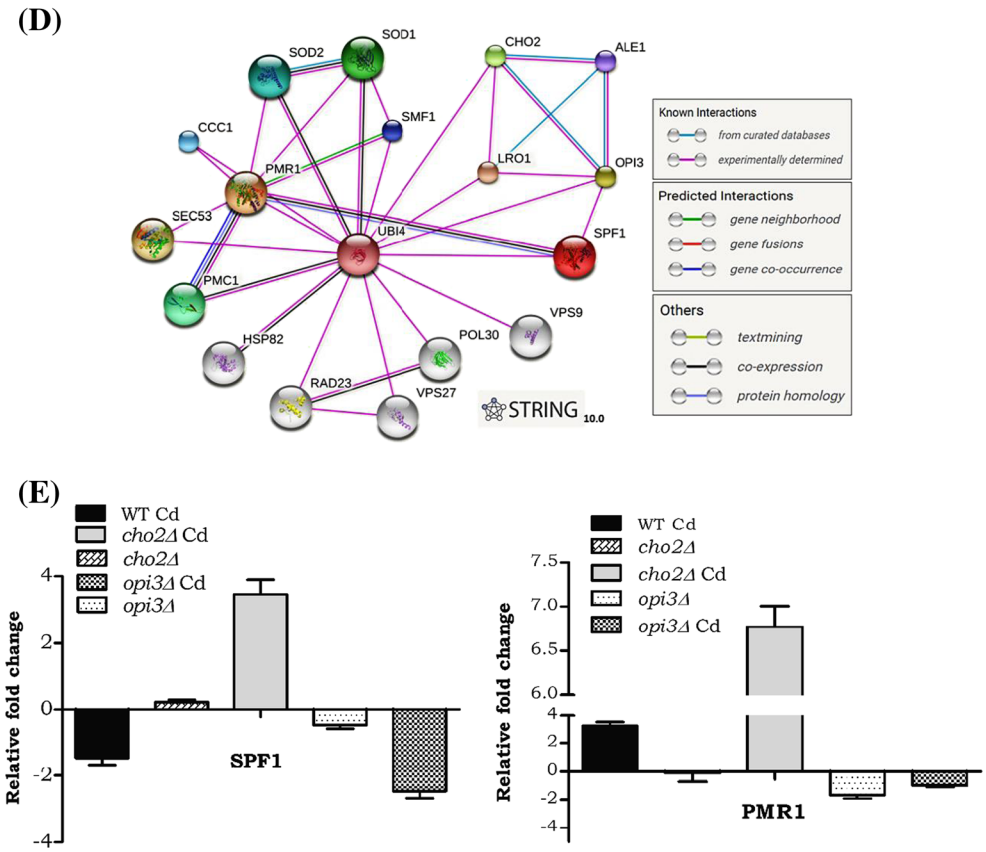


Fig. 4 (continued.)

so we analyzed the mitochondrial damage in the *cho2*Δ and *opi3*Δ genes during Cd stress by using *ACO1*-pGFP (aconitase) that worked as stress sensor for the protection of mtDNA. Our micrographs showed tubular mitochondria in wild-type and *cho2*Δ control strains, but *opi3*Δ strains showed fragmented mitochondria. On the other hand, Cd-treated cells displayed total mitochondrial fragmentation (Fig. 5C).

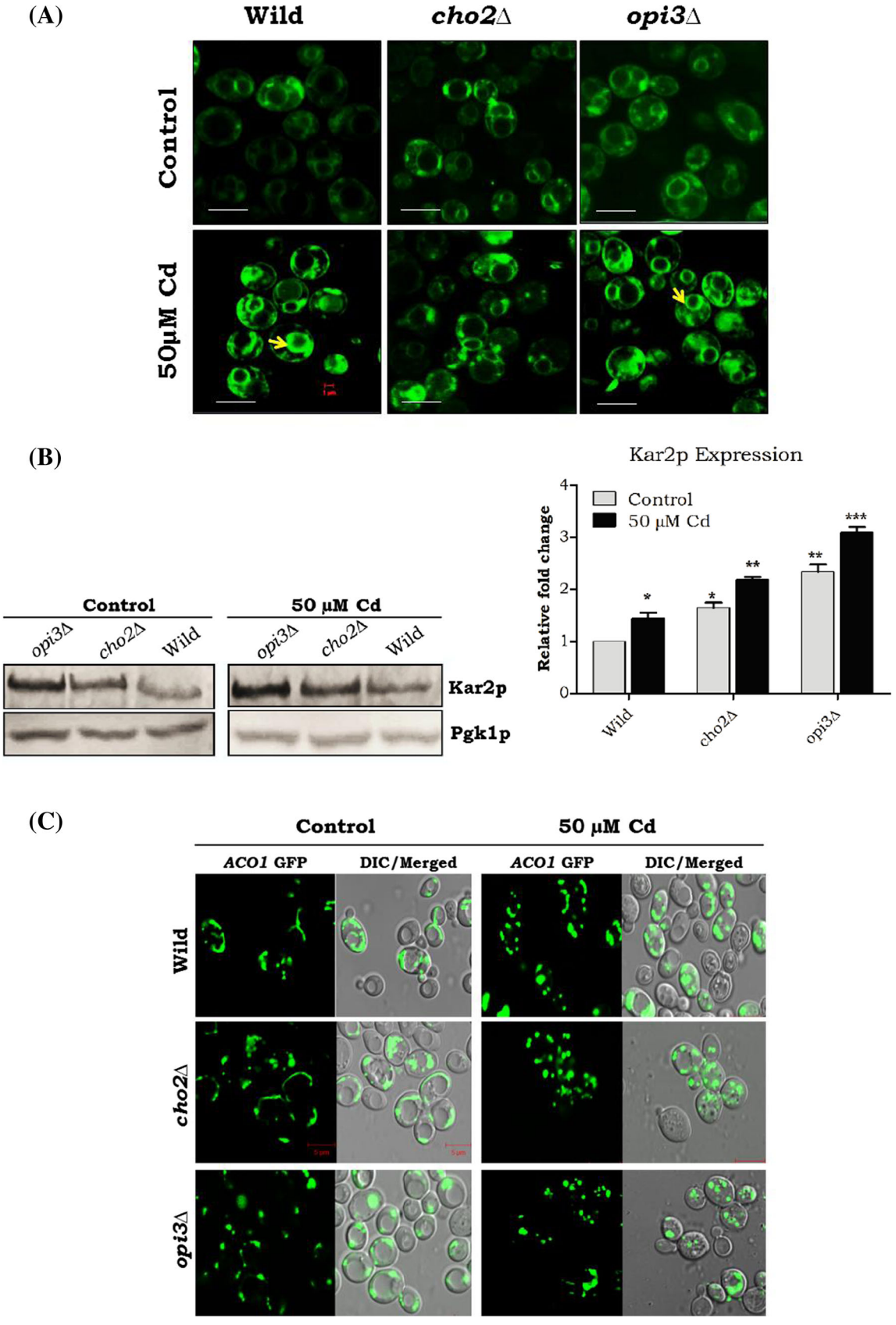
Discussion

The alteration of intracellular calcium and UPR activation are reported in several models of metabolic disease and lipid disequilibrium (Fu et al. 2011; Ozcan et al. 2004). ER calcium-dependent proteins have an important role in the maintenance of cellular integrity. Disruption of the calcium homeostasis in the ER is a potent trigger of ER stress and apoptosis (Groenendyk et al. 2010 and Schroder and Kaufman 2005). Recent studies revealed that lipid remodeling majorly contributes to adaptive stress response and acclimatization of ER stress but the mechanistic link between lipid aberrancy and Ca^{2+} alteration inducing ER stress is not yet elucidated.

In yeast, several lipid metabolic defects activate the UPR, suggesting a compensatory role when these pathways are disrupted (Jonikas et al. 2009 and Thibault et al. 2012). Our earlier reports have demonstrated that Cd exposure is induced

Table 1 Protein-protein interactions observed between Ca^{2+} homeostasis (Spf1), lipid (Cho2, Lro1, Ale1), and ubiquitin metabolic processes (Ubi4). Protein interaction summary of Opi3

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
OPI3	ALE1	YJR073C	YOR175C	Phospholipid methyltransferase	Broad specificity lysophospholipid acyltransferase	0.978
OPI3	CHO2	YJR073C	YGR157W	Phospholipid methyltransferase	Phosphatidylethanolamine methyltransferase (PEMT)	0.999
OPI3	LRO1	YJR073C	YNR008W	Phospholipid methyltransferase	Acyltransferase that catalyzes diacylglycerol esterification	0.958
OPI3	SPF1	YJR073C	YEL031W	Phospholipid methyltransferase	P-type ATPase, ion transporter of the ER membrane; ER function and Ca2+ homeostasis	0.665
OPI3	UBI4	YJR073C	YLL039C	Phospholipid methyltransferase	Ubiquitin, essential for the cellular stress response	0.79


(A)
Wild
cho2Δ
opi3Δ
Control
50μM Cd
(B)
Control
50 μM Cd
opi3Δ
cho2Δ
Wild
Kar2p
Pgk1p
Kar2p Expression
Control
50 μM Cd
Relative fold change
Wild
cho2Δ
opi3Δ
(C)
Control
50 μM Cd
ACO1 GFP
DIC/Merged
Wild
cho2Δ
opi3Δ

◀ **Fig. 5** Defects in PC methylation in inducing membrane proliferation, ER stress, and mitochondrial damage. Wild-type and mutant cells were grown up to mid-log phase on YPD medium with or without Cd at 30 °C. **a** Membrane proliferation was assessed by the incorporation of the lipophilic dye DiOC6 and viewed under the fluorescence microscope. The *bar* in the figures indicates the scale of 1.0 μm. **b** Cells were lysed, and 50 μg of protein was loaded and separated by 10 % SDS-PAGE followed by immunoblotting with kar2 antiserum. Band intensity was quantified by the ImageJ software and expressed as a ratio with pgk1. **c** The *ACO1*-pGFP was analyzed for mitochondria morphology. Strains wild-type and *cho2*Δ contain typical tubular mitochondria. *Opi3*Δ caused mitochondrial fragmentation gradually increased; Aco1p showed 100 % mitochondrial network fragmentation in all Cd-treated mutant yeast cells

ER stress (Muthukumar et al. 2011). In our current study, the phospholipids mainly PC and PE were increased in wild-type cells upon Cd exposure (Fig. 1A). The Kennedy (CKI1, EKI1, and CPT1) and the methylation pathway (CHO2 and OPI3) genes were upregulated during Cd exposure in the wild-type cells (Fig. 1B). It is also backed up by the DMSO treatment, facilitating the anomaly in gene expression of the phospholipid synthesis pathway, the relative quantities of phospholipids in yeast (Murata et al. 2003). These results are consistent and support our previous findings (Muthukumar et al. 2011 and Rajakumar et al. 2016). The de novo PC synthesis was affected in *cho2Δ* and *opi3Δ* because the cellular membranes have a high PC turnover due to a high rate of synthesis and breakdown (Jackowski 1996). The *cho2Δ* cell growth was greatly inhibited than *opi3Δ* and wild-type cells (Fig. S1) upon cadmium exposure. In contrary, the Kennedy pathway mutants (*cki1*Δ, *pct1*Δ, and *cpt1*Δ) showed more viability and also the significant increase in PC compared to the de novo pathway mutants (Fig. S1 and Fig. 2A). These might contribute to the upregulation of phospholipid methyl transferases (CHO2 and OPI3) during chemical stress (Murata et al. 2003). Intriguingly, *cho2Δ* reduced PC when compared to *opi3Δ* cells during Cd exposure (Figs. 3A and 5B) and also reduced Kar2p expression in *cho2Δ* than that in *opi3Δ* cells. It is in close grip with earlier studies; the reduction of PC suggests that Opi3p might partly compensate for *cho2Δ* (Kanipes and Henry 1997; Preitschopf et al. 1993). The *opi3Δ* showed more PC content during Cd exposure.

Our work extended to an integration of PC metabolic pathway and calcium homeostasis. The elevation of intracellular calcium leads to alteration of phospholipids, especially PC (Moore et al. 1984), and also the silencing of PEMT, accelerated the SERCA activity and reduced the ER stress (Jacobs et al. 2010). In yeast, the Biological General Repository for Interaction Datasets (BioGRIDs) showed a negative genetic interaction of OPI3 with SPF1 and PMR1 by high throughput screenings (Fig. 4D) (Schuldiner et al. 2005; Hoppins et al. 2011; Jonikas et al. 2009 and Surma et al. 2013). A problem that has yet to be resolved is why PC is regulated in response to ER calcium governance. We noticed an elevation of calcium in *cho2Δ* than *opi3Δ* cells during Cd exposure (Fig. 4A). Also, the calcium calmodulin kinase-encoding gene CMK1 expression was highly elevated, whereas SPF1 expression was reduced in *opi3Δ* when compared to WT Cd-treated cells (Fig. 4C and E). This result might suggest that cytosolic Ca^{2+} was increased, and ER calcium reduced. Inhibition of CaMKII (calcium calmodulin kinase) reduced the ER stress, mitochondrial integrity, and membrane potential in murine cells (Bracken et al. 2016). Previous studies also revealed that the ratio of phosphatidylcholine and phosphatidylethanolamine in membrane resulted in ER stress and Ca^{2+} imbalance in mice hepatocytes (Fu et al. 2011). Moreover, we observed higher expression of Kar2p level (ER chaperone) in *opi3Δ* control and Cd-treated strains and might be due to IRE1-mediated ER stress activation (Fig. 5B). These findings also support that activation of IRE1 in Opi3 deletion is likely to cause membrane or lipid-related aberrations (Promlek et al. 2011).

The lipophilic green fluorescent dye DiOC6 was used to identify the intercellular membrane abnormalities or membrane proliferation in yeast cells (Koning et al. 1996; Murata et al. 2003). Our microscopy results showed the aberrant membrane structures in Cd-treated WT and *opi3* mutant strains (Fig. 5A). This result also proves the ER stress triggers the UPR and is not constantly accompanied by accumulation of unfolded proteins in the ER, but is also activated by the aberrant lipid or membrane content (Promlek et al. 2011). Together, these results demonstrate the relationship between the UPR and lipid homeostasis. Also, we studied the mitochondrial dysfunctions during impairment of PC with Cd

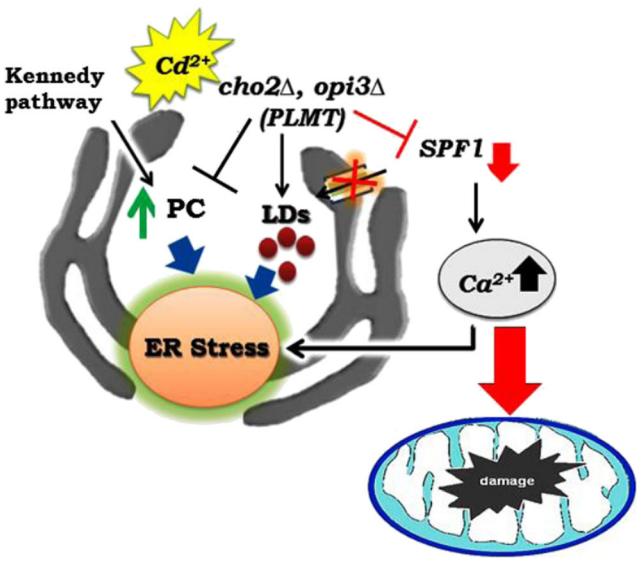


Fig. 6 A diagrammatic representation of phospholipid methylation and cadmium exposure alters calcium levels, induces ER stress mitochondrial dysfunction, and disrupts lipid homeostasis in *Saccharomyces cerevisiae*. (*Cd* cadmium, *Ca* calcium, *PC* Phosphatidylcholine, CHO2-phosphatidylethanolamine methyltransferase, OPI3-phospholipid methyltransferase, SPF1-ER Ca^{2+} ATPase, PMR1-ER & Golgi Ca^{2+} ATPase, *LDs* lipid droplets)

stress. ER and mitochondria interact both physiologically and functionally, and the association is referred as mitochondrial-associated membranes (Vance 1990). The main facets of this interaction are essential for calcium signaling, lipid transport, energy metabolism, and cell survival (Malhotra and Kaufman 2011: Stone and Vance 2000). In humans, congenital muscular dystrophy patients showed defects in the PC synthesis and associated mitochondrial abnormality, directing a role of PC in mitochondrial function (Mitsuhashi et al. 2011). Therefore, we studied mitochondrial morphology by using *ACO1*-pGFP in wild and mutant cells. The yeast mitochondrial Aco1p is a TCA cycle enzyme that converts citrate into isocitrate, and it is essential for mtDNA stability (Shadel 2005). Our microscopical data depicted tubular mitochondria and were observed in WT and *cho2*Δ control strains, but fragmented mitochondria was observed in *opi3*Δ and Cd-treated WT strains (Fig. 5C). Altogether, impairment of methylated PC synthesis specifically affects the function and stability of TOM and SAM complexes of mitochondria in yeast (Schuler et al. 2015). This data supports that PEMT pathway is also essential for mitochondrial function during ER stress and impairment of calcium. The defect of Pmr1p and Spf1p, implicated in protein glycosylation, influenced the conditions of ER stress stimulates LD formation in *Saccharomyces cerevisiae* (Fei et al. 2009; Cohen et al. 2013). Besides, the role of LD biogenesis and synthesis of TAG is a sign of cellular stress, and it was proposed that alterations in PC homeostasis might be the source of TAG (Al-Saffar et al. 2002 and Iorio et al. 2003). Our data showed increased number of LDs and accumulation TAG in *cho2Δ* and *opi3Δ* cells during Cd exposure (Fig. 3). Also, we reported that exposure to cadmium in yeast accumulates TAG and LDs (Rajakumar et al. 2016).

Finally, we can conclude that phospholipid has an important role in calcium homeostasis and ER stress during the Cd exposure (Fig. 6). The phospholipid methyltransferase encoding gene OPI3 and phosphatidylcholine are essential for Cd tolerance and are required for intracellular membrane maintenance and mitochondrial morphology.

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